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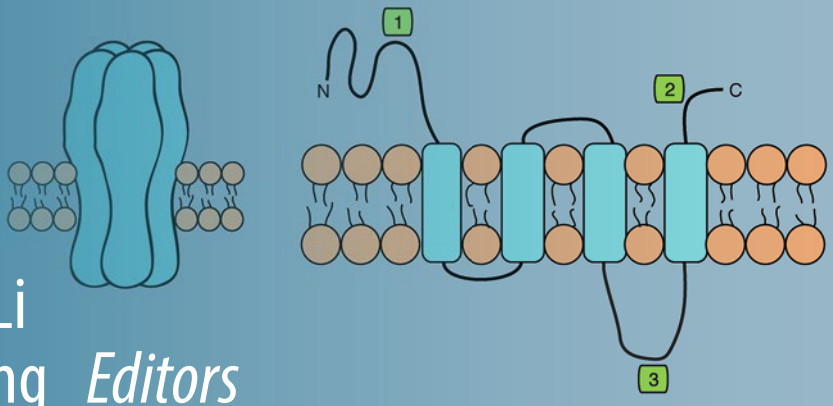

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Haojian Zhang *Editors*

Chronic Myeloid Leukemia

Methods and Protocols

Humana Press

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Methods and Protocols

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Preface

Cancer stem cells are responsible for the initiation and progression of many types of cancer and are in general less sensitive to chemotherapy. There is an urgent need to fully understand the biology of cancer stem cells for developing effective therapies, and the success in this approach relies on utilizing stem cell disease models and advanced technologies to study the molecular basis of cancer stem cells. It is generally believed that human chronic myeloid leukemia (CML) is derived from an abnormal hematopoietic stem cell harboring Philadelphia chromosome that results in the formation of the BCR-ABL oncogene. Leukemia stem cells (LSCs) in CML provide an ideal cellular system for studying how LSCs survive, proliferate, and evade from treatment with tyrosine kinase inhibitors. Technically, various molecular and cellular methods have been utilized to help understand the biology of LSCs in CML, and these methods have led to breakthrough discoveries useful in developing effective therapeutic strategies for CML. This book highlights the molecular and cellular methods used in studying CML pathogenesis and stem cell biology.

Massachusetts, USA — *Shaoguang Li*
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Chapter 1

Molecular Detection of BCR-ABL in Chronic Myeloid Leukemia

Ya-Zhen Qin and Xiao-Jun Huang

Abstract

All chronic myeloid leukemia (CML) patients have the BCR-ABL fusion gene. The constitutively activated BCR-ABL tyrosine kinase is a critical pathogenetic event in CML. Tyrosine kinase inhibitors (TKIs), such as imatinib, are synthesized small molecules that primarily target BCR-ABL tyrosine kinases and have become a first-line treatment for CML. Detection of BCR-ABL transcript level by real-time quantitative polymerase chain reaction (RQ-PCR) is a clinical routine for evaluating TKI treatment efficacy and predicting long-term response. Furthermore, because they are a main TKI resistance mechanism, the BCR-ABL tyrosine kinase domain (TKD) point mutations that are detected by Sanger sequencing can help clinicians make decisions on subsequent treatment selections. Here, we present protocols for the two abovementioned molecular methods for CML analysis.

Key words Chronic myeloid leukemia, BCR-ABL, Real-time quantitative polymerase chain reaction, Sanger sequencing, Tyrosine kinase inhibitor

1 Introduction

The Philadelphia chromosome (Ph) arises from a reciprocal translocation between chromosomes 22 and 9 [t(9,22)(q34;q11)] [1]. This translocation fuses the ABL1 (Abelson murine leukemia viral oncogene homolog) gene on chromosome 9 to the breakpoint cluster region (BCR) gene from chromosome 22 [2, 3]. The fusion of BCR with ABL creates the constitutively activated ABL tyrosine kinase, which is a critical pathogenetic event in chronic myeloid leukemia (CML), a myeloproliferative neoplasm that originates in an abnormal pluripotent bone marrow stem cell population [4, 5]. The overwhelming majority of CML patients have a P210 BCR-ABL gene (M-bcr), which encodes mRNA transcripts that have a b3a2 and/or a b2a2 junction [6]. Tyrosine kinase inhibitors (TKIs), such as imatinib, nilotinib, and dasatinib, are synthesized small molecules that primarily target BCR-ABL tyrosine kinases

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[7–9]. The introduction of TKIs has greatly revolutionized the treatment of CML [10, 11]. The majority of CML patients quickly achieve complete cytogenetic response (CCyR). Thereafter, in order to examine the leukemia burden, to evaluate treatment efficacy, and to predict the long-term response, a more sensitive and quantitative method, real-time quantitative polymerase chain reaction (RQ-PCR), is necessary to detect the BCR-ABL transcript levels [12, 13]. Furthermore, some patients show primary or acquired resistance to TKI during treatment, which is mainly caused by point mutations in the BCR-ABL tyrosine kinase domain (TKD) [14, 15]. Therefore, mutation testing could help clinicians make decisions on subsequent treatments, such as choosing the appropriate TKI or performing allogeneic hematologic stem cell transplantation [16]. Currently, the routine detection of BCR-ABL transcript levels by RQ-PCR and screening of BCR-ABL TKD point mutations in cases of a nonoptimal response have been recommended for CML diagnosis by both the European LeukemiaNet (ELN) recommendations and NCCN Guidelines [17, 18]. The procedures for detecting the BCR-ABL transcript and BCR-ABL TKD point mutations are described below.

2 Materials

2.1 Detection of BCR-ABL Transcript Levels by TaqMan-Based RQ-PCR

2.1.1 RNA Extraction

1. RNase-free water: Deionized water is treated with 0.1 % diethylpyrocarbonate (DEPC) overnight (*see* **Note 1**), autoclaved, and stored at room temperature.
2. Red blood cell (RBC) lysis buffer [19].
   (a) Stock solution.
      - 1.44 M NH_4Cl: Dissolve 38.52 g of NH_4Cl in water to a final volume of 500 mL. Autoclave and store at room temperature for 12 months. This should not be used in case of crystal separation.
      - 1.0 M NH_4HCO_3: Dissolve 39.55 g of NH_4HCO_3 in water to a final volume 500 mL. Store at room temperature for 12 months. Autoclaving is forbidden due to release of fumes.

   (b) Working solution. Add 50 mL 1.44 M NH_4Cl and 5 mL 1.0 M NH_4HCO_3 to water to make a final volume of 500 mL. The final concentrations of NH_4Cl and NH_4HCO_3 are 0.144 M and 0.01 M, respectively. Store at room temperature for 12 months. Autoclaving is forbidden.
3. TRIzol RNA isolation solution (Life technology, Product number 15596).
4. Chloroform: analytical reagent. Store at room temperature.

5. Isopropanol: analytical reagent. Store at −20 °C.
6. 80 % ethanol solution: Dilute pure ethanol with water and store at −20 °C.

2.1.2 cDNA Synthesis

1. High-Capacity cDNA Reverse Transcription Kits (Life technology, Product number 4368813).
2. RNase inhibitor (RNasin) (Promega, Product number N2112), 40 U/μL.

2.1.3 Preparation of Plasmid Standards for RQ-PCR

1. 2 × Taq PCR master mix: including Taq DNA polymerase, buffer, and dNTPs (Beijing Tiangen, Product number KT201).
2. Molecular cloning-related reagents, such as LB-ampicillin agar plates, X-gal and IPTG (isopropyl-β-d-thiogalactoside), restriction enzymes, TIANprep Mini Plasmid Kit (Beijing Tiangen, Product number DP103), and others.
3. Plasmid diluent: 50 μg/mL salmon sperm DNA in buffer with 1 mM Tris–HCl (pH 8.0) and 0.1 mM EDTA, stored at 4 °C or −20 °C.
4. Agarose: 6× loading buffer and DNA molecular weight marker for gel electrophoresis.

2.1.4 RQ-PCR

1. Primers and probes.
   The sequences of primers and probes for ABL and BCR-ABL are shown in Table 1. Both b2a2 and b3a2 types of BCR-ABL are detected using the same primers and probes. Primers and probes for ABL were referred from the report of the Europe against cancer program [20].
2. 2× TaqMan gene expression master mix (Life technology, Product number 4369016).

Table 1
RQ-PCR primer sequences and product sizes

Gene	Type	Sequences	Product sizes (bp)
ABL	Forward primer	5′-TGGAGATAACACTCTAAGCATAAC TAAAGGT-3′	124
	Reverse primer	5′-GATGTAGTTGCTTGGGACCCA -3′	
	Probe	5′-FAM-CCATTTTTGGTTTGGGCTTCACA CCATT-TAMRA-3′	
BCR-ABL (P210)	Forward primer	5′-CCGCTGACCATCAAYAAGGAA-3′	71(b2a2) 146(b3a2)
	Reverse primer	5′-CTCAGACCCTGAGGCTCAAAGT-3′	
	Probe	5′-FAM-AGCCCTTCAGCGGCCAGTA GCATCT-TAMRA-3′	

Table 2
Primer sequences and product sizes

PCR	Type	Sequences	Product sizes (bp)
1st round	Forward primer Reverse primer	5′-TGACCAACTCGTGTGTGAAACTC-3′ 5′-TCCACTTCGTCTGAGATACTGGATT-3′	1504(b2a2) 1579(b3a2)
2nd round	Forward primer Reverse primer	5′-CGCAACAAGCCCACTGTCT-3′ 5′-TCCACTTCGTCTGAGATACTGGATT-3′	863

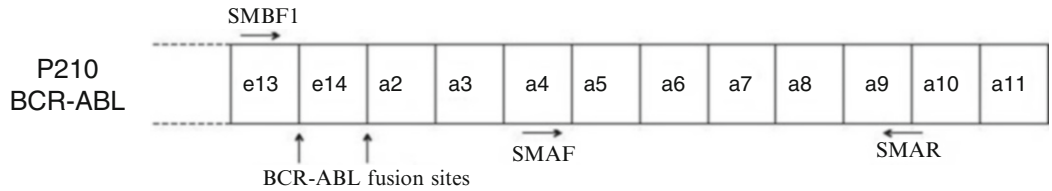

Fig. 1 Locations of primers for detecting BCR-ABL point mutations on BCR-ABL (P210) transcript

2.2 Detection of BCR-ABL TKD Point Mutations by Sanger Sequencing

1. Primers: semi-nested PCR is performed. Therefore, different forward primers and the same reverse primer are used. Sequences and PCR product sizes are shown in Table 2. Primer locations on BCR-ABL transcript are shown in Fig. 1.
2. 2× Pfu PCR master mix: including high-fidelity Pfu DNA polymerase, buffer, and dNTPs (Beijing Tiangen, Product number KP201).
3. 2.0 % agarose gel for electrophoresis.
4. Gel-loading buffer and DNA molecular weight marker.

3 Methods

3.1 Detection of BCR-ABL Transcript Levels by TaqMan-Based RQ-PCR

3.1.1 RNA Extraction

1. Sample collection.
   Patients with normal white blood cell (WBC) count have 8 mL of peripheral blood drawn into a vacuum tube with EDTA anticoagulant. The blood volume is adjusted according to WBC count. The sample is stored at 4 °C, and RNA is extracted within 24 h.
2. Acquisition of nucleated cells.
   (a) First, add 25 mL of RBC lysis buffer to a 50 mL tube.
   (b) Power the blood sample into buffer, and mix by inversion. Then, roll the tube on a rolling device for 10 min, and centrifuge at 500 × *g* for 5 min. The supernatant is

carefully poured out. If RBCs are not fully lysed, repeat lysis once more.

(c) Wash cells once with 5–10 mL PBS (phosphate buffered solution). After centrifugation, transfer cells to a 1.5 mL RNase-free Eppendorf tube. The cell number should be approximately 1×10^7 per tube.

3. RNA extraction.

(a) First, add 1 mL of TRIzol reagent to the tube, thoroughly mix with cells for homogenization, and incubate for 5 min at room temperature.

(b) Then, add 0.2 mL of chloroform to the tube, and vigorously shake by hand for at least 15 s. After 2–3 min of incubation at room temperature, centrifuge the tube at 12,000 × *g* for 15 min at 4 °C.

(c) Angle the tube at 45°, and pipette the upper colorless aqueous phase into a new tube containing 0.6 mL of ice-cold isopropanol, and mix through inversion. Take care not to draw any of the interphase and organic phase into the pipette. After incubating for 10 min at room temperature or overnight at −20 °C, centrifuge samples at 12,000 × *g* for 15 min at 4 °C.

(d) After centrifugation, RNA is precipitated at the bottom of the tube, and remove the supernatant. Then, add 1 mL of 80 % ethanol to wash the RNA, and centrifuge the mixture at 7500 × *g* for 5 min at 4 °C. Discard the supernatant.

(e) The RNA is air-dried for approximately 15 min. Dissolve the RNA pellet in 20–40 μL RNase-free water, and measure the absorbance at 260 nm and 280 nm. The ratio A260/A280 should be higher than 1.6. Calculate the RNA concentration.

3.1.2 cDNA Synthesis

1. Incubate the dissolved RNA at 65° for 5 min for denaturing, and then place it in an ice bath.
2. 2 μg RNA is used for 20 μL of reverse transcription. The total volume of water and RNA is 10 μL. Calculate the volume of RNA and an appropriate volume of water for each sample. Then, add the water to a 0.2 mL RNase-free Eppendorf tube.
3. Prepare the master mix for reverse transcription. The components for one sample are shown in Table 3. Mix the mixture by vortex and centrifuge, and pipette 10 μL into each tube with water in it. Two positive controls (high-level positive control (HLC) and low-level positive control (LLC)) and one no template control are included in each batch.

Table 3
The components of reverse transcription master mix

Component	Volume (μL)
10 × RT buffer	2.0
25 × dNTPs (each 100 mM)	0.8
10 × random hexamers	2.0
RNasin (40 U/μL)	0.5
MMLV reverse transcriptase[a]	1.0
dH_2O	3.7
Total volume	10.0

Moloney murine leukemia virus (MMLV) reverse transcriptase should be always stored at −20 °C; put it back immediately after using.

Table 4
PCR primer sequences and product sizes for the preparation of plasmid standards

Amplification region	Primer sequences	Product size (bp)
ABL	5′-CCTTCAGCGGCCAGTAGC-3′(F) 5′-GGACACAGGCCCATGGTAC-3′(R)	316
BCR-ABL (b3a2 type)	5′-AGGGTGCACAGCCGCAACGGC -3′(F) 5′-GGCTTCACTCAGACCCTGAGG -3′(R)	336

4. Add the appropriate volume of RNA into each tube, and mix by pipetting up and down; the mixture is then briefly centrifuged.
5. Next, set the reaction conditions. The reverse transcription conditions are as follows: 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min, then hold at 4 °C. Place the tubes on the thermal cycler, and start the reaction.
6. Briefly centrifuge the reaction tube after completion. cDNA may be stored at 4 °C for 1–2 days, or kept at −20 °C for long-term storage.

3.1.3 Preparation of Plasmid Standards for RQ-PCR

1. Preparation of recombinant plasmids containing b3a2 BCR-ABL transcript and ABL transcript.

   Design a pair of primers that cover the amplification region for RQ-PCR. The primer sequences for ABL and BCR-ABL (P210) are shown in Table 4. Amplify ABL and BCR-ABL (P210) from cDNA of the K562 cell line, which has the b3a2 BCR-ABL transcript. Purify the PCR products individually

from a gel, and insert into the pGEM-T plasmid vector. After the transformation of competent *E. coli* and screening by X-gal and IPTG, culture the colonies carrying recombinant plasmids, and extract the plasmid DNA, identify by sequencing, and linearize by digestion with the Sca1 restriction enzyme.

2. Preparation of plasmid standards (*see* **Note 2**).

   Determine the concentration of the plasmid DNA by measuring the absorbance at 260 nm and 280 nm. Calculate the copy number of the plasmid as follows:

   (a) Assume the concentration of the plasmid DNA is 100 ng/μL (0.1 g/L).

   (b) Plasmid molecular weight = (660, average molecular weight of double stranded DNA) × (vector size + insert size [bp]) = $660 \times (3015 + 316) = 2.199 \times 10^6$ g/mol.

   (c) 1 mol = 6.02336×10^{23} copies.

   (d) Plasmid concentration = 0.1 (g/L) ÷ [2.199×10^6(g/mol)] × 6.02336×10^{23}(copies/mol) = 2.739×10^{16} copies/L = 2.739×10^{10} copies/μL.

   To dilute the plasmid standards, add 10 μL plasmid DNA to 263.9 μL plasmid diluent, mix and centrifuge to obtain a plasmid DNA solution with a plasmid concentration of 1×10^9 copies/μL. Similarly, prepare a tenfold dilution series from 1×10^9 to 1×10^2 copies/μL. The solutions with plasmid concentration of 1×10^8 and 1×10^7 copies/μL are stock solutions for subsequent dilution. The plasmid solutions ranging from 1×10^6 to 1×10^2 copies/μL are used to construct a standard curve.

3.1.4 Preparation of Quality Controls for RQ-PCR

Both HLC and LLC are needed to monitor the quantitative PCR results. Either they are performed as an RNA extraction and the subsequent steps together with a batch of RNA samples, or the cDNAs are performed as a PCR together with a batch of cDNA samples.

Use the fresh cultured K562 cell line which contains b3a2 type of BCR-ABL to prepare the HLC. Use K562 cells diluted by the BCR-ABL (-) NB4 cell line with 1/1000 dilution (cell number) to prepare the LLC. After PBS wash and dilution, pipette approximately 1×10^7 cells into each 1.5 mL Eppendorf tube. Then, add 1 mL TRIzol, thoroughly pipette, and store at −70 °C.

3.1.5 RQ-PCR

1. Dissolve primers and probes, and prepare solutions. Centrifuge Eppendorf tubes that contain primers and probes at 500 × *g* for 1 min. Open the tube cap carefully. Add the appropriate volume of RNase-free water to obtain 150 mM primer and 100 mM probe stock solutions, pipette approximately 20 times

Table 5
The components of RQ-PCR master mix

Component	Volume (μL)
2× TaqMan gene expression master mix	10.0
Forward primer (7.5 mM)	0.8
Reverse primer (7.5 mM)	0.8
TaqMan probe (5 mM)	0.8
dH_2O	5.6
Total volume	18.0

for dissolution, and then briefly centrifuge. Then, add 1 μL stock solution to 19 μL water to make a working solution (final concentration, primer: 7.5 mM, probe: 5 mM).

2. Prepare the real-time PCR master mix for BCR-ABL and ABL, respectively. The components for each reaction are shown in Table 5. Mix the samples by vortex, centrifuge, and pipette as 18 μL/sample into PCR reaction plate wells or tube. Each PCR run should include HLC, LLC, no template control, and plasmids to construct a standard curve with concentrations ranging from 1×10^6 to 1×10^2 copies/μL in addition to samples. Each sample should perform the amplification of both BCR-ABL and ABL in duplicate. In addition, to construct good standard curves, perform plasmids with 1×10^2 copies/μL in duplicate and others as a single reaction.
3. Sample addition. After pipetting the master mix, add 2 μL of cDNA samples or controls to the BCR-ABL and ABL reaction wells individually, and mix by pipetting up and down. Add plasmids as the last step, and use ultraviolet irradiation immediately to avoid contamination by the plasmids. Briefly centrifuge the PCR plate or tube, and place into the 7500 sequence detection system (Applied Biosystems).
4. Set up real-time PCR reaction program. The reaction conditions are as follows: 95 °C for 10 min for 1 cycle; 95 °C for 15 s and 62 °C for 1 min for 40 cycles. Set the signal collection at the final step. Input sample name of each well and the copies of each plasmid standard. Then, save into a new file, and start amplification.
5. After the reaction is complete, remove the reaction plate/tubes to check the liquid volume. If evaporation has occurred in the well/tube, the reaction should be repeated.
6. Analyze the result. Select the "analysis settings" of "manual Ct," and input a threshold of 0.08 (the threshold should be

selected within the exponential amplification range). Select "auto baseline" and click "analyze". First, observe the standard curve. Discard the standard which obviously deviates from other standards, that is, select this deviated standard as "unknown" but not "standard," and reanalyze. An acceptable standard curve should have a slope value between −3.3 and −3.59, a y-intercept between 38.8 and 40.8, and a correlation coefficient higher than 0.997 [19, 21]. Next, observe in the "component" and "amplification plot" tab of each well to check for evaporation (Fig. 2) and nonreal amplification ("creep" curve, Fig. 3). If such phenomena are seen, repeat the corresponding amplification. Export the result as a comma-separated values (CSV) file, and open it in excel format.

7. Calculation and report. Calculate the average number of copies of BCR-ABL and ABL from the duplicate amplification. If the difference is great between the duplicates (>1.0 Ct), repeat the amplification, and take the average of the nearest two values. Calculate the raw BCR-ABL transcript level as BCR-ABL copies/ABL copies in percentage. Calculate the BCR-ABL transcript level on the international scale ($BCR\text{-}ABL^{IS}$) through the raw BCR-ABL transcript level multiplied by a conversion factor (CF) [22]. CF is derived and validated through sample exchange with the international reference laboratory, the Adelaide Institute of Medical and Veterinary Science (IMVS) laboratory in Australia [23, 24]. If $BCR\text{-}ABL^{IS}$ is greater than 10 %, CF is not appropriate, and only the raw BCR-ABL transcript level is reported.

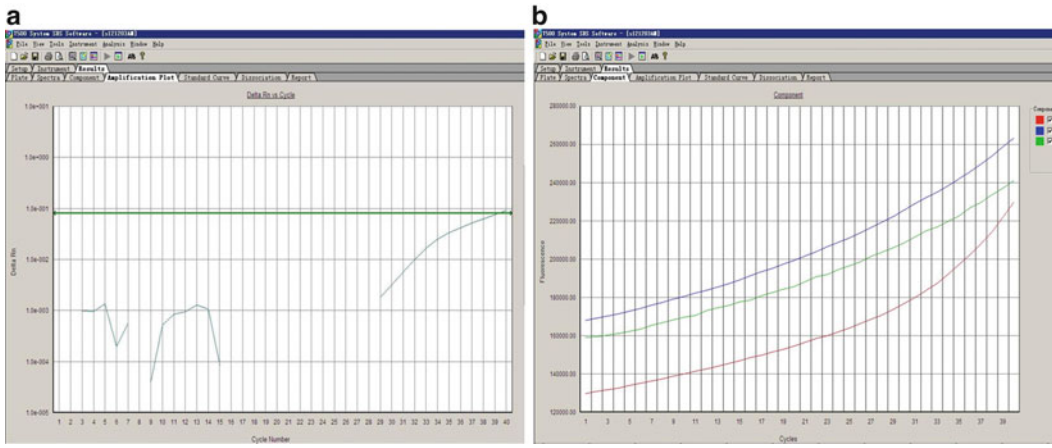

Fig. 2 Typical appearance of evaporation. (**a**) In "Amplification plot" tab, it shows exponential amplification but with a low relative fluorescence value (delta Rn). (**b**) In "Component" tab, it shows that the fluorescence of FAM, ROC, and TAMRA all increase

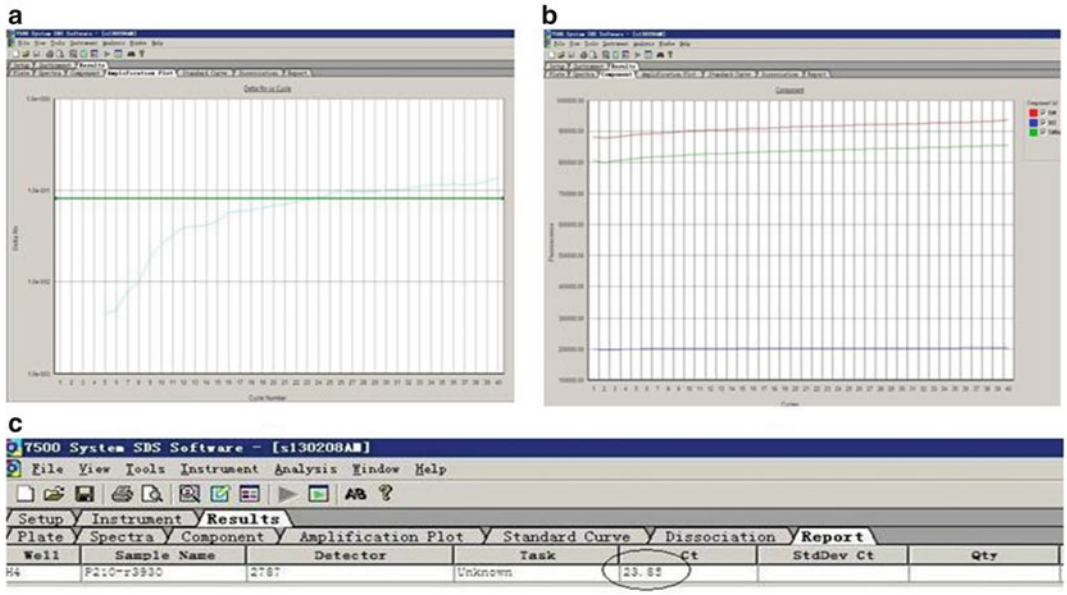

Fig. 3 Typical appearance of nonreal amplification. (**a**) In "Amplification plot" tab, it shows a "creep" curve, which reflects that it is a nonexponential amplification. (**b**) In "Component" tab, it shows no increase in fluorescence for FAM, ROC, and TAMRA. (**c**) In "Report" tab, it shows a Ct value, but it is not a real Ct because of nonexponential amplification

3.2 Detection of BCR-ABL Tyrosine Kinase Domain (TKD) Point Mutations by Sanger Sequencing (see Note 3)

1. RNA extraction and cDNA synthesis are the same as for the detection of BCR-ABL transcript levels. A high sample quality guarantees the successful amplification of BCR-ABL TKD region and accurate results. In general, the ABL copy number should be higher than 32,000.
2. Prepare the first round PCR master mix. The components are shown in Table 6. After pipetting 24 μL into each tube, add 1 μL cDNA of each sample, and mix with the master mix.
3. Set up PCR reaction file. The reaction conditions for the first round PCR are as follows: 95 °C for 5 min, 1 cycle; 95 °C for 40 s, 61 °C for 1 min, 72 °C for 2 min, 30 cycles; 72 °C for 7 min, and hold at 10 °C. Place the reaction tubes on the PCR machine, and start the program.
4. After completion, prepare the second round PCR master mix (shown in Table 6). Add 49 μL per tube, followed by 1 μL of the first PCR product. The reaction conditions for the second round PCR are as follows: 95 °C for 5 min, 1 cycle; 95 °C for 40 s, 62 °C for 1 min, 72 °C for 1.5 min, 30 cycles; 72 °C for 7 min, and hold at 10 °C.
5. Then, separate the second round of PCR products by 2.0 % agarose gel electrophoresis. If the correct size of the band

Table 6
The components of the 1st and 2nd PCR rounds

Components	Volume for the 1st round PCR (μL)	Volume for the 2nd round PCR (μL)	Final concentration
10× Pfu buffer (without Mg^{2+})	2.5	5.0	1×
dNTPs (each 2.5 mM)	2.0	4.0	Each 200 μM
$MgCl_2$ (25 mM)	1.5	3.0	1.5 mM
Forward primer (15 μM)	0.5	1.0	0.3 μM
Reverse primer (15 μM)	0.5	1.0	0.3 μM
Pfu DNA polymerase(5 U/μL)	0.5	1.0	0.1 U/μL
H_2O	16.5	34.0	–
cDNA	1.0	–	–
The 1st round PCR product	–	1.0	
Total volume (μL)	25.0	50.0	–

(863 bp) is clear and strong, perform gel extraction and purification to collect the PCR product.

6. Perform sequencing reactions using both forward and reverse primers on AB 3130XL (Life technology) genetic analyzers.
7. Analyze sequences by software (such as Mutation Surveyor) or via basic local alignment search tool (BLAST) on the following website (http://blast.ncbi.nlm.nih.gov/Blast). mRNA sequence X16416.1 is used as the ABL reference sequence. In addition, observe the whole chromatogram of forward and reverse directions of each sample carefully to avoid missing small mutations. Evaluate the relative proportion of the mutation according to the relative peak area between wild-type and mutant bases.
8. The following information should be included when reporting results (*see* **Note 4** and **5**).
   (a) Whether a mutation is detected.
   (b) The site and name of both wild-type and mutant nucleotides.
   (c) The site and name of both wild-type and mutant amino acids.
   (d) Mutant proportion.

4 Notes

1. DEPC is a suspected carcinogen that has high inhalation toxicity. Wearing a surgical mask while using the reagent and avoiding contact with the skin are recommended.
2. The preparations of plasmid standards must be performed in a different room from PCR set up and sample handling to avoid contamination.
3. BCR-ABL TKD point mutation analysis is not a routine follow-up to the detection of the BCR-ABL transcript levels but is performed when CML patients do not achieve an optimal response, as defined by European LeukemiaNet (ELN) recommendations or NCCN Guidelines [17, 18].
4. If it is difficult to judge whether a mutation occurs in the case of a low mutant proportion or an infrequent mutant, it is suggested to repeat the amplification and sequencing. If the mutant is highly related to TKI resistance, it is suggested to report the mutation accompanied by a note such as "dubious" or "suggest redetection."
5. The chromatogram of the mutant L248V has a specific characteristic (81 nucleotide deletion), which is caused by the formation of new donor splice site after mutation [25]. It may be observed based on the typical chromatogram (Fig. 4) or by clone sequencing.
6. Exon 7 deletion is frequently detected when performing BCR-ABL TKD mutation detection (Fig. 5). Although it was once reported to be related to TKI resistance [26], it is generally considered to have no clinical significance, because it commonly occurs and has poor repeatability [27, 28]. Therefore, it is suggested to not report this finding. Because it disturbs the mutation evaluation of other sites, if exon 7 deletion occurs, repeat amplification, and sequencing or clone sequencing is indicated.

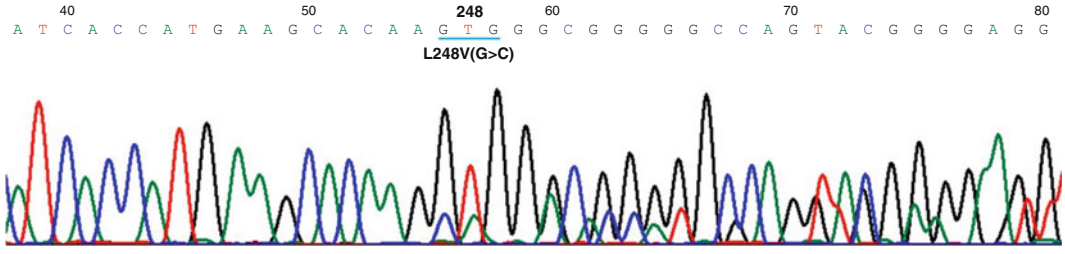

Fig. 4 Chromatogram of L248V mutant

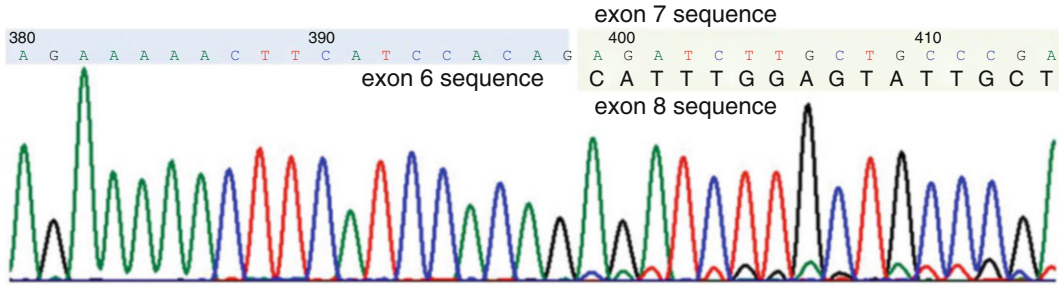

Fig. 5 Chromatogram of ABL with exon 7 deletion

Acknowledgment

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Chapter 2

Induction of Chronic Myeloid Leukemia in Mice

Haojian Zhang and Shaoguang Li

Abstract

Chronic myeloid leukemia (CML) is a myeloproliferative disorder derived from a hematopoietic stem cell (HSC), harboring Philadelphia chromosome (Ph chromosome). Formation of the Ph chromosome is caused by a reciprocal translocation between the chromosomes 9 and 22 t(9;22)(q34;q11), resulting in a fusion protein known as BCR-ABL which has constitutive tyrosine kinase activity and promotes the proliferation of leukemia cells via multiple mechanisms. Studies on CML have led to the identification of the first cancer-associated chromosomal abnormality and the subsequent development of tyrosine kinase inhibitors (TKIs) that inhibit BCR-ABL kinase activity in CML. It has become clear that leukemia stem cells (LSCs) in CML are insensitive to inhibition by TKIs, and eradication of LSCs appears to be difficult. Therefore, some of the major issues in current CML therapy are to understand the biology of LSCs and to investigate why LSCs are insensitive to TKIs for developing curative therapeutic strategies. In this regard, application of mouse models recapitulating human CML disease will be critical. In this chapter, we describe methods for induction of CML in mice with BCR-ABL.

Key words Chronic myeloid leukemia, Philadelphia chromosome, BCR-ABL, Mouse model, Transduction, Transplantation

1 Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disease that originates from a transformed hematopoietic stem cell (HSC), harboring the Philadelphia (Ph) chromosome (Fig. 1) [1–3]. Ph chromosome is generated by a reciprocal translocation between chromosomes 9 and 22 [t (9; 22)(q34; q11)], which results in the formation of the chimeric *BCR-ABL* gene that encodes a constitutively active tyrosine kinase [1, 4]. In general, CML has three distinct clinical stages: an initial indolent chronic phase, accelerated phase, and blast crisis. The chronic phase is normally characterized by accumulation of myeloid cells in the bone marrow, peripheral blood, and other sites in the body without a blockade of myeloid progenitor cell differentiation. The accelerated phase is characterized by an increase in disease burden and the frequency

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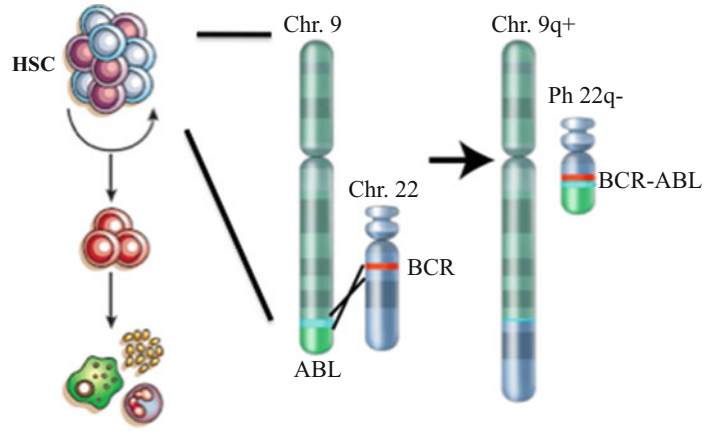

Fig. 1 Philadelphia chromosome and three different forms of the chimeric *BCR-ABL* oncogene. CML originates from an abnormal hematopoietic stem cell harboring Philadelphia chromosome. Due to chromosomal translocation, the *ABL* gene on chromosome 9 fuses the *BCR* gene on chromosome 22 to form the fusion oncogene *BCR-ABL*

of progenitor cells. In blast crisis, the disease displays arrested hematopoietic differentiation and accumulation of immature blast cells resembling acute leukemia. About 70 % of cases develop acute myeloblastic leukemia (AML), and approximately 20–30 % of patients develop lymphoblastic leukemia [1]. CML has an incidence of one or two cases per 100,000 people every year, occurs most commonly in the middle-aged and elderly, and accounts for 15–20 % of all cases of adult leukemia.

To study the biology of CML disease, two major mouse models have been established: BCR-ABL transgenic model and bone marrow transduction/transplantation model.

A few *BCR-ABL* transgenic mice strains have been generated using different regulatory elements to drive BCR-ABL expression [5, 6]. Specifically, the transgenic mice expressing *p210BCR-ABL* driven by the metallothionein (MT) promoter did not develop CML and instead developed T-cell leukemia [5]. Huettner et al. generated an inducible transgenic strain in which *BCR-ABL* expression was controlled by tetracycline transactivator (tTA) through the mouse mammary tumor virus-long terminal repeat (*MMTV-LTR*). Withdrawal of tetracycline administration in this transgenic strain allowed expression of BCR-ABL and resulted in mainly the development of acute B-cell leukemia in 100 % of the mice within a reasonable time frame [6]. These studies demonstrate that the disease phenotype of *BCR-ABL* transgenic mice is dependent on the expression pattern and cell-type specificity of the regulatory elements. It is likely that in the *MT-BCR-ABL* transgenic model, as the tumorigenicity of BCR-ABL chimeric protein is restricted to the hematopoietic tissues in vivo, the MT promoter

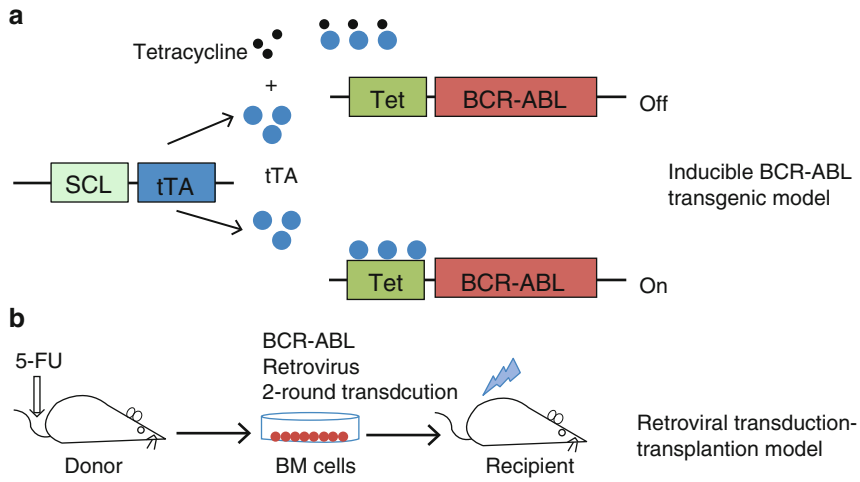

Fig. 2 CML mouse models. (**a**) BCR-ABL transgenic model. The *SCL* 3′ enhancer drives the expression of tetracycline dependent transcription factor (tTA) in hematopoietic stem/progenitor cells. The presence of tetracycline blocks the binding of tTA to Tet promoter, thus shutting off *BCR-ABL* expression. Reversely, tTA binding to Tet promoter in the absence of tetracycline activates transcription of *BCR-ABL* in hematopoietic stem/progenitor cells, leading to the induction of CML disease. (**b**) Retroviral transduction/transplantation model. Bone marrow cells from 5-FU (200 mg/kg) pretreated donor mice are transduced with BCR-ABL retrovirus twice and transplanted into lethally irradiated recipients for induction of CML

only drives *BCR-ABL* expression in the hematopoietic tissues, thymus, and spleen, thus accounting for the T-cell phenotype; in the *MMTV-LTR/tTA-BCR-ABL* inducible model, the MMTV-LTR directs expression of tTA to early B-cell progenitors within murine bone marrow, thus accounting for the B-cell phenotype. Given that CML is a stem cell disorder, it is reasonable that the regulatory elements derived from HSC-specific genes should allow generation of a more accurate model of human CML. The *SCL* (stem cell leukemia) gene encodes a basic helix-loop-helix (bHLH) transcription factor, which mainly expresses in hematopoietic cells, endothelial cells, and embryonic skeleton, and is critical for cell fate determination and differentiation [7–9]. An enhancer fragment within the 3′ region of *SCL* has been identified to restrict expression of the lacZ transgene in adult HSCs, progenitor cells, and megakaryocytes [10, 11]. Therefore, Koschmierder et al. generated the *SCLtTA/TRE-BCR-ABL* transgenic model (Fig. 2a), in which *tTA* was driven by the *SCL* 3′ enhancer. Upon induction of BCR-ABL, mice developed neutrophilia, splenomegaly, and myeloid invasion, mimicking the major features of human CML [12], with biological changes in stem cell and progenitor cell populations [13].

The most widely used CML mouse model is the retroviral transduction/transplantation model (Fig. 2b), which was developed and improved by several groups in the 1990s [14–18]. In this model, BCR-ABL induced myeloproliferative syndrome closely resembling human CML. In this model, 100 % of mice developed

CML-like syndrome in recipient mice after 2–4 weeks after transplantation of BCR-ABL-transduced bone marrow cells with high efficiency and consistency, allowing us to study the molecular mechanisms for CML development and the maintenance of CML stem cells. Below, we mainly focus on this retroviral transduction/transplantation model and describe in detail how to induce CML.

2 Materials

2.1 Mice

1. C57BL/6J mice (Cat: 000664; The Jackson Laboratory, Bar Harbor, Maine, USA), 8–12 weeks old. The gender of donor and recipient mice has to be matched.
2. Gamma irradiator: JL Shepherd 200 Ci Mark I Animal Irradiator.

2.2 Reagents for Generation of Retrovirus Stocks

1. Cell lines: 293T cells (ATCC, Cat# CRL-11268) and NIH3T3 cells (ATCC, Cat# CRL-1658).
2. Culture medium: DMEM, high glucose supplemented with 10 % fetal bovine serum (FBS), 1 % penicillin–streptomycin, 1 % glutamine solution, and 1 % MEM nonessential amino acid solution.
3. Trypsin–EDTA solution (0.05 %).
4. Calcium chloride ($CaCl_2$) solution: Prepare 2 M solution in sterile distilled water, then filter in hood, and store at room temperature.
5. 2× HBS solution: Weigh out 8 g NaCl, 0.37 g KCl, 106.5 mg Na_2HPO_4, 1 g dextrose, 5 g HEPES, add sterile distilled water to 500 mL, and adjust pH to 7.05 with 10 N sodium hydroxide (*see* **Note 1**).
6. Polybrene (Hexadimethrine bromide) (Sigma, Cat# H9268): dissolve in sterile distilled H_2O at 8 mg/mL, and store at −20 °C.
7. Filter: Millex-HP syringe filter unit (0.45 μm).

2.3 Bone Marrow Cell Collection, Culture, and Transduction Medium

1. Micro-dissecting scissor (Roboz, Cat# RS-5925) and micro-dissecting forceps (Roboz, Cat# RS-5190). 10 mL syringe with BD Luer-Lok® Tip and 27 G ½ PrecisionGlide needle.
2. DMEM, high glucose supplemented with 10 % fetal bovine serum, 1 % penicillin–streptomycin, 1 % glutamine solution, and 1 % MEM nonessential amino acid solution.
3. 5-Fluorouracil (Sigma, F6627): 10 mg/mL in sterile phosphate-buffered saline (PBS).
4. Hank's balanced salt solution (HBSS).

5. Red blood cell (RBC) lysis buffer: 150 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA (pH 7.4).
6. Bone marrow cells stimulation medium: 77 % DMEM (v/v), 15 % heat-inactivated fetal bovine serum, 5 % WEHI-3B conditioned medium, 1 % penicillin–streptomycin, 1.0 mg/mL ciprofloxacin, 200 mM L-glutamine, 6 ng/mL recombinant murine IL-3 (Peprotech, Cat# 213-13), 10 ng/mL recombinant murine IL-6 (Peprotech, Cat# 216-16), and 50 ng/mL recombinant murine stem cell factor (SCF; Peprotech, Cat# 250-03). The total volume is 10 mL for each sample.
7. Bone marrow cell transduction medium: 50 % retrovirus supernatant, 27 % DMEM, 15 % (v/v) heat-inactivated FBS, 5 % (v/v) WEHI-3B conditioned medium, penicillin–streptomycin, 1.0 μg/mL ciprofloxacin, 200 mM L-glutamine, 6 ng/mL recombinant murine IL-3 (Peprotech, Cat# 213-13), 10 ng/mL recombinant murine IL-6 (Peprotech, Cat# 216-16), 50–100 ng/mL recombinant murine stem cell factor (SCF; Peprotech, Cat# 250-03), 1 % (v/v) HEPES, and 2 ng/mL polybrene. The total volume is 4 mL for each sample.
8. Antibody: allophycocyanin (APC)-labeled anti-mouse Ly-6G (Gr-1), clone: RB6-8C5 (eBioscience, 17-5931-82).
9. Flow cytometry buffer: PBS supplemented with 1 % bovine serum albumin (BSA).

3 Methods

3.1 Generation of MSCV-BCR-ABL-GFP Retrovirus Supernatant

1. The 293T cells are passaged to 6 cm dish at 3–4 × 10^6 cells/dish 24 h before transfection (*see* **Note 2**).
2. Change 4 mL fresh 293T medium to each dish before transfection.
3. Prepare transfection cocktail per 6-cm dish: In a 15 mL tube, add 10 μg MIG-BCR-ABL-GFP plasmid, 5 μg EcoPack plasmid, 62 μL 2 M $CaCl_2$, and sterile water to 500 μL total volume. Briefly vortex.
4. Add 500 μL 2× HBS to the tube drop by drop, and mix by vortexing for 10 s (*see* **Note 3**).
5. Add DNA/HBS solution onto 293T cells drop by drop, and gently rock the dishes forward and backward a few times to achieve even distribution of DNA/$Ca_3(PO_4)_2$ particles, and then continue to culture.
6. Change medium: At 24 h post-transfection, remove the old medium, and carefully add 4 mL fresh 293T medium (*see* **Note 3**).

7. Collecting virus supernatant: At 48 h post-transfection, collect the supernatant by 10 mL BD syringe, and filter the supernatant through 0.45 μm syringe filter. Aliquot virus supernatant in 4 mL/tube, and store at −80 °C (*see* **Note 4**).

3.2 Testing of Viral Titer by Flow Cytometry

1. The NIH3T3 cells are passaged into 10 cm dish at 6×10^5 cells 24 h before infection.
2. At the day of infection, dilute virus supernatant with fresh culture medium at the ratio of 1:2, 1:8, and 1:16. Polybrene is added to the retroviral supernatant at the final concentration of 8 μg/mL.
3. Remove the NIH3T3 cell medium, and add premixed virus supernatant for infection. After 3 h culture, remove the virus supernatant, and change to 10 mL fresh medium.
4. After 48 h postinfection, digest cells with trypsin, and collect cells into 1 mL flow cytometry buffer. Take 300 μL cell suspension to run flow cytometry analysis for percentage of GFP^+ cells. Normally, the good retroviral supernatant means the % GFP can reach to 90–95 % at the 1:2 dilution, 75–85 % at 1:8 dilution, and 60–70 % at 1:16 dilution.

3.3 Bone Marrow Cell Transduction and Transplantation

3.3.1 Donor Mice Priming

1. Prepare 10 mg/mL 5-FU solution: dissolve 5-FU powder in PBS, incubate in 37 °C water bath for 10–30 min, and vortex the solution to help dissolve the powder (*see* **Note 5**).
2. Filter 5-FU solution before injection.
3. Inject 5-FU to donor mice via intravenous injection at 200 mg/kg (for example, 0.6 mL for 30 g mouse).

3.3.2 Bone Marrow Cell Collection

1. Sacrifice the primed donor mice with CO_2 at Day 4 after priming.
2. Sterilize the skin of the mice with 70 % ethanol, collect femurs and tibias, and place them in cold PBS.
3. Flush out bone marrow cells with 293T medium, and blow the cells with 10 mL pipette up–down to suspend cells (normally, $2–3 \times 10^6$ total bone marrow cells per donor mouse).
4. Spin down cells at $500 \times g$ for 10 min, and resuspend the cell pellet with bone marrow cells stimulation medium. Incubate cells at 37 °C for 24 h.

3.3.3 Bone Marrow Cell Transduction

1. Collect the cells, and spin down at $500 \times g$ for 10 min at room temperature.
2. During the same time, prepare bone marrow cell transduction medium.
3. Resuspend cells with 4 mL transduction medium, and then transfer the cells to a 6-well plate.

4. Spin the cells in the 6-well plate at 1,200 × *g* at room temperature for 90 min.
5. Incubate the cells at 37 °C, 5 % CO_2 for 3–4 h, and then change medium with fresh bone marrow cells stimulation medium, then incubate the cells at 37 °C overnight.
6. Repeat transduction by carefully replacing 2 mL of supernatant from each well with 2 mL of fresh transduction medium.
7. Spin the cells at 1,200 × *g* for 90 min, and incubate the cells at 37 °C, 5 % CO_2 for 3 h.
8. Collect the cells, and spin at 500 × *g* for 10 min, and resuspend the cell with a certain volume of HBSS solution. Now the cells are ready for transplantation.

3.3.4 Bone Marrow Cell Transplantation

1. Prepare recipient mice: Gender-matched, the strain mice are irradiated by two doses of 550-cGy gamma (C57BL/6J) separated by 3 h.
2. Adjust the cell concentration to 1.25 × 10^6 cells/mL in HBSS solution, then inject 5 × 10^5 cells (0.4 mL) to each mouse via intravenous injection (*see* **Note 6**).

3.4 Evaluation of CML Development by Counting White Blood Cell and Checking GFP^+ Myeloid Cells via Flow Cytometry

After transplantation, recipient mice are monitored daily for signs of morbidity, weight loss, and failure to thrive. Basically, we determine the development of CML by the following strategies (Fig. 3):

1. Retro-orbital bleed mice to collect about 100 μL peripheral blood.
2. Add 2 μL peripheral blood to 18 μL RBC lysis buffer, and mix well before keeping on ice for 10 min for lysing red blood cells.
3. Add 20 μL trypan blue, mix well, and drop 10 μL cells under the cover slip on a hemacytometer, and count the live cells using a microscope (dead cells will take up trypan blue, so they are darker than the live cells).

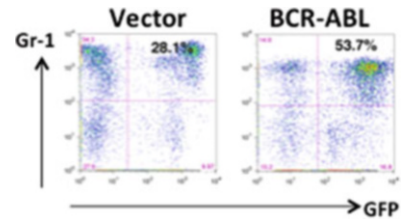

Fig. 3 Flow cytometry analysis showing the percentage (indicated in the quadrant) of GFP+Gr-1+ cells in the peripheral blood. Peripheral blood samples of recipient mice that received either vector-GFP or BCR-ABL-GFP retrovirus transduced bone marrow cells are harvested at day 14 post bone marrow transplantation, and stained with Gr-1 antibody. The percentage of retrovirus transduced granulocyte cells (GFP+Gr-1+) was significantly higher in the BCR-ABL group than that in the control mice

4. Add 1 mL of RBC buffer to the left peripheral blood, mix well, and lyse the red blood cell on ice 10 min.
5. Spin cells at 500 × *g* at 4 °C for 5 min.
6. Remove the supernatant, and resuspend the cell pellet in 1 mL of PBS to wash the cells.
7. Spin cells at 500 × *g* for 5 min again, and remove the supernatant.
8. Resuspend the cell pellet in fluorescence-activated cell sorting (FACS) buffer, and adjust cell concentration to 1×10^7 cells/mL.
9. Stain cells with fluorescence-labeled myeloid cell marker (Gr-1): add phycoerythrin (PE) or allophycocyanin (APC)-labeled Gr-1 antibody to 50 μL of cells. Mix well, and stain the cells at 4 °C for 15–30 min.
10. Add 1 mL of PBS buffer to the cells, and wash cells once by spinning at 500 × *g*, 4 °C for 5 min.
11. Resuspend the cell pellet in 300 μL of flow cytometry buffer. Now the samples are ready for running flow cytometry.

4 Notes

1. The use of high-titer BCR-ABL retrovirus is critical for successfully inducing CML in this mouse model, for which much attention should be paid to making 2× HBS with its pH adjusted exactly to 7.05.
2. The condition of 293T cells is also important for generating higher-titer virus. In addition, the confluence of the cells should reach ~90 %.
3. Virus-producing 293T cells are easy to detach from culture dish, medium should be changed carefully by adding it to the culture dish very slowly.
4. To avoid repeated freeze–thaw cycle, we recommend to aliquot virus supernatant after collection.
5. When making 5-FU solution for priming recipient mice, incubation in a 37 °C water bath followed by vortexing is recommended to help to fully dissolve 5-FU.
6. Because the number of cells transplanted per recipient affects the latency of CML development, counting cells accurately will be critical when comparing survival of CML mice between groups.

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Chapter 3

CML Mouse Model Generated from Leukemia Stem Cells

Yiguo Hu

Abstract

Chronic myeloid leukemia (CML) is a myeloproliferative disorder with a high number of well-differentiated neutrophils in peripheral blood and myeloid cells in bone marrow (BM). CML is derived from the hematopoietic stem cells (HSCs) with the Philadelphia chromosome (Ph^+, t(9;22)-(q34;q11)), resulting in generating a fusion oncogene, *BCR/ABL1*. HSCs with Ph^+ are defined as leukemia stem cells (LSCs), a subpopulation cell at the apex of hierarchies in leukemia cells and responsible for the disease continuous propagation. Several kinds of CML models have been developed to reveal the mechanism of CML pathogenesis and evaluate therapeutic drugs in the past three decades. Here, we describe the procedures to generate a CML mouse model by introducing *BCR/ABL1* into $Lin^-Sca1^+cKit^+$ population cells purified from mouse bone marrow. In CML retroviral transduction/transplantation mouse models, this modified model can mimic CML pathogenesis on high fidelity.

Key words Chronic myeloid leukemia (CML), Leukemia stem cells (LSCs), Mouse model, *BCR/ABL1*, Retroviral transduction/transplantation

1 Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by increased proliferation of mature and no function myeloid cells in peripheral blood, bone marrow (BM), and other immune organs. Most cases (~90 %) of CML are derived by a fusion oncogene, *BCR/ABL1*, which resulted from a reciprocal translocation between the chromosomes 9 and 22, t(9;22), also called Philadelphia chromosome (ph^+) [1]. In CML patients, the Ph chromosome is generated in a HSC for it can be clonally detected both in myeloid and lymphoid cells [2]. After clonal expansion, it forms a subpopulation of cells and causes CML. These cells which are responsible for the disease continuous propagation and relapse are defined as leukemia stem cell (LSC) [3]. Previous studies have demonstrated that the mechanism of LSC survival is different to its generation population [4]. These components include intracellular signaling pathways and extrinsic microenvironments [4]. It has

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become apparent that it is essential to eradicate LSCs for CML cure [5]. To achieve this goal, the first step is to characterize LSC and design the strategies to target LSC. In human, both of LSCs and normal HSCs reside in a rare cell population among $CD34^+CD38^-$cells [6, 7]. LSCs and normal HSCs can be distinguished with CD26 biomarker [8]. In a murine model of *BCR/ABL1*-induced CML-like disease, $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ (LSK) cells have been demonstrated to function as LSCs [5].

In the past 30 years, several *BCR/ABL1*-induced CML-like mouse models were developed for CML studies [9, 10]. Among these models, the retroviral transduction/transplantation model is currently the most widely used model for its advantages:

1. It is a more faithful model of *BCR-ABL1*-induced CML.
2. All recipients can develop CML of a short and similar latency. It is suitable for drug validation or rare material collection.
3. Most inbred mouse strains could be used for inducing CML.
4. Specific knockout genes are easily applied for CML pathogenesis studies.

In a traditional *BCR/ABL1*-induced CML mouse model, 5-FU-primed mouse BM cells are cocultured with retrovirus containing *BCR/ABL1* gene, and infected BM cells are transplanted into lethally irradiated syngeneic recipient mice. In this model, although HSCs are significantly enriched in donor mice post-5-FU primed, still, some disease cells initiate from hematopoietic progenitors, such as common myeloid progenitors (CMP)s. For this reason, this model cannot reveal a gene role in CML LSCs in the primary transplantation if the gene does not strongly affect LSC function. In this condition, a series of disease BM cell transplantations need to be performed. Secondary, this is not a real stem cell disease model in first transplantation.

To overcome these deficiencies, we modified model to make a LSC initiation CML model (the processes are briefly shown in Fig. 1). The modified model can mimic CML pathogenesis on high fidelity, and more biological characterizations of CML LSCs can be studied in this model. In conclusion, LSC initiation CML mouse model provides a powerful tool for studying CML LSCs and investigating potential drugs for targeting LSCs.

2 Materials

2.1 Equipment

Gamma irradiator: Gamma cell 40 (Nordion International, Kanata, Ontario, Canada)

Centrifuge: Eppendorf 5810R

BD FACSAria™ III sorter

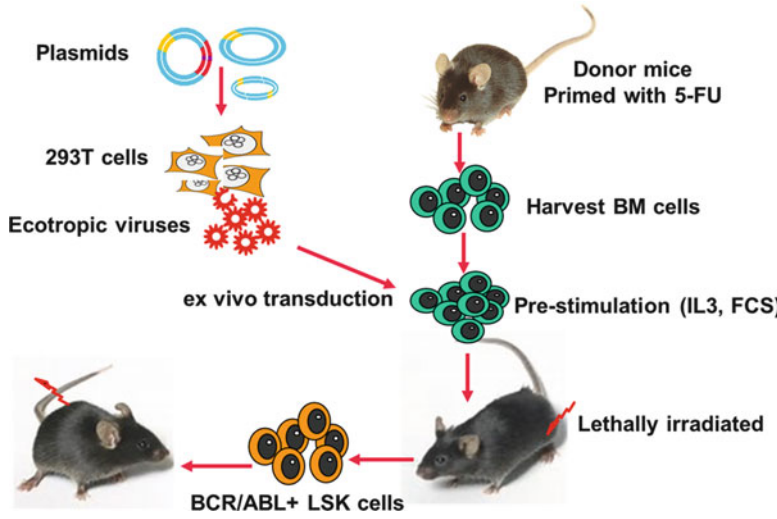

Fig. 1 The workflow for developing CML LSC mouse model. Retrovirus containing *BCR/ABL1* is produced with 293T cells. Donor mice are premised with 5-FU, and collected bone marrow cells are stimulated with cytokines in vitro. BM cells infected twice with retrovirus are transplanted into lethally irradiated syngeneic recipients for induction of CML. BM cells are collected from primary CML recipients at day 12 post BMT, and LSK cells are sorted and re-transplanted into lethally irradiated syngeneic recipients

BD FACSCanto™ II
Scissors: microdissection scissor
Forceps: microdissection forceps
Needle: precision glide needle 27G1/2
Syringe: 10 ml BD Luer-Lok™ tip syringe
Syringe filters: hydrophilic PVDF membrane, 0.45 μm
15 ml centrifuge tube
T75 cell culture flask

2.2 Medium and Reagents

OPTI-MEM
Lipofectamine 2000
DMEM with high glucose, L-glutamine, and phenol red
Fetal bovine serum
New born calf serum
100× penicillin/streptomycin solution
100× L-glutamine solution
Polybrene (hexadimethrine bromide): 1 mg/ml stock solution in H_2O and keep at −20 °C
1× trypsin–EDTA solution (0.025 %)

2.3 Cell Lines

293T cells

NIH3T3 cells

2.4 Mice

C57BL/6J or BALB/cJ mice (The Jackson Laboratory, Bar Harbor, Maine, USA), age at 6–10 weeks

3 Methods

3.1 Generation of Retroviral Stocks

1. Plated ~7.5 × 10^6 293T cells in 75 cm^2 flask with 15 ml 293T medium (DMEM medium with 10 % BCS, 1 % penicillin/streptomycin, 1 % L-glutamine) the day before.
2. Next day cells ~85 % confluent.
3. Aspirate medium from each flask.
4. Feed each flask with 10 ml of fresh 293T medium.
5. Add 1 ml OPTI-MEM medium and 50 μl of Lipofectamine 2000 into a 15 ml conical tube.
6. Vortex the tube and sit for 5 min at room temperature.
7. Label another tube and add 1 ml of OPTI-MEM, 8 μg of pCL-ecotropic plasmid, and 16 μg appropriate viral plasmid into it; vortex the tube.
8. Smartly add the first tube liquid (OPTI-MEM + Lipofectamine 2000) into the second tube (OPTI-MEM pCL-ecotropic + viral plasmid), vortex second tube at same time, and then add the first tube liquid.
9. Sit the tube for 20 min at room temperature.
10. Add the appropriate tube with 2 ml mixture to its correspondingly labeled 75 cm^2 flask.
11. Place in 37 °C incubator.
12. After 16 h, change medium and replace with 15 ml fresh 293T medium.

    ***Note**: Remember to wear proper personal protective equipment. Some active virus might be present after 16 h.*
13. After another 24 h, collect supernatant from the flask and filter with 0.45 μm filter.
14. Aliquot the virus and freeze at −80 °C freezer.

3.2 Defining Viral Titer with Flow Cytometry

1. Culture NIH3T3 cells with NIH3T3 medium (DMEM medium with 10 % BCS, 1 % penicillin/streptomycin, 1 % L-glutamine) in 10 cm dish.
2. When cells reach 90 % confluence, remove the medium and wash cells once with 10 ml 1× PBS. Remove PBS, add 1 ml pre-warmed 0.5 % trypsin–EDTA solution to cover all the

bottom, and incubate the dish at 37 °C for 5 min to release cells, then add 9 ml NIH3T3 medium to stop the enzymatic reaction. Collect cells carefully in 15 ml centrifuge tube and spin at 300 × *g* for 10 min at room temperature.

3. The NIH3T3 cells are passaged to 10 cm dish at 0.6×10^5 cells/dish at the day before infection.
4. At the day of infection, serially dilute virus supernatant with 293T medium at 1:2, 1:8, and 1:16. Final volume of each dilution is 5 ml with 10 μg polybrene and 10 mM HEPES (pH = 7.4).
5. Remove NIH3T3 cell culture medium and add infection medium.
6. After 3 h infection in cell culture incubator, change infection medium with 10 ml fresh NIH3T3 medium.
7. After another 48 h culture, collect cells and resuspend cells with 1× PBS; aliquot ~1×10^5 cells into FACS tubes for flow cytometry analysis. Virus titer is determined with the percentage of GFP-expressing cells. If GFP% reaches to 90–95 % at 1:2 dilution, 75–85 % at 1:8 dilution, and 60–70 % at 1:16 dilution, the virus titer is high enough for BM cell transduction.

3.3 Inducing Primary CML Mouse Model

3.3.1 Priming Donor Mice with 5-FU (4 Days Before BM Cell Collection)

1. Dissolving 5-FU in PBS at 37 °C at 10 mg/ml in dark.
2. Injecting 5-FU into donor mice via tail vein at dose of 200 mg/kg (0.2 ml per 10 g weight).

***Note**: 5-FU will remove S phage pre-lymphocyte and circular lymphocyte to increase the percentage of HSCs in BM.*

3.3.2 Collecting BM Cells from Primed Donor Mice

1. Sacrifice primed donor mice with CO_2 and sterilize them with 70 % of ethanol.
2. Take the femurs and tibias in cold 293T culture medium (DMEM with 10 % FCS, 1 % penicillin/streptomycin, and 1 % glutamine).
3. Elute BM cells from the femurs and tibias with 293T medium by 10 ml syringe with 27G1/2 needle into 50 ml tubes.
4. Suspend cells with 10 ml pipette up-down to dissociate cells, then filter cells with 100 μm cell filter.
5. Determine total cell number: take 5 μl suspended cells into 20 μl RBC lysis buffer in 0.5 ml Eppendorf tube, put tube on ice for no less than 10 min, add 25 μl 1 % trypan blue, count cells with microscope, and calculate total cell number.
6. Spin down cells at 300 × *g* for 10 min.

7. Loose cell pellet by flicking the tube, and resuspend cells with 10 ml stimulation medium and transfer them into 10 cm cell culture dish.

   ***Note**: less than 3×10^7 cells/well in 10 ml pre-stimulating solution.*

   10 ml stimulation: DMEM 7.7 ml, FBS 1.5 ml, HEPES 10 mM (pH = 7.4), WEHI-3B condition medium 0.5 ml, 100× penicillin/strep, 100 μl, 100× L-glutamine 100 μl, Ciproxin 10 μg, IL-3 10 μg, Il-6 10 μg, and SCF 10 μg.
8. Incubating cells in 10 ml dishes at 37 °C, 5 % CO_2 overnight.

3.3.3 First Round of Viral Transfection

1. Making transfection solution – **4 ml transfection medium**: BCR/ABL1 virus 2 ml, DMEM 1.33 ml, polybrene 2.5 μg, FBS 0.33 ml, 40 μl HEPES (1 M, pH = 7.4), WEHI-3B condition medium 0.2 ml, 100× penicillin/strep, 40 μl, 100× L-glutamine 40 μl, Ciproxin 4 μg, IL-3 4 μg, Il-6 4 μg, and SCF 4 μg.
2. Collect the cells and determine total cell number.
3. Spin down cells at 300 × *g* for 10 min.
4. Aspirate the supernatant and gently flicking tube to make pellet loose.
5. Add 4 ml infection solution in the tube to resuspend cells and transfer them to 6-well plate.
6. Low-speed centrifuge cells and virus at 37 °C, 1000 × *g* for 90 min.
7. Incubate cells in incubator at 37 °C, 5 % CO_2 for 3 h.

   ***Note**: If the cells accumulate in the corner, gently shake or blow the clot to make it loose.*
8. After 3 h incubation, gently remove supernatant with pipette to new tubes and centrifuge at 300 × *g* for 10 min to return the removed cells; continually culture the cells with 4 ml fresh stimulation medium overnight.

   4 ml stimulation medium: DMEM 3.13 ml, FBS 0.6 ml, 40 μl HEPES (1 M, pH = 7.4), WEHI-3B condition medium 0.2 ml, 100× penicillin/strep, 40 μl, 100× L-glutamine 40 μl, Ciproxin 4 μg, IL-3 4 μg, Il-6 4 μg, and SCF 4 μg.

   ***Note**: You could make the culture medium half hour before changing medium.*

3.3.4 Second Round of Viral Transfection

1. Carefully remove 2 ml supernatant from each well, then add 2 ml virus with 2.5 μg polybrene and 40 μl HEPES (1 M, pH = 7.4).
2. Centrifuge cells and virus at 37 °C, 1000 × *g* for 90 min.
3. Incubate cell at 37 °C, 5 % CO_2 for another 3 h.

4. Collect the cells into tube, wash the well with 2 ml 1× PBS once.
5. Centrifuge cells at 300 × *g* for 10 min.
6. Resuspend the pellet with 5 ml Hank's solution and determine cell number.
7. Centrifuge cell at 300 × *g* for 10 min.
8. Adjust cell concentration at 2.5×10^6 cells/ml.
9. Inject 0.2 ml cells into syngeneic recipient mouse via tail vein with 27G1/2 syringe.

3.3.5 Recipient Irradiation

Recipient mice are placed in an acrylic container (28 cm diameter, 8 cm depth) in a continuous airflow between two opposing ^{137}Cs γ-ray source. Recipient mice are lethally irradiated with two doses of 550 cGy for C57BL/6J mice or 450 cGy for Balb/cJ mice with a 3 h interval.

3.3.6 Flow Cytometric Analyses

The recipient mice are eye-bled at 12 days post BMT and mononuclear cells collected. The cells were stained for anti-mouse GR-1 antibody conjugated with phycoerythrin (**PE**). The stained cells were analyzed by a FACScan (Becton Dickinson, Mountain View, CA, USA).

3.4 Develop LSC CML Mouse Model

3.4.1 CML Mouse BM Cell Collection

1. Sacrifice primary CML mice with CO_2 and sterilize them with 70 % of ethanol.
2. Take the femurs and tibias in cold 293T culture medium.
3. Elute BM cells from the femurs and tibias with 293T medium by 10 ml syringe with 27G1/2 needle into 50 ml tubes.
4. Suspend cells with 10 ml pipette up-down to dissociate cells, and filter cells with 100 μm cell filter.
5. Determine total cell number: take 5 μl suspended cells into 20 μl RBC lysis buffer in 0.5 ml Eppendorf tube, put tube on ice for no less than 10 min, add 25 μl 1 % trypan blue, count cells with microscope, and calculate total cell number.
6. Spin down cells at 300 × *g* for 10 min.
7. Loose cell pellet by flicking the tube, and resuspend cells with RBC lysis buffer at 5×10^6 cells/ml, and put on ice for 10 min.
8. Spin down cells at 300 × *g* for 10 min.
9. Wash cells with binding solution (PBS with 0.5 % FBS and 2 mM EDTA).
10. Determine cell numbers.

3.4.2 CML LSC Purification

1. Resuspend cell at 2 × 10^7 cells/ml with binding buffer, and add 40 μl biotin-labeled lineage antibody cocktail containing a mixture of antibodies against CD3, CD4, CD8, B220, Gr-1, Mac-1, and Ter119.
2. Mixing well and incubating for 15 min in dark at 4 °C, flick tube every 5 min or rotate the tube with hand.
3. Wash cells to remove unbound primary antibody by adding 1–2 ml buffer per 10^7 cells and centrifuge at 300 × *g* for 10 min.
4. Repeat washing step.
5. Aspirate supernatant completely and resuspend cell pellet in 1 ml buffer per 2 × 10^7 total cells.
6. Add 20 μl APC-Cy7-conjugated streptavidin to recognize biotin, 20 μl PE-conjugated antibody to c-Kit (clone ACK2), and 20 μl APC-conjugated antibody to Sca-1 (clone D7) per 10^7 cells.
7. Mixing well and incubating for 20 min in dark at 4 °C, flick tube every 5 min or rotate the tube with hand.
8. Wash cells to remove unbound primary antibody by adding 1–2 ml buffer per 10^7 cells, and centrifuge at 300 × *g* for 10 min twice.
9. Resuspend cell at 1 × 10^7 cells/ml with binding buffer and sort cells with BD FACSAria™ III sorter and collect $GFP^+Lin^-Sca1^+c\text{-}Kit^+$ LSCs (Fig. 2).

3.4.3 Recipient Irradiation: The Same as Above

Recipient mice are placed in an acrylic container (28 cm diameter, 8 cm depth) in a continuous airflow between two opposing 137Cs γ-ray source. Recipient mice are lethally irradiated with two doses of 550 cGy for C57BL/6J mice or 450 cGy for Balb/cJ mice with a 3 h interval.

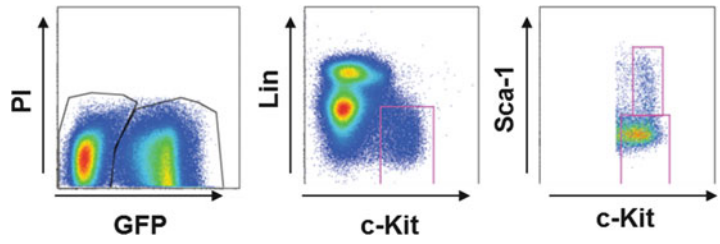

Fig. 2 LSK sorting. BM cells from primary CML recipients are staining with antibodies to linage markers, Sca-1 and c-Kit. $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ cells are collected

3.4.4 CML LSC Injection

1. Base on sorted cell number, adjust LSC cell concentration at 2.5×10^4 cells/ml with normal BM cell at 2.5×10^6 cells/ml for recipient rescue.
2. Inject 0.2 ml cells into syngeneic recipient mouse via tail vein with 27G1/2 syringe.

3.4.5 Flow Cytometric Analyses

The recipient mice are eye-bled at 14 days post BMT and mononuclear cells collected. Cells are stained with anti-mouse Gr-1 antibody conjugated with phycoerythrin (**PE**) and analyzed with BD FACSCanto™ II for disease monitor. If high WBC and percentage GFP + Gr-1^+ cells are detected in peripheral blood, this indicates that the CML mouse model initiated from LSC is successfully developed.

3.4.6 Histological Examination

Various tissues of the recipient mice, such as spleen, lung, bone, and liver, can be fixed with 10 % formalin and sectioned. The sections are stained with hematoxylin/eosin (HE).

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Chapter 4

Immunological Analyses of Leukemia Stem Cells

Kazuhito Naka and Yoshihiro Takihara

Abstract

Traditionally, the intracellular localization and expression levels of specific proteins in CML Leukemia stem cells (LSCs) have been evaluated by fluorescence immunohistochemistry (FIHC). More recently, Duolink® in situ PLA technology has opened up a new and more quantitative way to evaluate signal transduction, posttranslational modification, and protein-protein interaction at the single-stem-cell level. This novel methodology, which employs two antibody-based probes, has already increased our understanding of the biology of the rare CML LSC population. In the future, the use of this approach may contribute to the development of novel therapeutics aimed at eradicating CML LSCs in CML patients.

Key words Fluorescence immunohistochemistry (FIHC), Duolink® in situ PLA, Single CML stem cell, Posttranslational modification, Protein-protein interaction

1 Introduction

CML leukemia stem cells (LSCs) are the cells of origin of most mature CML cells [1–5]. Although the development of tyrosine kinase inhibitors (TKIs) has significantly improved the prognoses of CML patients, several studies have demonstrated that a complete cure is not possible by the use of TKI therapy alone [6–12]. It is now generally accepted that CML LSCs are responsible for the relapse of CML disease post-TKI therapy [1–5, 13, 14]. Because TKIs affect only actively proliferating CML cells, dormant CML LSCs escape TKI-mediated killing. After TKI therapy is discontinued, CML LSCs can emerge from quiescence and give rise to large numbers of differentiated CML cells. Thus, to eliminate CML relapses, the molecular mechanism(s) used by quiescent CML LSCs to preserve their self-renewal capacity even in the presence of TKIs must be targeted. Recent technical advances have made it possible to identify molecules and pathways of biological significance in the rare CML LSC population. In this chapter, we outline two immunological techniques, one traditional and one novel, for dissecting molecular mechanisms in CML LSCs. Fluorescence

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immunohistochemistry (FIHC) allows the intracellular localization and expression of a given protein to be determined in the CML LSC population, while Duolink® in situ PLA technology permits more precise examinations of signal transduction, posttranslational modification, and protein-protein interaction at the level of the single CML LSC.

2 Materials

For the work described below, we used CML LSCs or CML-multipotent progenitors (MPPs) that were isolated from a *BCR-ABL1* transduction/transplantation-based CML mouse model [15], or from *Scl/Tal1-tTA* and *TRE-BCR-ABL1* double transgenic (tg) mice [16–20] (*see* Subheadings 2.1 and 2.2 and **Note 1**).

2.1 Transduction-/Transplantation-Based CML Mouse Model

Our *BCR-ABL1* transduction-/transplantation-based CML mouse model was established by infection of mouse HSCs with retrovirus carrying the human *BCR-ABL1* oncogene tagged with GFP [15]. Briefly, retroviral packaging cells were transiently transfected with MSCV-*BCR-ABL1* plasmid using FuGene6 (Roche) [15]. HSCs transduced with retrovirus were transplanted into irradiated (9.5 Gy) congenic recipient mice [15].

2.2 Scl/Tal1-tTA x TRE-BCR-ABL1 Double Transgenic (tg)-CML Mouse Model

1. Doxycycline (Dox; Sigma-Aldrich) (*see* **Note 2**).
2. *Scl/Tal1-tTA* (JAX mice: 6209) [17] http://jaxmice.jax.org/strain/006209.html.
3. *TRE-BCR-ABL1* (JAX mice: 6202) [16] http://jaxmice.jax.org/strain/006202.html.
4. Interbreed *Scl/Tal1-tTA* and *TRE-BCR-ABL1* tg mice to generate *Scl/Tal1-tTA* × *TRE-BCR-ABL1* double tg progeny.
5. Maintain these animals in cages supplied with drinking water containing 20 μg/ml Dox (*see* **Note 3**).
6. At 5 weeks after birth, induce expression of the *BCR-ABL1* oncogene by replacing the Dox-containing drinking water with normal drinking water.

2.3 FIHC

1. Serum-free S-Clone SF-03 medium (Sanko Junyaku) supplemented with penicillin/streptomycin (Gibco) and 0.1 % bovine serum albumin (BSA) (Stem Cell Technology #09300).
2. Poly-L-lysine (Sigma-Aldrich, P4832).
3. 4 % paraformaldehyde (PFA; Sigma-Aldrich, P6148) in PBS or TBS.
   Add 2 g PFA to 45 ml water, add 10 μl 10 N NaOH, and warm to 40–50 °C.

After the PFA dissolves, add 5 ml 10× PBS or 10× TBS.
Pass through a 0.45 μm PVDF filter (Millipore).

4. 0.25 % Triton-X100 (Sigma-Aldrich) in PBS or TBS.
5. 2 % BSA in PBS or TBS.
   Add 1 g BSA (Sigma-Aldrich) to 45 ml water and rotate.
   After BSA dissolves, add 5 ml 10× PBS or 10× TBS.
   Pass through a 0.45 μm PVDF filter (Millipore).

2.4 Duolink® In Situ PLA analysis (See Note 4)

1. 4 % PFA in PBS or TBS (see above).
2. 0.25 % Triton-X100 in PBS or TBS.
3. 2 % BSA in PBS or TBS.
4. Duolink® in situ Detection Reagents (Sigma-Aldrich).
5. Duolink® in situ PLA probe PLUS (Sigma-Aldrich).
6. Duolink® in situ PLA probe MINUS (Sigma-Aldrich).
7. Duolink® in situ Mounting Medium with DAPI (Sigma-Aldrich).

3 Methods

3.1 Isolation of CML-MPPs from the Transduction-/Transplantation-Based CML Mouse Model

1. Isolate mononuclear cells (MNCs) from bone marrow (BM) of *BCR-ABL1* transduction-/transplantation-based CML-affected recipient mice.
2. Stain these MNCs with anti-Sca-1 (E13-161.7)-PE, anti-CD4 (L3T4)-biotin, anti-CD8 (53–6.7)-biotin, anti-B220 (RA3-6B2)-biotin, anti-TER119 (Ly-76)-biotin, anti-Gr-1 (RB6-8C5)-biotin, and anti-Mac1 (M1/70)–biotin Abs (all from BD Biosciences), followed by addition of Streptavidin-PECy7 (BD Biosciences) and anti-c-Kit (ACK2)-APC Ab (eBiosciences).
   Use 5–10 μl of each Ab solution for 1×10^7 cells suspended in 1 ml PBS containing 5 % FBS.
3. Use a flow cytometry such as a FACS Aria III instrument (BD Biosciences) or a similar instrument to isolate the cell fraction that is BCR-ABL1-GFP positive, cKit positive, lineage (CD4, CD8, B220, Mac1, Gr-1, and Ter119) negative, and Sca-1 positive. These cells constitute the CML-MPP (KLS) cell population, which includes CML LSCs.

3.2 Isolation of Murine LT-CML LSCs from the Double tg-CML Mouse Model

1. For the prospective isolation of CML LSCs, obtain BM MNCs from the two hind limbs of tetracycline-inducible tg-CML-affected mice at 5 weeks after Dox withdrawal [18] (*see* **Note 5**).
2. Immunostain these cells with anti-Sca-1 (E13-161.7)-PE, anti-CD4 (L3T4)-FITC, anti-CD8 (53–6.7)-FITC, anti-B220 (RA3-6B2)-FITC, anti-TER119 (Ly-76)-FITC, anti-Gr-1

(RB6-8C5)-FITC, and anti-Mac1 (M1/70)-FITC antibodies (Abs) (all from BD Biosciences), anti-CD48 (HM48-1)-APC-Cy7 and anti-CD150/SLAM (TC15-12F12.2)-Pacific blue Abs (both from BioLegend), and anti-cKit (ACK2)-APC, and anti-CD135/Flk2/Flt3 (A2F10)-biotin Abs (both from eBiosciences), followed by addition of Streptavidin-PE-Cy7 (BD Biosciences).

Use 5–10 μl of each Ab solution for 1×10^7 cells suspended in 1 ml PBS containing 5 % FBS.

3. Using a FACS Aria III flow cytometry (BD Biosciences) or a similar instrument, sort the cell fraction containing CD150/SLAM-positive, CD135/Flk2/Flt3-negative, CD48-negative, cKit-positive, Lin (CD4, CD8, B220, Mac1, Gr-1, and Ter119)-negative, and Sca1-positive LT-CML LSCs, i.e., $CD150^+CD135^-CD48^-KLS^+$ cells [18].

3.3 Fluorescence Immunostaining (FIHC)

FIHC is the traditional means of evaluating the subcellular localization and expression levels of specific proteins in CML LSCs. As an example, we show here an examination of the intracellular localization of Foxo3a (forkhead O transcription factor 3a) and the expression levels of the cell proliferation marker Ki67. Freshly isolated CML LSCs plated on a poly-L-lysine-coated glass slide were subjected to FIHC (Fig. 1). As can be clearly seen in Fig. 2, some CML LSCs are actively dividing while others are not. Whereas both groups express Foxo3a, only the actively dividing CML LSCs show high expression of Ki67 [15]. Notably, these proliferating $Ki67^+$ CML LSCs exhibit cytoplasmic localization of Foxo3a. In contrast, Foxo3a is present in the nuclei of quiescent CML LSCs, which do not express Ki67 [15].

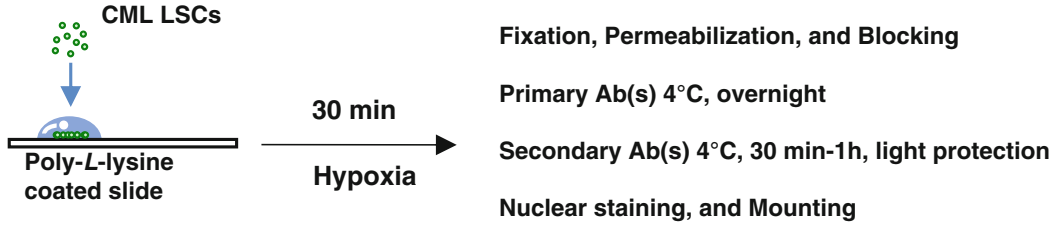

Fig. 1 Schematic diagram of preparation of murine CML LSC samples for FIHC analysis. Freshly isolated CML LSCs are plated on poly-L-lysine-coated slides and incubated for 30 min in serum-free stem cell medium under hypoxic conditions (3 % O_2). After fixation and permeabilization, cells are stained with primary Ab overnight. Primary Ab binding is visualized by incubating cells with secondary Ab conjugated to fluorochrome, as indicated. Nuclei are stained with a DNA marker prior to inspection of slides using a laser confocal microscope

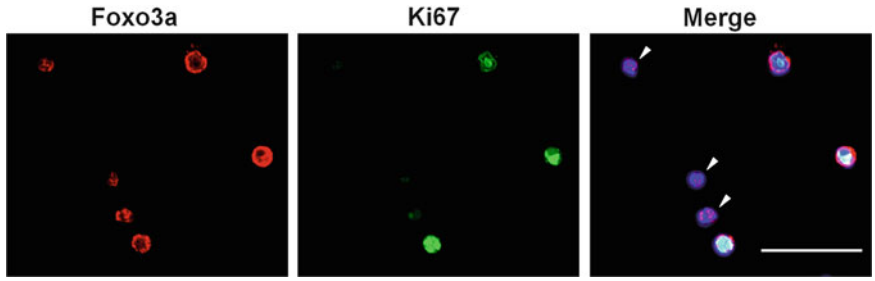

Fig. 2 Example of FIHC to evaluate protein intracellular localization and expression in murine CML LSCs. Freshly isolated CML-MPPs were subjected to FIHC using anti-Foxo3a (*red*) and anti-Ki67 antigen (*green*) primary Abs. Within the total CML-MPPs population, those CML-MPPs that are actively dividing express high levels of the cell proliferation marker Ki67 and show cytoplasmic localization of Foxo3a. In contrast, quiescent CML-MPPs do not express Ki67 and Foxo3a is present in the nucleus (*white arrowheads*). Nuclei are stained with the DNA marker DAPI (*blue*). Scale bar, 50 μm

1. Incubate 100–10,000 freshly isolated CML LSCs suspended in 20–30 μl SF-03 stem cell medium on poly-L-lysine-coated slides under hypoxic conditions (3 % O_2) [21] at 37 °C for 30 min–2 h (Fig. 1) (*see* **Note 5**).
2. Fix cells with freshly prepared 4 % PFA at room temperature for 30 min.
3. Permeabilize cells with 0.25 % Triton-X100 for 5 min.
4. Block permeabilized cells by incubation in 2 % BSA/PBS for 1 h.
5. Incubate the blocked and permeabilized cells with appropriate primary Ab(s) (1/50–1/100 dilution) in 2 % BSA/PBS at 4 °C for 12 h.
6. Wash cells in 2 % BSA/PBS three times for 5 min each.
7. Incubate the cells with secondary Ab(s) tagged with a fluorescent marker (1/200–1/500 dilution) in 2 % BSA/PBS at 4 °C for 30 min–1 h (*see* **Note 6**).
8. Stain nuclei with a DNA marker diluted 1/500–1/1000, such as DAPI (Sigma-Aldrich) or TOTO®-3 Iodide (Life Technologies) (*see* **Note 7**).
9. Wash cells in 2 % BSA/PBS three times for 5 min each.
10. Mount cells using Fluoromount Plus (Diagnostic Biosystems) or Prolong Diamond® (Life Technologies).
11. Acquire fluorescent images using a laser confocal microscope and Photoshop software (Adobe).

3.4 Duolink® In Situ PLA Technology

Duolink® in situ PLA technology is a highly sensitive approach based on the use of two Abs that originate from different species (i.e., mouse Ab combined with rabbit Ab). The proximate binding of these Abs to a protein(s) of interest is then detected using the Duolink® system (Sigma-Aldrich), which employs a set of

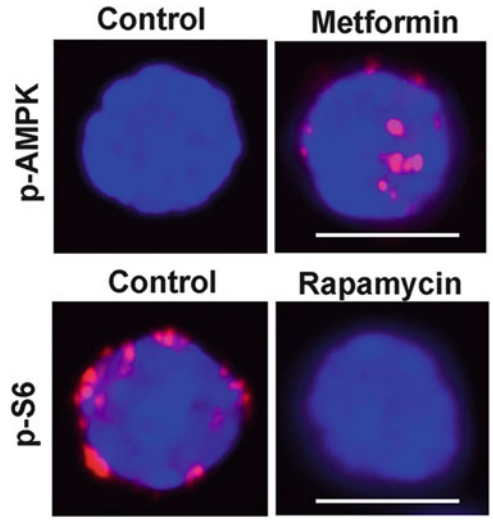

Fig. 3 Duolink® in situ PLA analyses of signal transduction in murine CML LSCs. Freshly isolated LT-CML LSCs were treated in vitro without (control) or with metformin (10 mM, AMPK activator) or rapamycin (100 nM, mTORC1 inhibitor) for 30 min under hypoxic conditions (3 % O_2). AMPK activity (*top*) as determined by the appearance of phospho-AMPK was detected by a combination of anti-phospho-AMPK and anti-AMPK Abs. Inhibition of mTORC1 activity (*bottom*) as determined by the disappearance of phospho-S6 ribosomal protein was detected by a combination of anti-phospho-S6 and anti-S6 Abs. The proximate binding of these antibodies was visualized using the Duolink® system (Sigma-Aldrich). Nuclei are stained with DAPI. Scale bar, 10 μm

secondary antibodies conjugated to PLA probes (i.e., anti-mouse IgG conjugated to a minus strand PLA probe and anti-rabbit IgG conjugated to a plus strand PLA probe). For example, AMPK activity can be assessed by combining rabbit anti-phospho-AMPK Ab with mouse anti-AMPK Ab [22] (Fig. 3). Similarly, mTORC1 activity can be evaluated by combining rabbit anti-phospho-S6 Ab and mouse anti-S6 ribosomal protein Ab [23, 24] (Fig. 3). Figure 4 shows another example: the evaluation of phosphorylation levels of Smad3, as well as binding between Smad3 and Foxo, as determined by combining anti-Smad3 plus anti-phospho-Smad3 Abs, and anti-Smad3 plus anti-Foxo3a Abs, respectively [25].

1. Immediately fix 100–10,000 freshly isolated CML LSCs in 4 % PFA/TBS for 30 min on ice.
2. Permeabilize cells with 0.25 % Triton-X100/TBS for 5 min.
3. Block permeabilized cells by incubation in 2 % BSA/TBS for 1 h.
4. Collect cells on a glass slide by centrifugation at (120 × *g*) for 5 min using a Cytospin™ centrifuge (Thermo Scientific).
5. Incubate the permeabilized and blocked cells at 4 °C for 12 h with a dual species primary Ab set (e.g., mouse Ab plus rabbit Ab), each diluted 1/25–1/100 in 2 % BSA/TBS (*see* **Note 8**).

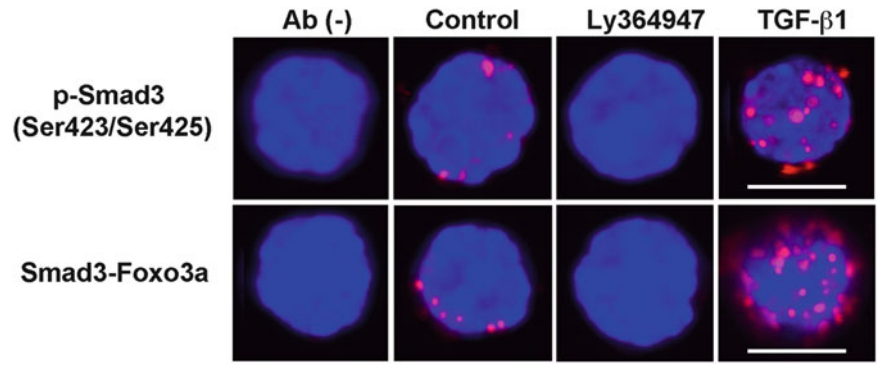

Fig. 4 Duolink® in situ PLA analyses of Smad3 phosphorylation and Smad3-Foxo3a interaction in murine CML LSCs. Freshly isolated CML LSCs were treated in vitro without (Control) or with Ly364947 (5 μM, TGF-β type I receptor kinase inhibitor) or TGF-β1 (10 ng/ml) for 30 min under hypoxic conditions (3 % O_2). Phospho-Smad3 (Ser423/Ser425) (*top*) and Smad3-Foxo3a interaction (*bottom*) were evaluated using a combination of anti-phospho-Smad3 and anti-Smad3 Abs, or anti-Smad3 and anti-Foxo3a Abs, respectively. The proximate binding of these Abs was detected using the Duolink® system (Sigma-Aldrich). Ab (-), a technical negative control using a single mouse anti-Smad3 Ab alone. Nuclei are stained with DAPI. Scale bar, 10 μm

6. Wash cells in 2 % BSA/TBS three times for 5 min each.
7. Incubate the cells at 37 °C for 1 h with a secondary Ab set conjugated to PLA probes in 2 % BSA/TBS, for example, anti-mouse IgG conjugated to a minus strand PLA probe and anti-rabbit IgG conjugated to a plus strand PLA probe.
8. Detect the proximate binding of these Abs using the Duolink® in situ PLA system (Sigma-Aldrich) following the manufacturer's instructions.
9. Stain nuclei with the DNA marker DAPI (Sigma-Aldrich).
10. Acquire fluorescent images using a laser confocal microscope and Photoshop software (Adobe).
11. Quantify the number of fluorescent foci per single CML LSC using the Duolink® Image software tool (Sigma-Aldrich).

4 Notes

1. **Terminology for CML LSCs.**
   For the *BCR-ABL1* transduction-/transplantation-based CML mouse model (*see* Subheading 2.1) [15], or the double tg-CML mouse model (*see* Subheading 2.2) [18], we consider the most primitive murine "long-term (LT)-CML LSCs" to be $CD150^+CD135^-CD48^-KLS^+$ cells and murine "CML-multipotent progenitor cells (CML-MPPs)" to be KLS^+ cells.

2. **Dox solutions.**
   Prepare a 200 mg/ml Dox (Sigma-Aldrich) stock solution in water and protect from light. Store the Dox stock solution at −30 °C or −80 °C.
3. For mouse experiments, dilute the 200 mg/ml Dox stock solution to a final concentration of 20 μg/ml in drinking water in a light-protected bottle. Replace the Dox-containing drinking water every third day.
4. Selection of Duolink® in situ Detection Reagents.
   Choose an appropriate combination of secondary Abs conjugated with plus or minus strand PLA probes (i.e., anti-mouse IgG PLA probe Minus and anti-rabbit IgG PLA probe Plus, or anti-goat IgG PLA probe Minus and anti-rabbit IgG PLA probe Plus). Choose appropriate Duolink® in situ Detection Reagents (i.e., Red, Orange, FarRed, or Green).
5. We recommend the isolation of BM MNCs under hypoxia (3 % O_2) because these conditions dramatically improve the retention of self-renewal capacity by HSCs [21]. CML LSCs are known to originate from normal HSCs [1–4], and so the isolation of CML LSCs under hypoxia may help to maintain CML LSC activity.
6. Select the species for the secondary Ab set. For example, use AlexaFluor 555- or AlexaFluor 647-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Molecular Probes).
   Be aware of potential cross-reactions among secondary Abs. We recommend that both secondary Abs in a set originate from the same species (i.e., "goat" anti-mouse IgG and "goat" anti-rabbit IgG, or "donkey" anti-mouse IgG and "donkey" anti-goat IgG).
   To avoid nonspecific cross-reactions, we also recommend that the secondary Abs be highly cross-adsorbed.
7. Select the DNA marker such that the wavelength of its fluorescence is distinct from those of the secondary Ab set so as to avoid obscuring their detection.
8. As a technical negative control, treat CML LSCs in vitro with a single antibody alone and confirm that no fluorescent foci can be detected.

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Chapter 5

Cell Cycle Analysis of CML Stem Cells Using Hoechst 33342 and Propidium Iodide

Ngoc DeSouza, Megan Zhou, and Yi Shan

Abstract

Chronic myeloid leukemia (CML) is a myeloproliferative disease with an expansion of white blood cells. The current treatments for CML are shown not to be long-term effective because of CML stem cells' insensitivity to tyrosine kinase inhibitors. Therefore, studying more about CML stem cells is essential to understand the pathways of CML stem cell development and proliferation and finally lead to effective treatments to eliminate CML stem cells and eradicate CML. This chapter describes two methods to analyze cell cycle of CML stem cells. The rare population of CML stem cells can be identified by staining with cell surface markers, and then DNA-binding dyes Hoechst 33342 and propidium iodide (PI) are added to stain the DNA content which is changed when cells go through different phases of the cell cycle. Samples are run through the flow cytometer to be analyzed based on different absorbance and emission wavelengths of different florescent colors.

Key words Chronic myeloid leukemia (CML) stem cells, Cell cycle analysis, Hoechst 33342, Ki67, Propidium iodide (PI), Flow cytometry

1 Introduction

Hematopoietic stem cells (HSCs) found in bone marrow are a rare population with the capacity of self-renewal and differentiation into long-term self-renewing HSCs, short-term self-renewing HSCs, and multipotent progenitors which give rise to lymphoid and myeloid progenitors [1]. Pathways to regulate stem cell renewals, differentiation, and proliferation must be tightly controlled by different mechanisms, many of which are still remained unknown. Once any of these pathways encounters abnormal development or expression, it can cause uncontrolled proliferation of immature cells thus can lead to diseases such as blood cancer.

Chronic myeloid leukemia (CML) is a myeloproliferative disease characterized by the presence of an oncogenic *BCR-ABL* fusion gene resulting from a reciprocal translocation, t(9;22)-(q34;q11), between the long arms of chromosomes 9 and 22 [2].

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BCR-ABL is a constitutively active tyrosine kinase that causes activation of downstream signaling pathways and inhibition of apoptosis, resulting in the abnormal proliferation of myeloid cells. This hallmark of CML is found in majority of patients, and a tyrosine inhibitor, such as imatinib, is often used to treat CML [3–5]. Although imatinib can successfully eliminate progenitor cells, it is not the cure for CML because CML stem cells are insensitive to imatinib [6, 7]. Therefore, it is necessary to study about CML stem cell properties and development to find a pathway to inhibit and eliminate these CML stem cells.

Both HSCs and CML stem cells are rare populations. The most reliable method to identify them is by using several cell surface markers. The in vitro and in vivo studies have shown that mouse HSCs express high level of cell surface markers c-Kit, Sca-1, low level of CD34, and Thy1.1, but lack expression of lineage (Lin) [8–10]. The study of CML stem cells can be done in vitro with different cell lines that express *BCR-ABL*. To study it in vivo, we induce CML in mice through bone marrow transplantation: mice are lethally irradiated and injected with donor cells that are previously transduced with retrovirus containing *BCR-ABL-GFP*; we can identify the CML cells by the expression of GFP from non-GFP-expressing cells which are normal hematopoietic cells [11].

To renew and proliferate, stem cells are also required to go through cell cycle in which they divide into daughter cells. However, when cell damage occurs, it causes cell division dysregulation that increases the risk of giving rise to abnormal cells. Therefore, it is important to compare the cell cycle profiles between normal HSCs and CML stem cells to identify the phases in cell cycle are interrupted. The method of cell cycle analysis is using flow cytometry to identify different cell phases by the amount of DNA content which is stained with florescent dyes [12]. There are three cell cycle stages: quiescence/senescence, interphase, and cell division. Quiescent phase or G_0 is the resting phase where cell is not dividing and should have low DNA content at this stage. Interphase includes G_1, cells going through checkpoint to get ready for DNA synthesis; S phase, where DNA replication occurs; and G2, which is the second checkpoint after replication before cells go to mitosis. High DNA content is recorded at this stage. Finally cell division or mitosis is where cell stops growing and divides into two daughter cells [13].

This chapter describes the methods of cell cycle analysis for CML stem cells by using two different ways of staining DNA content: Hoeschst 33342 with Ki-67 and propidium iodide (PI) [14–16]. These two methods both incorporate florescent molecules into the nucleic acid to quantitatively access the DNA content using flow cytometry.

2 Materials

1. Flow cytometer.
2. The software used to analyze data collected from the flow cytometer is FlowJo (Version 8.4.4).
3. Centrifuge that can accommodate tubes up to 50 mL.
4. RBC (red blood cell) lysis buffer (1×): 150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, adjust pH to 7.4.
5. Hanks' balanced salt solution—HBSS (Cellgro).
6. Fetal Bovine Serum—FBS (HyClone).
7. RPMI 1640 (CORNING Cellgro).
8. Antibodies: anti-mouse c-Kit-PE, anti-mouse Sca-1-APC, anti-mouse Gr-1-FITC, anti-mouse Gr-1-APC, anti-mouse Gr-1-APC-Cy7, anti-mouse B220-PE-Cy7, anti-mouse Cd11b-PE, Streptavidin-APC-eFluor780, anti-mouse Ki67-PerCP-Cy5.5, anti-mouse IgG-PerCP-Cy5.5 (eBiosciences).
9. Mouse lineage cell detection cocktail-biotin kit with lineage detection cocktail and microbeads (Miltenyl Biotec).
10. AutoMACS Pro Separator machine from MACS Miltenyl Biotec.
11. 7-AAD (BD Pharmingen).
12. Hoeschst 33342, 20 mM solution in water (AnaSpec Inc).
13. Paraformaldehyde 20 % solution (Electron Microscopy Science). Dilute this solution with distilled water to make 4 % PFA.
14. BD Perm and Wash Buffer for permeabilization (BD Biosciences).
15. Propidium iodide (PI) $\geq$ 94 % (Sigma).
16. Staining buffer: HBSS solution containing 2 % FBS, and keep solution cold.

3 Methods

3.1 Collecting Cells

1. Sacrifice the mouse and clean it with 70 % ethanol.
2. Collect the femurs and tibias bones, plate them on a dish containing cold HBSS, and flush the bone marrow cells out using 27.5 gauge needle with RBC lysis buffer into 15 or 50 mL falcon tube.
3. Incubate on ice for 10 min; then centrifuge at 300 × *g* for 10 min at 4 °C.

4. Discard the supernatant and resuspend the pellet with 10 mL of HBSS. Filter the mixture through a cell strainer.
5. Take an aliquot to count the cell number. Centrifuge again at 300 × *g* for 10 min.

3.2 Hoechst 33342 and Ki67 Staining of CML Stem Cells

Hoechst 33342 is a nucleic acid dye that emits blue florescence when bound to DNA. Hoechst 33342 signal is changed between different phases of cell cycle due to the amount of DNA. Hoechst 33342 is strongest during interphase when DNA synthesis takes place. Ki-67 is a cellular marker for proliferation. Ki-67 should be present in G_1, S, and G_2/M phases; however, resting or non-cycling cells (G_0 phase) lack Ki-67 expression. Together, Ki-67 is used to cells in resting phase, and Hoechst 33342 is used to separate other phases of cell cycle.

1. Make Hoechst 33342 solution containing: warm RPMI1640 medium, 2 % FBS, and 2.5 μL Hoechst 33342 per 10 mL medium.
2. Resuspend 10^6 cells in 1 mL of Hoechst 33342 solution and incubate it at 37 °C for 90 min. Note: wrap tube with aluminum foil to avoid direct light.
3. Prepare the stem cell antibody cocktail (for 10^6 cells, adjust the appropriate amount of antibodies with a number of cells): in 100 μL of cold HBSS, add 1 μL biotin lineage cocktail, 1 μL c-Kit-PE, and 0.5 μL Sca-1-APC.
4. Add 10 mL staining buffer (HBSS + 2 % FBS). Aliquot a small amount into another tube for Hoechst single control that has no stem cell staining and fixation.
5. Centrifuge sample for 10 min at 300 × *g*.
6. Add 100 μL of above stem cell antibody cocktail into sample and incubate at 4 °C for 30 min.
7. Wash cells by adding staining buffer. Centrifuge and discard the supernatant.
8. Resuspend cell pellet with 100 μL of HBSS containing second antibody anti-biotin Streptavidin-APC-eFluor780 (1 μL per 10^6 cells). Incubate at 4 °C for 15 min.
9. Wash cells. Spin down and fix cells in 100 μL 4 % PFA for 20 min at 4 °C. Aliquot a small sample for Hoechst + Fix control.
10. Wash cells one time and resuspend cells in staining buffer. Keep this sample on ice overnight, or directly proceed to staining with Ki-67.
11. Centrifuge and resuspend cells in BD Perm/Wash buffer for 15 min at 4 °C for permeabilization.

12. Take out a small aliquot for isotope IgG control (no Ki-67). Centrifuge and add 50 μL of BD Perm/Wash buffer containing 5 μL of Ki-67 (5 μL of IgG for the control sample) for 30 min at 4 °C in the dark (wrap with aluminum foil).
13. Wash cells twice with BD Perm/Wash Buffer. Centrifuge, and resuspend cells in 500 μL of HBSS + 2 % FBS.
14. Keep sample on ice until running the flow cytometry.
15. Single control color samples need to be made for compensation for multicolor flow cytometry to better distinguish different parameters between colors. For bone marrow cell controls, take bone marrow cells from the same strain mouse but does not have GFP (no bone marrow transplantation done in this mouse). Make the following controls by staining each sample with different antibodies for 15 min at 4 °C, and wash with staining buffer:
    (a) No staining (with no antibody or anything).
    (b) FITC-Gr-1 (or a small aliquot from CML mouse that has not gone through any staining can also be used for this control because a portion of CML bone marrow cells is $FITC^+/GFP^+$).
    (c) PE-Cd11b.
    (d) APC-Gr-1.
    (e) APC-Cy7-Gr-1.
    (f) 7-ADD.
    (g) Hoechst alone (mentioned above).
    (h) Hoechst + Fix.
    (i) Hoechst + Stem cell + IgG.
    (j) Hoechst + Stem cell + Ki-67.

3.3 Propidium Iodide (PI) Staining of CML Stem Cells

PI is a DNA-binding dye that needs to enter the cells by fixation and permeabilization. A common problem usually found with some DNA dyes is that if cell surface markers are added together with PI, they are usually not compatible which leads to poor profile signal on the flow cytometer. The best way to improve this problem while using PI is sorting out the stem cell population and then proceeding to PI staining. This method provides another way to analyze cell cycle of CML stem cells. It takes longer; however, it gives much clearer profile on the flow cytometer.

1. Resuspend cell pellet in 40 μL of staining buffer (HBSS + 2 % FBS) per 10^7 cells.
2. Add 10 μL of biotin-antibody cocktail per 10^7 cells. Mix by pipetting and incubate for 10 min at 4 °C.

3. Add 30 μL of staining buffer per 10^7 cells. Add 20 μL of Anti-Biotin Microbeads per 10^7 cells. Mix and incubate for 15 min at 4 °C.
4. Add 2 mL of staining buffer to wash cells. Centrifuge for 10 min at 300 × *g* at 4 °C.
5. Resuspend cells in 1 mL of staining buffer and proceed to lineage cell depletion step either by using manual magnetic separation or the automatic MACS machine.
6. After lineage deletion step, 5–10 % cells should be collected. Count cell number again.
7. Stain cell with stem cell antibody: add 100 μL of staining buffer containing 1 μL c-Kit-PE, 0.5 μL Sca-1-APC and 1 μL anti-biotin Streptavidin-APC-eFluor780 (per 10^6 cells). Incubate for 30 min at 4 °C.
8. Wash cell with 2 mL staining buffer. Centrifuge and decant supernatant.
9. Resuspend cells in 1 mL of HBSS; add 5 μL of 7-AAD.
10. Prepare single color control with a mouse that has the same strain and no GFP cells.
    (a) No staining.
    (b) FITC-Gr-1 (or no-staining cells from CML mouse).
    (c) APC-Gr-1.
    (d) APC-Cy7-Gr-1.
    (e) PE-Cd11b or Gr-1.
    (f) 7-AAD.
11. Proceed to sorting cells. Use the flow cytometer that can collect cells after analysis; a bigger collecting nozzle is preferred to avoid breaking cells.
12. Collect the population with 7-AAD-negative (alive cells), APC-Cy7-negative (lineage$^-$), c-Kit-positive, and Sca-1-positive from both GFP-negative (normal stem cells) and GFP-positive cells (CML stem cells).
13. After collecting the GFP-negative and GFP-positive stem cell samples, proceed to PI staining. Centrifuge cells at 300 × *g* for 10 min at 4 °C. Discard supernatant carefully because there is only small cell pellet.
14. While vortexing the cell pellet, add drop wise cold 70 % ethanol for cell fixing.
15. Incubate for 30 min at 4 °C.
16. Wash cell twice with HBSS. Centrifuge at 300 × *g* for 10 min each time. Remove supernatant carefully.

17. Optional: add 50 μL of 100 μg/mL of RNase to remove RNA.
18. Resuspend cells in 200 μL of 50 μg/mL PI.
19. Proceed to flow cytometry analysis.

3.4 Flow Cytometric Analysis of Stained Cell Samples

1. Measure the forward scatter (FSC) and side scatter (SSC) to gate out single cells (Fig. 1a).
2. Gate out alive cells which are 7-AAD negative (Fig. 1b) because 7-AAD is a marker that intercalates into double-stranded DNA and mark dead cells.
3. Gate out the GFP^- and GFP^+ populations. GFP^+ cells have *BCR-ABL* expression and supposed to be CML cells, while GFP^- cells are normal bone marrow cells (Fig. 2a).
4. Gate out lineage-negative cells (Fig. 2b, c) that contain stem cells and multipotent progenitor cells. Identify the stem cell populations ($Lin^-c\text{-}Kit^+Sca\text{-}1^+$) from each GFP^- and GFP^+ group (Fig. 2d, e).
5. Identify the G0 population by Ki-67-PerCP-Cy5.5 positive which is not shown in the negative control (Fig. 3).
6. Use DAPI channel to detect Hoechst 33342, and select Histogram in the software to display cell cycle curves (Fig. 4).
7. For PI detection, use channel for conjugated color of PI antibody; also select Histogram to display cell cycle curves (Fig. 5).

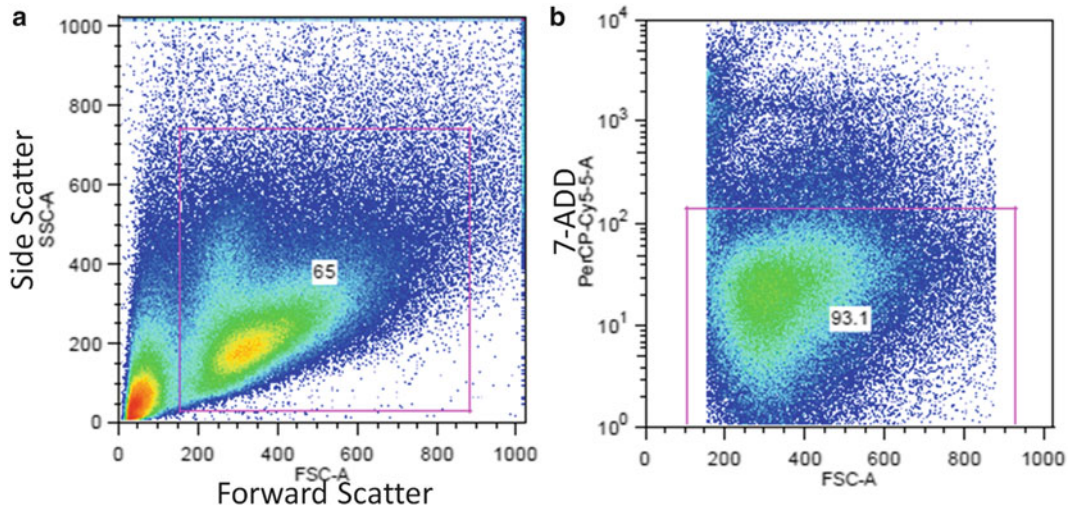

Fig. 1 Flow cytometry analysis for single and alive cells. (**a**) Using forward and side scatter to gate out single cells. (**b**) Gate out alive cells by identifying the population that lacks 7-ADD expression

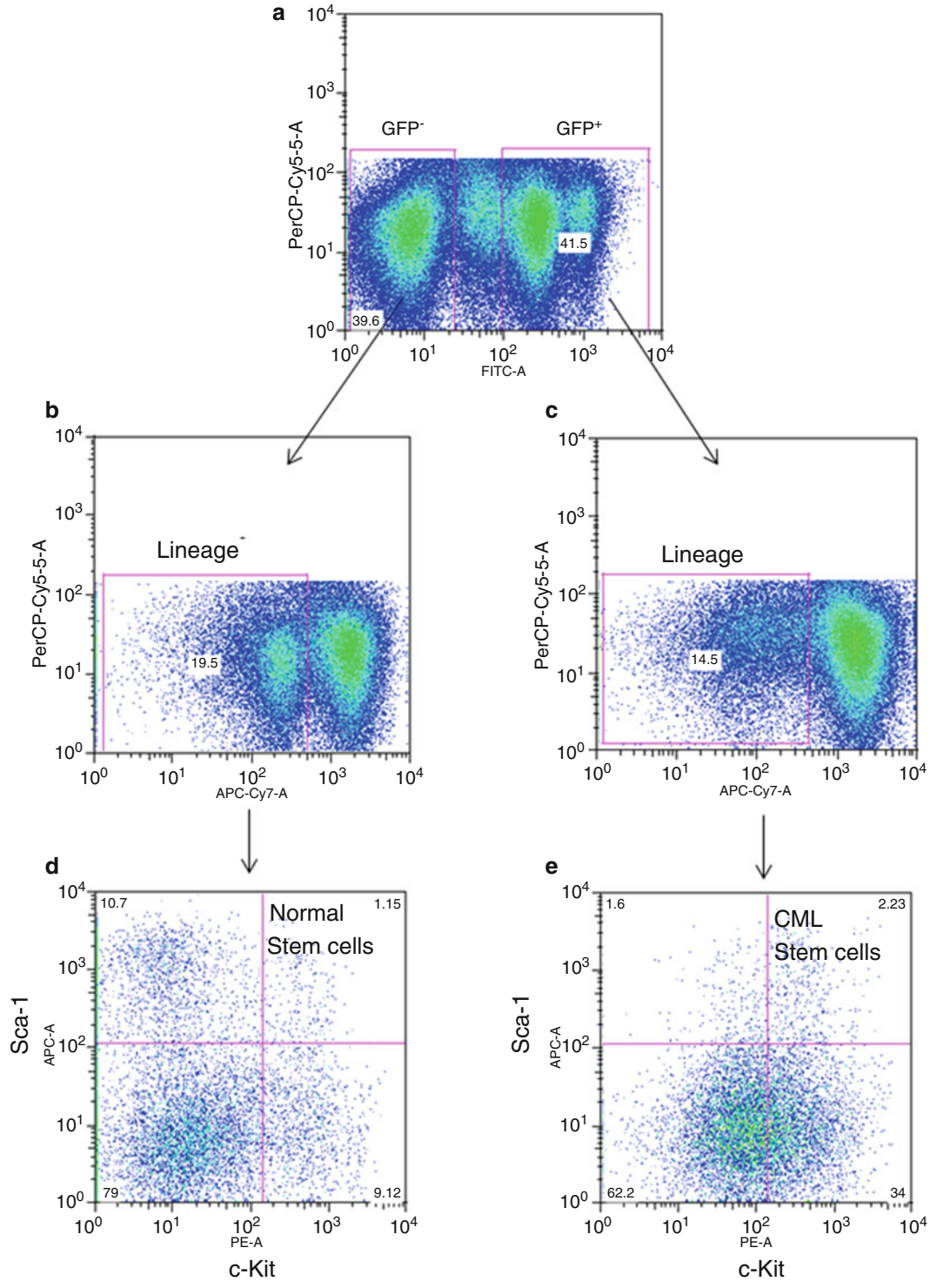

Fig. 2 Flow cytometry analysis to identify the stem cell population. (**a**) Gate out populations with FITC negative and positive. FITC negative is the GFP-negative population which contains normal cells with no *BCR-ABL* expression, whereas FITC positive or GFP positive is the leukemia cell population with *BCR-ABL* expression. (**b**, **c**) Progenitor cells are stained with lineage markers which are APC-Cy7 positive. Gate out the lineage-negative population which contains the stem cells from either normal or leukemia cells. (**d**, **e**) The stem cells are those expressing both c-Kit and Sca-1 markers

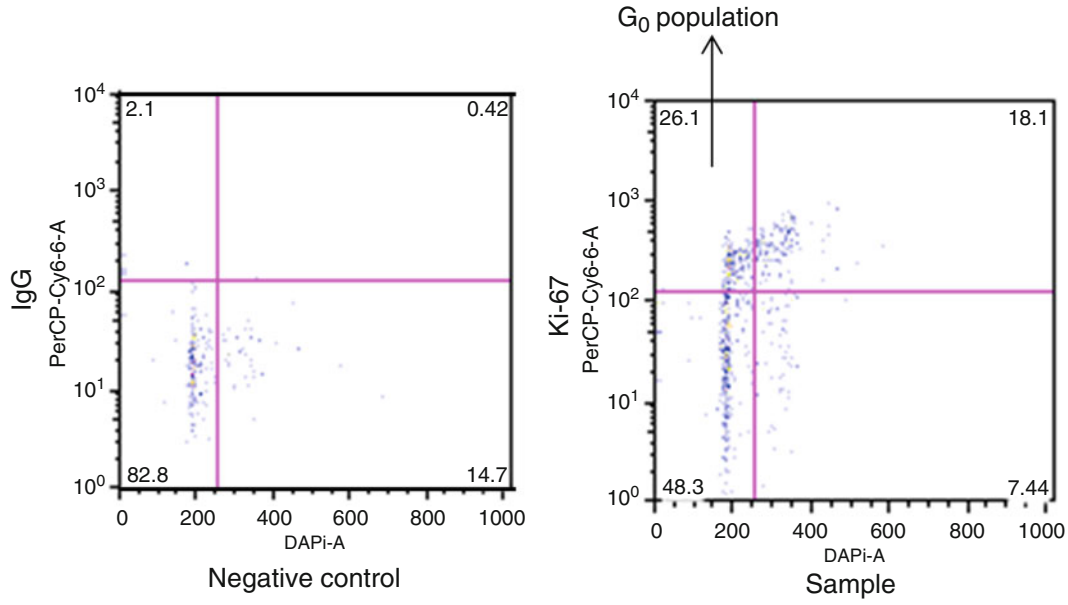

Fig. 3 Flow cytometry analysis for G_0 population. The quiescent G_0 population is positive for Ki-67 and negative for DAPI expression. Using the negative control lacking Ki-67 to draw the gate for Ki-67 positive

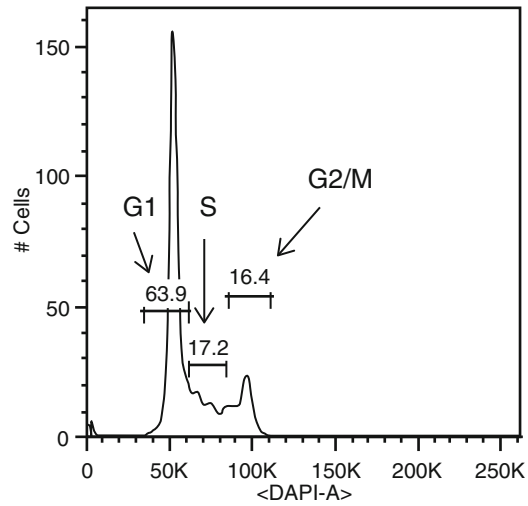

Fig. 4 Flow cytometry analysis for cell cycle using Hoechst 33342. Hoechst 33342 can be detected using laser that detect DAPI. Use histogram display to see the expression of DAPI. There are two peaks: the area under the first peak is G1 population and the area under second peak is G2/M population. The area between these two peaks is the S population

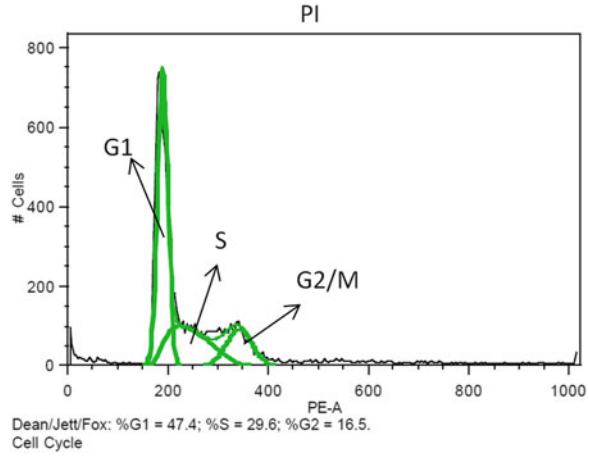

Fig. 5 Flow cytometry analysis for cell cycle using Propidium iodide (PI). Instead of using Hoechst 33342, PI can also be used to detect different cell cycles. In this case, PI is conjugated with PE color. With the histogram display of PE expression, there are also two peaks: G1 population is under the first peak and G2/M population is under the second peak. The area in between the two peaks is the S population

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Chapter 6

Histological and In Vivo Microscopic Analysis of the Bone Marrow Microenvironment in a Murine Model of Chronic Myelogenous Leukemia

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Abstract

Imaging of the leukemic bone marrow microenvironment, also called the leukemic bone marrow niche, is an essential method to determine and to evaluate the progression of chronic myelogenous leukemia (CML) and other leukemias in murine models. In this chapter we introduce the murine model of CML primarily used in our laboratory by describing blood and bone marrow analysis as well as the method of histological sectioning and immunohistochemistry in combination with various stainings that can help to understand the complex interaction between leukemic cells, their normal hematopoietic counterparts, and the bone marrow microenvironment. We conclude with describing how to image the bone marrow niche using in vivo microscopy.

Key words Histological sectioning, Immunohistochemistry, In vivo microscopy, Bone marrow niche, Leukemic stem cell niche, Murine models of hematological malignancies, Chronic myelogenous leukemia

1 Introduction

Chronic myelogenous leukemia (CML) is a hematological malignancy caused by reciprocal translocation of chromosomes 9 and 22 that results in the generation of the *BCR/ABL1* fusion gene. This gene encodes for a dysregulated cytoplasmic tyrosine kinase with a size of 210 kDa and is therefore named p210-BCR/ABL1 [1].

In humans, CML is mainly characterized by elevated leukocytes (leukocytosis) in the peripheral blood, most of them being myeloid cells of different maturation stages, basophilia, eosinophilia, and splenomegaly. If untreated, CML progresses from a chronic phase (<5 % blasts) to an accelerated phase (5–19 % blasts) and blast crisis [1], which resembles an acute myeloid or lymphoblastic leukemia (>20 % blasts) [2].

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The characterization of a disease like CML requires specific laboratory techniques and a complex, faithful mouse model. There have been different approaches [3] on how to investigate the pathophysiology of CML so far, and most approaches have their own merit.

Some experiments can be modeled in vitro, for instance, with the BCR/ABL1+ cell line K562 (derived from a patient with CML in blast crisis), but an in-depth examination of the mechanisms of CML, especially when it pertains to the bone marrow microenvironment (BMM) [4], cannot be fully addressed with in vitro experiments only. While some promising in vitro methods to model the BMM are being developed, many lack factors such as shear forces, pH, oxygen status, the cytokine milieu, etc., all of which are thought to play a role in the BMM. For more detailed research and research on the BMM in particular, in vivo models probably provide a more comprehensive way to analyze disease development, progression, and response to therapy. One method to induce CML-like myeloproliferative neoplasia (MPN) in vivo is to transplant human CML cells into immunocompromised mice, for instance, NOD SCID interleukin-2 receptor gamma knockout (NSG) mice, in a so-called xenotransplantation [5]. However, this model is limited as the engraftment of BCR/ABL1+ cells is poor. Another attempt is the development of murine models that express the *BCR/ABL1* oncogene, for instance, in the form of a transgene [6] or in the way of transplantation of donor bone marrow retrovirally transduced with BCR/ABL1 [7, 8].

The murine CML model with the most history, which has also been used to model other hematological malignancies, is the murine retroviral transduction/transplantation model of BCR/ABL1+ CML [7, 8]. In this model, depending on the mouse strain, CML-like leukemia can be induced in 100 % of recipient mice within 30 days after transplantation. As the disease progresses, the malignant cells infiltrate the spleen and liver, causing hepatosplenomegaly (massive enlargement of both the spleen and liver). Due to infiltration of the lungs by myeloid cells, death occurs rapidly due to respiratory failure and pulmonary hemorrhages (acute bleeding affecting the lung tissue).

In order to test disease establishment, progression, and interaction with the BMM, histological analysis and immunohistochemistry can be performed on bone sections embedded in paraffin or on cryopreserved sections. In addition, in vivo microscopy is capable of imaging the microanatomy of the bone marrow niche in a very profound and detailed way.

2 Materials

2.1 Components for Blood Analysis

Microtainer tube with dipotassium EDTA (BD, Heidelberg, Baden-Württemberg, Germany).

Hematocrit capillaries (Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Baden-Württemberg, Germany).

FACS tubes for flow cytometry (Sarstedt AG & Co., Nümbrecht, Nordrhein-Westfalen, Germany).

ACK Lysing Buffer (red cell lysis buffer) (Life Technologies, Darmstadt, Hessen, Germany).

PE anti-mouse/human Mac-1/CD11b antibody (BD Pharmingen, Heidelberg, Baden-Württemberg, Germany).

FACS buffer with 3 % fetal calf serum (FCS) and 2 mM EDTA: Add 15 ml FCS to 485 ml of DPBS so that the final concentration of FCS is 3 %. Add 2 ml of EDTA (stock solution 0.5 M) and mix the solutions by inverting the bottle a few times. Store at 4 °C.

2.2 Cytospins

Cytospin Cytocentrifuge (Thermo Scientific/Fisher Scientific, Schwerte, Nordrhein-Westfalen, Germany) or THARMACspin Cytocentrifuge (Tharmac GmbH, Marburg, Hessen, Germany).

DPBS containing 2 % FCS: Add 10 ml of FCS to a 490 ml of DPBS. Mix the solution by inverting the bottle a few times, store at 4 °C.

2.3 Histological Sections

10 % formalin, neutral buffered (Sigma-Aldrich Chemie GmbH, Taufkirchen, Bayern, Germany).

Xylene (AppliChem GmbH, Darmstadt, Hessen, Germany).

Paraffin wax/Paraplast® (Sigma-Aldrich Chemie GmbH, Taufkirchen, Bayern, Germany).

2.4 Histological Staining

Aluminum potassium sulfate, sodium iodate, and acetic acid (Sigma-Aldrich Chemie GmbH, Taufkirchen, Bayern, Germany).

Hematoxylin: either buy the Hematoxylin Solution (Mayer's) directly from Sigma-Aldrich (this is 100 mg of hematoxylin already dissolved in 100 ml of dH_2O) or buy the powdered form from Leica (Leica Mikrosysteme Vertrieb GmbH Mikroskopie und Histologie, Wetzlar, Hessen, Germany).

For mounting of your slides: Entellan (Merck Millipore, Darmstadt, Hessen, Germany).

2.5 Immunohistochemistry

0.01 M sodium citrate buffer pH 6.0: Combine 3 ml 100 mM citric acid and 3 ml 100 mM sodium citrate in a measuring cylinder. Fill up to 500 ml with ddH_2O. Check pH with a pH meter and adjust to a pH of 6.0, if necessary.

1× PBS/0.1 % Triton X-100 solution: For 500 ml, take one bottle of PBS and add 500 µl of Triton X-100, and double the amounts if necessary.

Whatman 3MM.

1× PBS/0.1 % Triton X-100 + 10 % goat serum: Prepare as above, adding 50 ml of normal goat serum.

2.6 Immunohistochemical Methods

Tartrate-resistant acid phosphatase/acid phosphatase leukocyte kit (Sigma-Aldrich Chemie GmbH, Taufkirchen, Bayern, Germany).

2.7 In Vivo Microscopy

C57/BL6N mice.

Lineage Cell Depletion Kit for mice (Miltenyi Biotec GmbH, Bergisch Gladbach, Nordrhein-Westfalen, Germany).

Lineage Cocktail: anti-CD5, B220, Gr-1, Ter119, F4/80 (BD Pharmingen, Heidelberg, Baden-Württemberg, Germany).

Methocel (Dow Wolff Cellulosics GmbH, Bomlitz, Niedersachsen, Germany).

3 Methods

Before explaining our most commonly used methods for analyzing disease progression in mice with CML, we would like to point out another alternative to the retroviral transduction/transplantation model: the transgenic mouse model. This system allows the user to model a BCR-ABL1-positive CML in mice (SCLtTA/BCR-ABL mice) without the need to irradiate and transplant [6]. It is a less time-consuming model which has the advantage that you need fewer mice. The disadvantage on the other hand is that you are limited to the use of the SCLtTA/BCR-ABL mice, while in the retroviral transduction/transplantation model, you can chose various genetically modified mice as donors or recipients depending on the scientific question being asked.

For the induction of CML, the "tet-off" system is used: tetracycline, an antibiotic, is put in the drinking water of the mice. Tetracycline will constantly suppress the translation of the *BCR-ABL1* oncogene in these mice when consumed. When removed from the water, tetracycline will no longer have a preventing effect, BCR-ABL1 can be translated, and leukemia will be induced about 4 weeks after tetracycline withdrawal. Death will occur within 50–120 days.

In order to closely follow disease progression in mice in the CML model (retroviral or transgenic), blood and bone marrow analysis are essential, as discussed below.

3.1 Blood Analysis via CBC

1. Collect blood (around 75 μl is sufficient) in an EDTA- or heparin-coated tube to stop the sample from clotting (*see* **Note 1**).
2. Analyze the sample by using an animal blood counter (the machine we use is the scil Vet abc, scil animal care company GmbH, Viernheim, Hessen, Germany, *see* **Note 2**).

3.2 Blood Analysis via FACS

1. Immediately after analyzing your sample by the animal blood counter, add 500–800 μl ACK Lysing Buffer to lyse the erythrocytes. Let the mixture sit on ice for at least 5 min.
2. Transfer your samples to polystyrene FACS tubes. Add 1 ml PBS and pellet at 320 × *g* for 5 min, preferably at 4 °C.
3. While the tubes are spinning, prepare the Mac-1 antibody. We generally dilute the appropriate antibody (if, for instance, phycoerythrin conjugated) 1:10 in FACS buffer and then take 3.3 μl from this dilution to reach a final dilution of 1:300. Incubate for 15 min on ice in the dark.
4. Add 1 ml DPBS to wash away unbound antibody before spinning with the same conditions again.
5. Remove supernatant and resuspend the pellet in 300 μl FACS buffer.
6. Keep dark and cold until the FACS analysis.

3.3 Cytospins

1. To assess the morphology of cells that may only be present in small numbers cytospins can be performed. Assemble your slide, filtercard, and the TPX cell funnel and put them into the metal clip provided with your machine (mostly, the TPX cell funnel system for the Shandon® Cytospin® is used). Perform the following steps on ice if not stated otherwise: Resuspend your cells in DPBS with 2 % FCS so that the final concentration is approximately 1–5 × 10^5 cells per ml.
2. Pipette 100 μl of your sample into the corresponding funnel. Spin at 70 × *g* for 3–5 min (*see* **Note 3**).
3. Remove the filter without disturbing the cells on the slide. Let the slide dry.
4. Check cell viability and morphology under a light microscope. One should aim for a rather confluent single layer distribution with cells lying flat on the slide.
5. Let the slide dry at RT or in a desiccation chamber, if at hand. Non-fixed slides can be stored at RT for up to 2 days.
6. The slides can now be stained with May-Grünwald or Giemsa stains.

3.4 Histological Sections

1. For a more detailed microscopic view of the bone marrow, its cellular constituents, and its surrounding tissues, histological sections can be very helpful. First, the femur of a deceased mouse must be isolated and then placed into 10 % buffered formalin, thereby preventing decay like autolysis. The formalin fixation works best if you keep the bone in the solution for at least 48 h. Then the bone needs to be decalcified by putting it in 0.5 M EDTA for 24 h. After 24 h, change the EDTA to fresh 0.5 M EDTA and let the bone sit until it is flexible without breaking. This might take up to 5 consecutive days.
2. Then the samples must be dehydrated, if the sample is to be embedded in paraffin. This is achieved by using a series of ascending ethanol concentrations:
   - 70 % ethanol for ½ h
   - 70 % ethanol for 1 h
   - 95 % ethanol for 1 h
   - 100 % ethanol for 1 h
   - 100 % ethanol for 1½ h
   - 100 % ethanol for 2 h
3. After incubating your samples in the different alcoholic baths, a clearing agent needs to be used to free the sample from the ethanol. Place the sample into Xylene for 1 h. Repeat this step (*see* **Note 4**).
4. The tissue can now be immersed in paraffin wax that has been heated to about 56–58 °C (this varies depending on the company the paraffin has been ordered from, we generally use Paraplast®). Let the sample sit in paraffin for at least 1 h and repeat in another paraffin bath.
5. When hard, the paraffin block can be further processed and sectioned into 2–4 μm thin slices that should be immediately placed into a 40–45 °C water bath for this will smoothen its surface.
6. After letting the thin paraffin section float in the water bath for 1–3 min, gently slide a glass object holder underneath it and let your specimen attach.
7. The sample is now ready for further procedures, e.g., different staining methods.

3.5 Histological Stainings

1. Hematoxylin and eosin (H&E) stain: This stain is the most widely used stain for histological sections [9, 10]. Properly done, it provides an excellent overview of your sample and its morphological structures. First, deparaffinize all specimen in two different xylene chambers for 5 min each. Prepare a

humidified chamber and put it in a 37 °C incubator while rehydrating your slides in EtOH baths:

- 100 % ethanol for 5 min
- 100 % ethanol for 5 min
- 95 % ethanol for 5 min
- 70 % ethanol for 5 min
- Distilled water for 5 min

2. Meanwhile, prepare your Hematoxylin Solution (Mayer):
   - Dissolve 50 g of aluminum potassium sulfate (= alum) in 1 l of distilled H_2O.
   - Add 1 g of Hematoxylin and wait for it to dissolve.
   - Add 0.2 mg of sodium iodate and 20 ml of acetic acid.
   - Heat the solution until it boils, letting it cool down afterward.
   - Filter into an appropriate container.
3. Add your Hematoxylin Solution dropwise on your slides. Incubate for 5 min (*see* **Note 5**).
4. Let any excessive Hematoxylin drip down the side of your slide by holding it in a slight angle, then wash the slide with dH_2O.
5. Rinse with tap water for 10 min (*see* **Note 6**).
6. Stain with 1 % eosin (make up this solution in distilled water; we recommend to make at least 100 ml) for 2–5 min; this depends on the thickness of your sample (*see* **Note 7**).
7. Rinse again with tap water for 5 min.
8. End your differentiation process and begin dehydrating your sample in ethanol baths of increasing concentrations, starting from 95 % for 2 min and then moving on to 100 % ethanol in two more containers. Finish with two different xylene baths, letting your slide sit for 5 min in each one.
9. Mount your slides.
10. Your samples are ready to be analyzed microscopically.

3.6 Immunohistochemistry

Immunohistochemistry (IHC) was first described by Coons et al. in 1941 when they found a method to detect antigens in mammalian tissue sections by using immunofluorescence. Now, a few decades later, IHC has become the go-to method in clinical diagnosis as well as in research in general. The technique behind IHC is fairly simple: antigens are detected with specific antibodies that are coupled with a detection system (reporter molecules, mostly used are enzymes like peroxidases, etc.) [11, 12]. These complexes that are either direct (antigen + coupled antibody) or indirect (antigen + primary antibody + coupled secondary antibody) can then be visualized under a

light microscope. In the following, we will describe an indirect method using avidin-biotin complexes (ABC) [13]:

1. Deparaffinize sections as done for the H&E stain, placing them into dH_2O afterward.
2. Retrieval of your antigen using the heat-inducing method (*see* **Note 8**): It is said that inducing heat to a sectioned tissue sample can reverse the interaction between proteins and formalin, at least slightly. High temperatures can also reverse the mask effect on some epitopes by hydrolysis of methylene cross-links; other scientists state that the retrieval happens by extraction of blocking proteins or by rehydration of the specimen which then eases antibody penetration. Most commonly used for heat-induced epitope retrieval (HIER) is a 0.01 M sodium citrate buffer with a pH of 6.0: Put your samples in a jar filled with the buffer and heat in a steamer at 90–95 °C for 20 min. Let the slides cool on bench for 20–30 min.
3. Transfer your samples back to dH_2O.
4. To block endogenous peroxidase activity in your specimen place your samples in a jar with 0.003–3 % H_2O_2 in 70 % methanol (prepare this solution fresh in ddH_2O every time) for 5 min (*see* **Note 9**).
5. Wash your sections again in dH_2O.
6. Blocking of unspecific antigen-antibody reactions: Incubate your sections for 15 min in a 1× PBS/0.1 % Triton X-100 solution.
7. Meanwhile, prepare wet chambers using, e.g. Whatman 3MM Paper that has been dipped into ddH_2O and put a stage for your slides above the wet paper.
8. Let the excess amount of the PBS/Triton solution drip off from your specimen and place them onto your stage. Add a few drops of your blocking solution (1× PBS/0.1 % Triton X-100 + 10 % goat serum) and incubate for 1 h at room temperature.
9. Take your sections and let the excess liquid drip off again. Add some of your primary antibody solution dropwise onto your samples and let them incubate in the fridge at 4 °C. Seal your container with your slides with Parafilm to prevent evaporation.
10. Get your samples out of the container, let the excess liquid drip off, and wash them in 1× PBS/0.1 % Triton X-100 for 10 min. Repeat.
11. Get rid of the excess liquid by dipping the edge of your slide gently on a paper towel. Place your slides onto the stage of your wet chamber again and add a sufficient amount of secondary

antibody to your specimen. Let them sit for 30–35 min at room temperature.

12. Prepare your ABC solution (avidin-biotin-complex solution) according to the manufacturer's instructions.
13. Wash your slides twice with 1× PBS/0.1 % Triton X-100, then wash them with 1× PBS for 5 min.
14. Place your slide back onto your stage after letting the excess liquid drip off again. Add the ABC solution dropwise to your slide. Incubate for 30–45 min at room temperature.
15. Put your slide into a jar filled with 1× PBS and wash them for 5 min. Repeat this step three times. Meanwhile, prepare your 3,3′-diaminobenzidine (DAB) solution according to the manufacturer's instructions.
16. Take the first slide, let the excess of the PBS drip off, and add the DAB dropwise onto your sample. Monitor this staining step closely under a microscope for 1–4 min. If you are satisfied with the result, place your slide into a container filled with ddH_2O. This will stop the staining process. Repeat this step for all your slides, keeping the finished ones in 1× PBS.
17. Wash your samples in 1× PBS for 5 min.
18. Wash again in ddH_2O for 5 min. Meanwhile, inactivate the excessive DAB-solution by adding 3 ml of 2 M H_2SO_4 and 3 ml of 0.2 M $KMnO_4$. Incubate overnight and then dispose of it.
19. Now that you stained for DAB, you have to make sure that you will not confuse DAB+ cells with normal/unstained cells. To achieve this, it is common to use a hematoxylin and eosin (H&E) counterstain (*see* Subheading 3.5).
20. Mount your slides. Your slides are ready for microscopic analysis.

3.7 TRAP Staining

1. TRAP staining using the SIGMA kit to visualize osteoclasts: First, deparaffinize specimen (blank sections) in two different xylene baths for 5 min each. Prepare a humidified chamber and put it in a 37 °C incubator while rehydrating your slides in EtOH baths:
   - 100 % ethanol for 5 min
   - 100 % ethanol for 5 min
   - 95 % ethanol for 5 min
   - 70 % ethanol for 5 min
   - Distilled water for 5 min
2. Preheat around 10 ml of distilled H_2O in a 37 °C water bath.
3. To a tube, add 0.5 ml Fast Garnet GBC Base solution and 0.5 ml sodium nitrite solution from the Sigma Kit, mix by

gently inverting the tube for 30 s, and let it incubate for 2 min at room temperature.

4. In a 100 ml beaker or Coplin jar, mix the following solutions:
   - 45 ml of distilled 37 °C H_2O
   - 1.0 ml of the Fast Garnet/sodium nitrite solution that was made up in **step 3**
   - 0.5 ml naphthol AS-BI phosphate solution
   - 2.0 ml acetate solution
   - 1.0 ml tartrate solution
5. Heat up your jar in a 37 °C water bath. Add your samples to the jar.
6. Incubate for up to 1 h but at least for 10–30 min and keep your slides protected from light.
7. Wash your slides in dH_2O for about 5 min.
8. Counterstain with Hematoxylin Solution, Gill No. 3 from the SIGMA kit for 2 min.
9. Rinse your slides for about 5 min with tap water and check for color changes (from red to blue/violet).
10. Let your slides air-dry. If desired, mount your slide with Aqua-Mount (be aware that the dye might fade after a certain period of time). Your samples are ready for microscopic analysis.

3.8 In Vivo Microscopy

In vivo microscopy entails the microscopy of the bone marrow cavity, the calvarium, in live mice [14]. While traditional 2D microscopy and histological sections provide information about cellular structures and tissues, they are not very suitable for more detailed observations on a single-cell level or for 3D imaging. 2D imaging has also not been performed on live mice; hence, traditional microscopy has been limited in imaging interactions between cells and their microenvironment in real time. Therefore the new method of in vivo microscopy, which is gaining increased visibility and importance, also due to its increasing sophistication, is described here: In vivo microscopy can visualize how leukemic cells are distributed in the osteoblastic or vascular niches of a diseased mouse compared to a healthy equivalent or how transplanted hematopoietic stem and progenitor cells (HSPCs) interact with their microenvironment upon homing into the niche. Osteoblastic cells, which are involved in bone formation, are part of the osteoblastic niche in the bone marrow microenvironment, and they are involved in the lodging of hematopoietic stem cells, as well as their regulation with regard to cell number, quiescence, and differentiation. The bone marrow microenvironment also consists of endothelial cells in the vascular niche, mesenchymal stem cells, neurons, and many other cell types. To further investigate the

relationship between bone marrow and injected leukemia cells, in vivo imaging of the calvarium of a live mouse can be performed using two-photon (or, ideally, confocal two-photon) microscopy as was shown by Lo Celso et al. in 2010 [14]. The obvious advantages of in vivo microscopy compared to other methods are single-cell observations that can be obtained in a live animal with minimally invasive techniques. However, there are some limitations to this method as the maximum depth of observation is only 100 μm with most microscopes. Although this, therefore, may pose a bias with regard to the structures being imaged, this method may help to understand complex interactions between different cell types that occur, e.g. while homing or during first stages of engraftment of hematopoietic stem and progenitor cells, as well as leukemia (-initiating) cells.

Depending on the scientific question, it is possible to image the homing of leukemia-initiating cells within 2–4 h of transplantation or to image leukemic growth at later time points. For imaging of short-term homing, leukemia-initiating cells can be labeled with the lipophilic dye DiD or DiR. For longer-term experiments (>72 h after injection), it is recommended to use permanently labeled cells, e.g., bone marrow cells from an actin-DsRed mouse. The following imaging protocol describes imaging in a short-term homing experiment in the retroviral transduction/transplantation model. Please, note that leukemic mice as donors of leukemia-initiating cells have to be generated first, as insufficient numbers of leukemia-initiating cells are retrieved from the 48-h in vitro culture system:

1. For your experimental setup with the retroviral transduction/transplantation model to generate donors of leukemia-initiating cells, follow common bone marrow transplant procedures involving irradiation of your recipient mice (generally, 450 cGy twice with a period of at least 2 h in between for C57/BL6N mice), harvest of donor bone marrow, and transduction with retrovirus. Between days 17–19 after transplantation, when the CML is fully established, sort for your desired cells by flow cytometry. In this model, the Lin^- c-Kit^+ $Sca\text{-}1^+$ ($CD48^-$ $CD150^+$) fraction will harbor the leukemia-initiating cells (*see* **Note 10**).
2. Label your cells (ideally, approximately 10,000 cells) with DiD (Thermo Fisher, Darmstadt, Hessen, Germany).
3. Anesthetize the recipient mouse, ideally a reporter mouse, with fluorescent bone marrow niche cells, e.g., Col2.3 kb GFP (osteoblastic cells), Tie2-GFP (endothelial cells), or nestin-GFP (mesenchymal stem cells) mice with ketamine/xylazine (ketamine 120 mg/kg body weight, xylazine 16 mg/kg body weight) by intraperitoneal injection. Place Methocel on the mouse's eyes to prevent them from drying out.

4. Remove the fur on the scalp. Cut the scalp open using a sterile pair of scissors. The incision should be made starting between the ears and descending in a straight line toward the nose on one side past the eye. Flap over the scalp and wash remaining hair off the calvarium with sterile PBS. Place Methocel onto the calvarium to allow an airtight seal between the calvarium and coverslip. Place the mouse onto your microscopic stage to hold the mouse's calvarium in a flat position. It is important to keep the mouse warm, either by fixing the mouse in a heatable device or by placing the mouse in an incubator-like structure while under the microscope.
5. Align the lasers on the intersection between the central and the frontal sinus and find the correct plane for imaging. Then find the intersection between the central and the coronal sinus (bottom reference), record those coordinates, and then find the intersection between the central and the frontal sinus (top reference) by following the course of the central sinus and record the coordinates. Start observing and imaging your labeled cells by meandering through the bone marrow cavity and taking stacks of up to 50 images (2 μm sections) of the cells. Vascular dyes can be injected to visualize the vasculature. Reporter mice make it easier to discern the location of the injected leukemia-initiating cells in relation to GFP^+ "landmarks" in the calvarium.
6. After finishing your imaging process either euthanize the animal or close the wound by using sutures or dermal glue. Up to one or possibly two more imaging sessions can be performed on the same mouse, but the scarring of the tissue can decrease the quality of the imaging process (*see* **Note 10**).
7. The analysis of the images in the way of measurement of distances between leukemia-initiating cells to bone, osteoblastic cells, or the vasculature, the counting of leukemic cells per leukemic "nest," or measuring the diameter of such a leukemic "nest" is performed by ImageJ software.

4 Notes

1. This can be done by different techniques: by puncturing the retro-orbital venous plexus or the facial vein or by making a small cut in the tail and then collecting the blood in a microtainer tube. Make sure to mix the blood with the EDTA or heparin in the tube to prevent coagulation.
2. In order to quantitate the tumor burden, it is recommended to focus on the white blood count (WBC, an elevated leukocyte number is a hallmark of CML (and possibly other leukemias))

and on the percentage of granulocytes (% GRA). The reference range for the WBC lies between 3000 and 15,000 cells per μl; the % GRA should be around 10–20 % for a healthy mouse. With disease progression, the WBC normally increases to more than 50,000 cells per μl (we even observe numbers above 400,000 cells per μl, but this is dependent on the type of leukemia and the mouse strain). The % GRA can be used to judge the frequency of granulocytes in comparison to lymphocytes. With disease progression, the % GRA increases due to the proliferation of myeloid cells of various maturation stages. However, the most quantitative assessment of tumor burden is performed by flow cytometry of GFP^+ (BCR-ABL1+) myeloid cells.

3. If you are concerned that your cell numbers are low, place 100 μl of the cold DPBS-FCS mixture into each funnel and spin for 1–2 min, as this will wet the filter and should allow more cells to be pelleted on the slide.
4. Performing this step is necessary because ethanol is polar compared to paraffin. Therefore, both solutions cannot be mixed and a clearing agent like xylene is necessary prior to further processing.
5. As hematoxylin is a basic dye and positively charged, it will react with negatively charged compounds. In this case, it will stain the nucleic acids found in the nucleus blue.
6. Tap water has a basic pH and will therefore change the color of the staining from red to blue. This step will also stabilize the hematoxylin.
7. Most proteins that are present in the cytoplasm are basic (positively charged); the dye will bind to those structures because of its own negative charge, eventually turning them red/pink. You can check the staining and your differentiation process by observing the sample under a microscope: If the cytoplasm has a pale/pink color, repeat your eosin-staining step for another 2–3 min. Check again under the microscope before moving on to the next step.
8. Formalin fixation changes the tertiary structure of antigens, therefore making your antibodies unable to detect them. Antigen retrieval will help in optimizing the antigen-antibody reaction. There are several methods and techniques that can be used for this purpose: using enzymes (protease-induced epitope retrieval (PIER)), which was introduced by Huang in 1975, or by using heat-induced epitope retrieval (HIER), first described in 1991 by Shi et al.
9. The LSAB method is an immunological stain that is based on enzyme-substrate reactions that transform chromogens into

colored end products. The LSAB method can be classified as a "three-step indirect method":

(a) Unconjugated primary antibody

(b) Biotinylated secondary antibody

(c) Peroxidase-labeled streptavidin

(d) Substrate/chromogen

(e) Visualization of color change.
If DAB (acts as an electron-donor) is present, the enzyme (peroxidase) and the substrate-chromogen mix will create a complex that will then, after oxidation of the DAB, change its color to an unsoluble brown end product. In most laboratories, horseradish peroxidase (HRP) is used as an enzyme to catalyze the chromogenic reaction. Streptavidin (derived from *Streptomyces avidinii*) is widely used because of its affinity to biotin.

10. Closing the wound by suturing should be performed if further imaging sessions with this animal are planned as this will lead to less scar tissue than after using dermal glue.

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Chapter 7

The Culture Repopulation Ability (CRA) Assay and Incubation in Low Oxygen to Test Antileukemic Drugs on Imatinib-Resistant CML Stem-Like Cells

Giulia Cheloni and Michele Tanturli

Abstract

Chronic myeloid leukemia (CML) is a stem cell-driven disorder caused by the BCR/Abl oncoprotein, a constitutively active tyrosine kinase (TK). Chronic-phase CML patients are treated with impressive efficacy with TK inhibitors (TKi) such as imatinib mesylate (IM). However, rather than definitively curing CML, TKi induces a state of minimal residual disease, due to the persistence of leukemia stem cells (LSC) which are insensitive to this class of drugs. LSC persistence may be due to different reasons, including the suppression of BCR/Abl oncoprotein. It has been shown that this suppression follows incubation in low oxygen under appropriate culture conditions and incubation times.

Here we describe the culture repopulation ability (CRA) assay, a non-clonogenic assay capable – together with incubation in low oxygen – to reveal in vitro stem cells endowed with marrow repopulation ability (MRA) in vivo. The CRA assay can be used, before moving to animal tests, as a simple and reliable method for the prescreening of drugs potentially active on CML and other leukemias with respect to their activity on the more immature leukemia cell subsets.

Key words Hypoxia, Incubation in low oxygen, Leukemia stem cells, Imatinib mesylate, Minimal residual disease

1 Introduction

The culture repopulation ability (CRA) assay (Fig. 1) was originally developed to measure in vitro the bone marrow repopulation ability (MRA) potential of normal hematopoietic cells and later adapted to study leukemia cell populations [1, 2]. The CRA assay is based on cell transfer from a selective primary liquid culture (LC1) to a growth-permissive secondary liquid culture (LC2), where cells are counted periodically to estimate their kinetics of expansion therein. During LC1, an environmental pressure is applied capable to suppress most of the bulk of cell population but not cells with progenitor/stem cell properties. Such an enrichment is usually obtained by

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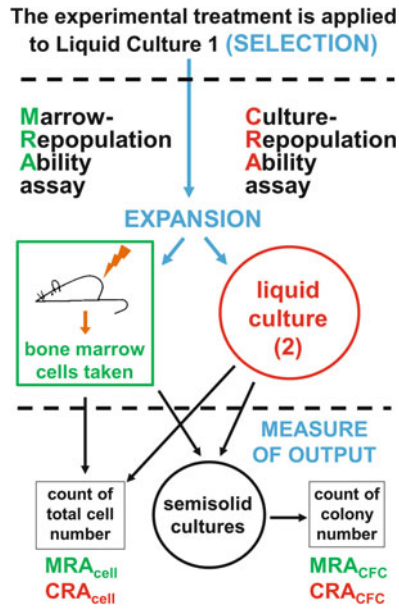

Fig. 1 The culture repopulation ability (CRA) and the marrow repopulation ability (MRA) assays. *CFC* colony-forming cells

incubating LC1 at low-oxygen tension, since hematopoietic and leukemia stem cells are better maintained in "hypoxia" than "normoxia," according to the well-established concept of the "hypoxic stem cell niche" [3–5] where stem cells physiologically reside in vivo (Fig. 2).

Following its adaptation to the study of leukemia cell populations, the CRA assay turned out capable to detect different immature cell subsets, each with peculiar features and abilities, within the same leukemic population. In particular, cells incubated in LC1 in low oxygen for different times repopulate nonselective LC2 (incubated in "normoxia") with different kinetics, which reflect the hierarchical level of stem or progenitor cells enriched in LC1. The kinetics of LC2 repopulation represents, de facto, the estimate of CRA. In chronic myeloid leukemia (CML) cells, incubation in low oxygen is paralleled by the suppression of BCR/Abl oncoprotein [6, 7], so that, after appropriate incubation times, it is possible to select cell subsets completely insensitive to imatinib mesylate (IM). Most likely, these cells constitute, or at least are a substantial fraction of, the leukemia cell subset responsible for minimal residual disease (MRD).

We standardized the application of CRA assay to study CML cell populations by using the K562 cell line. Cells are incubated at 3×10^5 cells/ml in low oxygen (~0.3 % O_2) for 2 or 7 days (LC1). Cells recovered from LC1 at day 2 or day 7 show different phenotypic features (the most remarkable regards the expression

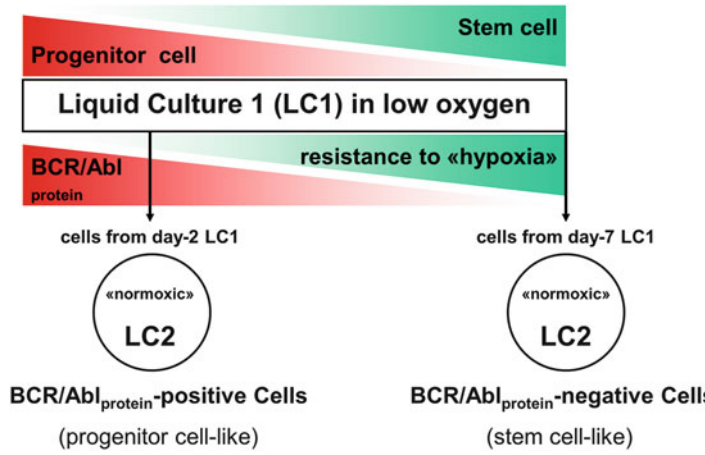

Fig. 2 Relationship of the hierarchical level of CML progenitor and stem cells to resistance to low oxygen

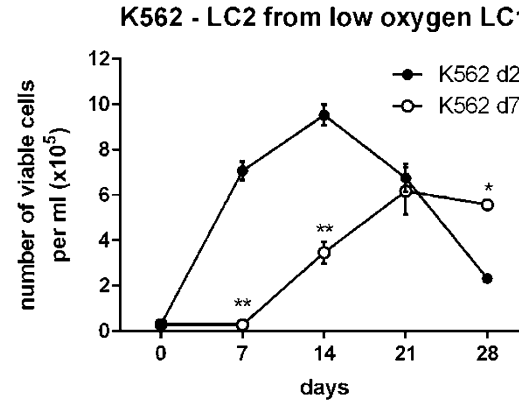

Fig. 3 Culture repopulation ability assay of CML cells incubated in low oxygen. Time courses of the number of viable cells in LC2. Exponentially growing K562 cells were incubated in LC1 in atmosphere containing ~0.3 % O_2 and transferred at day 2 (filled circle) or 7 (circle) into LC2 incubated in air, to determine the maintenance of progenitor- or stem- like cells, respectively, in LC1. Values are means ± S.E.M. of data obtained from four independent experiments. *$p < 0.05$, **$p < 0.01$

of BCR/Abl protein) and different, reproducible, LC2 repopulation kinetics (Fig. 3). CML cells recovered from day-2 LC1 (short-term adaptation to "hypoxia") still express BCR/Abl oncoprotein, so that they *immediately* repopulate LC2, taking advantage of BCR/Abl-dependent growth-promoting signaling ("progenitor cell-like" kinetics). The CRA of these cells is IM sensitive. On the contrary, CML cells recovered from day-7 (*or later*) LC1 (long-term adaptation to "hypoxia") do not express BCR/Abl oncoprotein, so that they repopulate LC2 *after* a 7-day-long lag phase

Table 1
Key features of cell kinetics in the secondary liquid culture (LC2)

LC2 established from	Lag phase (days)	Peak of expansion (days)	Type of culture repopulation	Type of selected cells	$BCR/Abl_{protein}$ at day 1 of LC2
Day 2 of hypoxic LC1	0	7 (range 7–14)	Immediate	Progenitor-like	Expressed
Day 7 of hypoxic LC1	7 (range 7–14)	21 (range 21–28)	Late	Stem cell-like	Not expressed

("stem cell-like" kinetics). The CRA of these cells is completely IM insensitive [6, 7]. The hallmarks of these two types of LC2 kinetics are reported in Table 1 and Fig. 3. On this basis, we describe here how to perform the CRA assay as an in vitro screening method for drugs targeting IM-resistant CML cells. The information on the drug's activity obtained from the CRA assay (yield in LC2) is, of course, usually integrated with that coming from LC1 (kinetics of total cell number in culture, cell viability/apoptosis, cell distribution through cell cycle, etc.).

2 Materials

1. Cells: stabilized CML cell lines (i.e., K562) or primary CML cells (*see* **Note 1**).
2. Culture medium: RPMI 1640 medium supplemented with 10 % heat-inactivated fetal bovine serum, 1 % L-glutamine solution, and 1 % penicillin-streptomycin.
3. Trypan blue solution 0.4 % in PBS.
4. Bürker hemocytometer.
5. "Anaerobic workstation" for cell incubation and manipulation under controlled atmosphere.
6. Tank containing a gas mixture composed of 0.3 % O_2, 94.7 % N_2, and 5 % CO_2.

3 Methods

3.1 Preliminary Steps

1. Maintain K562 cells in tissue flasks at 3×10^5 cells/ml in RPMI medium supplemented as indicated above (*see* **Note 1** for the use of CML cell lines other than K562 and of primary

Table 2
Modalities and type of drug treatment in primary liquid culture (LC1) to target specific subpopulations

Day of treatment in LC1	Target cell population		Other targets	
	IM sensitive (LC2 established with cells recovered at day 2 of LC1)	**IM insensitive** (LC2 established with cells recovered at day 7 of LC1)	**Resistance to hypoxia**	**BCR/Abl pathway or signaling**
0	√	√	X	√
1	√	√	X	√
6	X	√	√	X

CML cells). Renew the medium every 3 days by spinning down cells at 200 × *g* for 5 min at room temperature (RT).

2. Replate cells from maintenance cultures at 5×10^5 cells/ml in fresh medium and incubate for 24 h.

3.2 LC1

1. Establish LC1 by plating cells at 3×10^5 cells/ml in fresh medium (*see* **Note 2**). We recommend to plate cells in at least 10 ml per 25 cm^2 tissue culture flask.
2. Treat LC1 with drug(s) at time 0 or at different incubation times (*see* Table 2 for details).
3. Incubate untreated and treated cultures at 37 °C in a water-saturated low-oxygen atmosphere (containing 0.3 % O_2, 94.7 % N_2, and 5 % CO_2) for 2 or 7 days. Do not renew the medium during LC1 (*see* **Note 3**).
4. Count viable cells by the trypan blue exclusion assay at least at days 2, 3, and 7 of incubation. The number of viable cells at day 7 is critical: a cell density lower than 10^5 cells/ml indicates a successful selection; if necessary, the selective LC1 incubation can be carried on until day 9 (*see* **Note 4**).
5. If needed, collect part of LC1 cells for analyses not directly related to the CRA assay (e.g., estimate of BCR/Abl protein expression, protein and/or nucleic acid extraction, flow cytometry, etc.).

3.3 LC2

1. Recover cells from LC1 at day 2 or day 7 of incubation. Wash the cells twice with fresh medium by spinning at 100 × *g* for 10 min at RT. This step is necessary to reduce cell debris and to remove drugs administered during LC1.

2. Establish LC2 by plating cells at 3×10^4 cells/ml in fresh medium.
3. Incubate the cultures at 37 °C in a water-saturated atmosphere containing 20 % O_2, 75 % N_2, and 5 % CO_2. Do not renew the medium during LC2.
4. Count viable cells by the trypan blue assay every 2 or 3 days at least until cell number reaches the peak of expansion, to determine the LC2 repopulation pattern: peak levels and kinetics (*see* **Note 5**).

3.4 Example #1: Effects of Imatinib, Administered at Time 0 of LC1, on Stem-Like Cells

3.4.1 LC1

1. K562 cells were replated from maintenance cultures at 5×10^5 cells/ml in 20 ml of RPMI medium supplemented as described above in a T75 flask and incubated for 24 h.
2. LC1 were established by plating cells at 3×10^5 cells/ml in 10 ml of fresh medium/flask in two T25 flasks (*see* **Note 2**). Cultures were treated or not (Not Treated, NT) with IM 1 μM at time zero (t0) of incubation.
3. Untreated and treated cultures were incubated in low oxygen (0.3 % O_2, 94.7 % N_2, and 5 % CO_2) for 7 days. The medium was not renewed during LC1 (*see* **Note 3**).

3.4.2 LC2

1. After 7 days of incubation in LC1, the cells were collected into 15 ml tubes and viable cells were counted by the trypan blue assay.
2. Cells were washed twice with fresh medium by spinning at $100 \times g$ for 10 min at RT.
3. LC2 were established by plating cells at 3×10^4 cells/ml in 10 ml of fresh medium/flask in one T25 flask per experimental condition.
4. LC2 were incubated at 37 °C in a water-saturated atmosphere containing 20 % O_2, 75 % N_2, and 5 % CO_2. The medium was not renewed during LC2.
5. During LC2, viable cells were counted by the trypan blue assay every 2 or 3 days, to determine the LC2 repopulation pattern: peak levels and kinetics (Fig. 4) (*see* **Note 5**).

Figure 4 shows the effects of IM added at t0 of LC1 incubation on the CRA of K562 cells. IM did not suppress LC2 repopulation of cells recovered from day-7 LC1, having their kinetics (stem cell-like) equal to that of control cells. Thus, cells long-term adapted to "hypoxia" and capable of repopulation with a "stem cell-like" kinetics were resistant to IM (Fig. 4). Translated to the in vivo scenario, these results are in agreement with the notion of refractoriness of LSC responsible of MRD to IM.

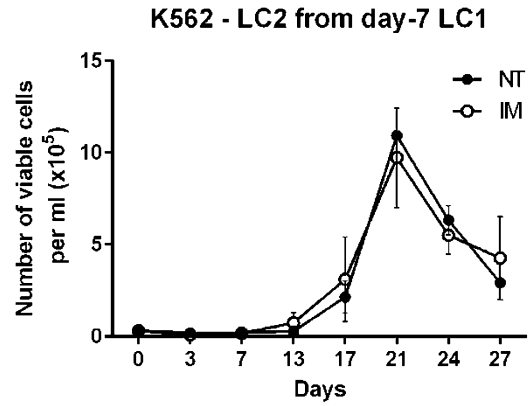

Fig. 4 Effects of imatinib on the culture repopulation ability of CML cells incubated in low oxygen. Time courses of the number of viable cells in LC2. Exponentially growing K562 cells treated (IM, *circle*) or not (NT, *filled circle*) with IM 1 μM were incubated in LC1 in atmosphere containing ~0.3 % O_2 and transferred at day 7 into LC2 incubated in air. Values are means ± S.E.M. of data obtained from four independent experiments

3.5 Example #2: Effects of Bortezomib, Administered at Time 0 of LC1, on Progenitor- or Stem-Like Cells

3.5.1 LC1

1. K562 cells were replated from maintenance cultures at 5×10^5 cells/ml in 20 ml of RPMI medium supplemented as described above in a T75 flask and incubated for 24 h.
2. LC1 were established by plating cells at 3×10^5 cells/ml in 10 ml of fresh medium/flask in two T25 flasks (*see* **Note 2**). Cultures were treated or not (not treated, NT) with bortezomib (BTZ) 5 nM at t0 of incubation.
3. Untreated and treated cultures were incubated in low oxygen (0.3 % O_2, 94.7 % N_2, and 5 % CO_2) for 7 days. The medium was not renewed during LC1 (*see* **Note 3**).
4. Viable cells were counted by the trypan blue assay at days 1, 2, 3, and 7 (Fig. 5a).

3.5.2 LC2

1. After 2 days of incubation in LC1, about 1/3 (3 ml) of LC1 cells (NT and BTZ) was collected into 15 ml tubes and viable cells were counted by the trypan blue assay.
2. Cells were washed twice with fresh medium by spinning at $100 \times g$ for 10 min at RT.
3. LC2 were established by plating cells at 3×10^4 cells/ml in 10 ml of fresh medium/flask in one T25 flask per experimental condition.
4. LC2 were incubated at 37 °C in a water-saturated atmosphere containing 20 % O_2, 75 % N_2, and 5 % CO_2. The medium was not renewed during LC2.

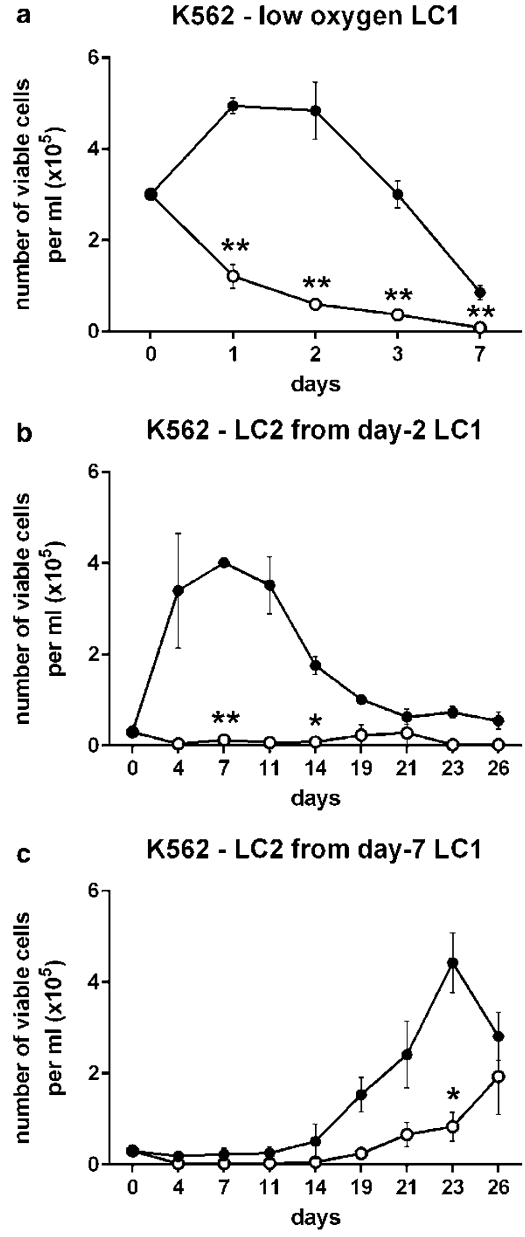

Fig. 5 Effects of bortezomib on the culture repopulation ability of CML cells incubated in low oxygen. (**a**) Exponentially growing K562 cells were treated (BTZ, *circle*) or not (NT, *filled circle*) with BTZ 5 nM in LC1 and incubated in atmosphere containing ~0.3 % O_2. (**b**) Time courses of the number of viable cells in LC2 established with cells recovered from day-2 LC1. (**c**) Time courses of the number of viable cells in LC2 established with cells recovered from day-7 LC1. Values are means $\pm$ S.E.M. of data obtained from four independent experiments. *p value < 0.05, **p value < 0.01

5. After 7 days of incubation in LC1, the remaining LC1 cells were collected to establish LC2 as described above (**steps 1–4**).
6. During LC2 (established with either day-2 or day-7 LC1 cells), viable cells were counted by the trypan blue assay every 2 or 3 days, to determine the LC2 repopulation pattern: peak levels and kinetics (Fig. 5b, c) (*see* **Note 5**).

Here, we show the effects of BTZ (a proteasome inhibitor) added at t0 to K562 cells incubated in low oxygen for a short (2 days) or a long (7 days) period. In this study, published in 2011 [8], CRA assay revealed that the same pharmacological treatment affected two CML cell subsets differently. BTZ determined a marked reduction of cell viability, so that after 7 days of incubation, the number of viable cells in LC1 was close to zero (Fig. 5a); however, it was possible to collect from LC1 a number of viable cells sufficient to establish LC2. BTZ-treated cells recovered from day-2 LC1 were unable to repopulate LC2, indicating that cells short-term adapted to "hypoxia" and capable of repopulating LC2 with a "progenitor cell-like" kinetics were extremely sensitive to the effect of BTZ (Fig. 5b). On the contrary, BTZ-treated cells recovered from day-7 LC1 were able to repopulate LC2, although following a delayed kinetics. This indicates that cells long-term adapted to "hypoxia" and capable of repopulation with a "stem cell-like" kinetics were at least in part resistant to BTZ (Fig. 5c). Translated to the in vivo scenario, these results predict that BTZ would be inactive on leukemia stem cells (LSC) responsible for MRD and refractory to IM.

3.6 Example #3: Effects of Salarin C, Administered at Time 0 or Day 1 of LC1, on IM-Insensitive Cells

3.6.1 LC1

1. K562 cells were replated from maintenance cultures at 5×10^5 cells/ml in 20 ml of RPMI medium supplemented as described above in a T75 flask and incubated for 24 h.
2. LC1 were established by plating cells at 3×10^5 cells/ml in 10 ml of fresh medium/flask in three T25 flasks (*see* **Note 2**). Cultures were treated or not (not treated, NT) with salarin C (SalC) 1 μM at t0 of incubation or after 24 h long preincubation in LC1 (day 1, d1).
3. Untreated and treated cultures were incubated in low oxygen (0.3 % O_2, 94.7 % N_2, and 5 % CO_2) for 7 days. The medium was not renewed during LC1 (*see* **Note 3**).
4. Viable cells were counted by the trypan blue assay at days 1, 2, 3, and 7 (Fig. 6a).

3.6.2 LC2

1. After 7 days of incubation in LC1, cells were collected into 15 ml tubes and viable cells were counted by the trypan blue assay.

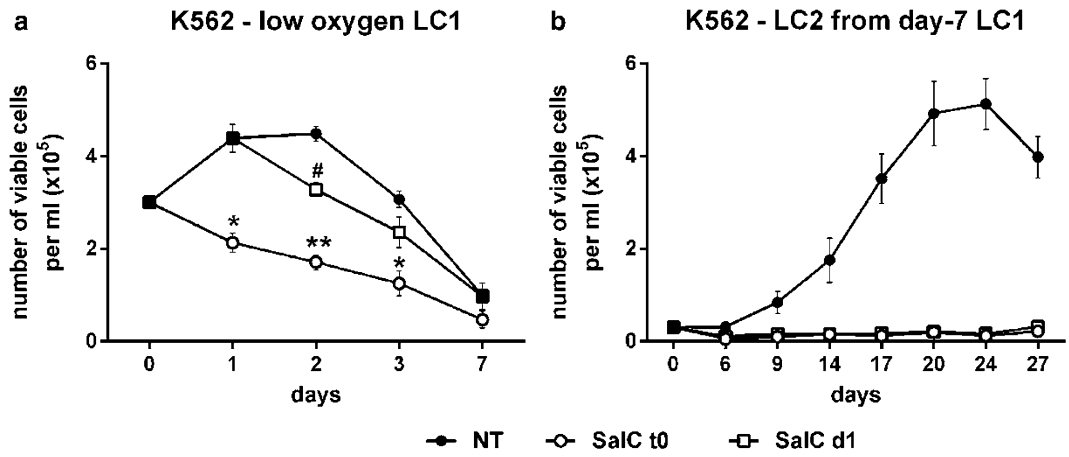

Fig. 6 Effects of salarin C on the culture repopulation ability of CML cells incubated in low oxygen. (**a**) Exponentially growing K562 cells were incubated in atmosphere containing ~0.3 % O_2 (LC1) and treated (SalC) or not (NT, *filled circle*) with SalC 1 μM at time 0 (t0, *circle*) or day 1 (d1, square) of LC1. (**b**) Time courses of the number of viable cells in LC2 established with cells recovered from day-7 LC1. Values are means ± S.E.M. of data obtained from four independent experiments. **p* value < 0.05 (t0 treatment versus NT), ***p* value < 0.01 (t0 treatment versus NT), #*p* value < 0.05 (d1 treatment versus NT)

2. Cells were washed twice with fresh medium by spinning at 100 × *g* for 10 min at RT.
3. LC2 were established by plating cells at 3×10^4 cells/ml in 10 ml of fresh medium/flask in one T25 flask per experimental condition.
4. LC2 were incubated at 37 °C in a water-saturated atmosphere containing 20 % O_2, 75 % N_2, and 5 % CO_2. The medium was not renewed during LC2.
5. During LC2, viable cells were counted by the trypan blue assay every 2 or 3 days, to determine the LC2 repopulation pattern: peak levels and kinetics (Fig. 6b) (*see* **Note 5**).

Figure 6 shows the effects of SalC, a novel proapoptotic compound, on K562 cells treated with the drug at time 0 or day 1 of incubation in low oxygen [9]. SalC administration at day 1 was used to better mimic the scenario most likely occurring in vivo. It is indeed straightforward to think that, in vivo, LSC are already established in the stem cell niches, i.e., adapted to low oxygen, before the beginning of treatment. Accordingly, it has been demonstrated that hypoxia can protect cancer stem cells from drug treatments [5–8, 10, 11]. However, independently of the time of addition to LC1, SalC is able to suppress completely LC2 repopulation driven by cells rescued from LC1 at day 7. Translated to the in vivo scenario, these results predict that SalC would be effective in suppressing LSC responsible for MRD and refractory to IM.

4 Notes

1. **The use of CML cell lines other than K562 and of primary CML cells**. The CRA assay is applicable to any CML cell populations, including stabilized cell lines and primary cells [1, 2, 6–9]. However, different cell lines may behave slightly differently. For example, KCL22 CML cells have slower growth kinetics in low oxygen and consequently a slower glucose consumption than K562 cells. Thus, the suppression of $BCR/Abl_{protein}$ is delayed in KCL22 with respect to K562 cells. The use, in preliminary experiments, of different cell densities at time zero and of different times of incubation in low oxygen is suggested when starting to use a different cell line. The use of primary CML cells requires the addition of stem cell-active cytokines. Add to LC1 10 ng/ml interleukin 3 (IL3), 50 ng/ml stem cell factor (SCF), 50 ng/ml flt3 ligand, 20 ng/ml thrombopoietin, and 10 μg/ml insulin. Add to LC2 10 ng/ml IL3, 50 ng/ml SCF, 20 ng/ml interleukin 6, and 100 ng/ml granulocyte colony-stimulating factor. It is suggested to perform the CRA assay using mononuclear cells obtained by centrifuging the blood or bone marrow sample on a Ficoll-Hypaque gradient.
2. **Cell density at time zero of LC1**. Since a different time-zero cell density corresponds to different kinetics of glucose consumption [7], it is important to establish LC1 with the correct cell density (3×10^5 cells/ml) to maintain the standard kinetics of $BCR/Abl_{protein}$ suppression. Indeed, time-zero cell densities higher than 3×10^5 cells/ml result in a faster consumption of glucose and $BCR/Abl_{protein}$ suppression. An early exhaustion of glucose makes the separate rescue of leukemia progenitor- or stem-like cells from LC1 more difficult. On the contrary, at one-log-lower low cell density (3×10^4 cells/ml), the relatively slow glucose consumption and the consequently delayed $BCR/Abl_{protein}$ suppression allowed a clear identification of three different hypoxia-resistant CML cell subsets: $BCR/Abl_{protein}$-positive cells, capable of *immediate* LC2 repopulation; $BCR/Abl_{protein}$-negative cells, capable of *early* LC2 repopulation; and $BCR/Abl_{protein}$-negative stem-type cells, capable of *late* LC2 repopulation [7].
3. **Do not renew the medium during LC1**. In low-oxygen LC1, $BCR/Abl_{protein}$ expression is also regulated by glucose concentration. $BCR/Abl_{protein}$ expression survives a fairly long incubation at not-limiting residual glucose concentrations in culture medium, in spite of being in low oxygen [7]. Thus, the addition of fresh medium delays the suppression of $BCR/Abl_{protein}$.

4. **Cell density at the end of hypoxic LC1 (day 7 of LC1)**. The number of viable cells at day 7 of LC1 is critical because is an index of cell enrichment/selection in low oxygen. A number of viable cells (trypan blue negative) lower than 10^5 cells/ml indicate a successful selection, whereas a number higher than 10^5 cells/ml indicate a defective selection. To avoid such a defective selection, we recommend counting cells with trypan blue before removing the cultures from the incubator at controlled atmosphere (low oxygen). Indeed, if viable cell number at day 7 is higher than 10^5 cells/ml, it is possible to carry on the LC1 until day 9.
5. **LC2 repopulation: lag phase**. As pointed out above (*see* Table 1), cells transplanted into normoxic LC2 after 7 days of incubation in standard low-oxygen LC1 repopulate the culture after an initial lag phase of 7–14 days. We believe that this kinetics is consequence of the fact that $BCR/Abl_{protein}$ is suppressed in day-7 LC1 and that the delay in LC2 repopulation driven by stem-type culture-repopulating cells is due to the time necessary for the re-expression of $BCR/Abl_{protein}$ following transfer to normoxia [6, 7]. Thus, the absence of the initial lag phase in LC2 indicates a defective selection in LC1.

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Chapter 8

Tumor Suppressor Analysis in CML

Oliver Herrmann and Mirle Schemionek

Abstract

Retroviral models have tremendously contributed to our understanding of CML development and have been indispensable for preclinical drug testing which facilitated the implementation of a targeted therapy. The retroviral insertion of Bcr-Abl into mice that are genetically depleted for a potential tumor suppressor is a tool to test for a specific gene function in Bcr-Abl disease. Here we describe how to generate a Bcr-Abl retrovirus that is subsequently used for infection of primary murine BM cells, which are genetically depleted for a potential tumor suppressor gene. We will suggest control experiments and outline further methods that are required to allow for assessment of disease development upon tumor suppressor knockout in CML.

Key words Retroviral transduction, Knockout cells, Retronectin

1 Introduction

Reactivation of tumor suppressors in CML has previously been shown to strongly affect malignant cell biology. In order to identify and characterize potential tumor suppressor function, a vast number of in vitro and in vivo techniques are available. Depending on the type of tumor suppressor, assays that allow for the detection of cell proliferation or cell cycle, differentiation, survival, or apoptosis can be applied in vitro as well as in vivo. Beyond, the capacity of a respective tumor suppressor protein to alter CML development in vivo can be tested by means of mouse models. Various approaches may be used to generate CML-like disease in mice. These include retroviral approaches [1] or injection of murine or human cell lines or primary cells from CML patients into syngeneic or immunocompromised recipients [2, 3]. Conditional transgenic Bcr-Abl mouse models allow for a cell type-specific Bcr-Abl expression that is depending on the applied promoter/enhancer construct [4, 5]. These models have previously been shown to reduce the variability of retrovirally induced CML. However, generation and breeding of these mice is time consuming. CML models can be manipulated aiming to overexpress or inactivate a potential tumor

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suppressor gene. While overexpression can be achieved, e.g., by viral integration into lineage depleted bone marrow (BM) cells or purified stem cells, inactivation requires the administration of siRNA/shRNA or the utilization of knockout mice [6, 7]. Aiming to identify potential tumor suppressor gene function in vivo, we here describe a method that combines a retroviral approach with the application of a "loss of function" knockout model that had been modified to genetically deplete the potential tumor suppressor gene of interest.

2 Material

All solutions have to be prepared using ultrapure water and analytical grade reagents. Store all solutions and cell culture media at 4 °C (unless indicated otherwise).

2.1 Transfection

1. Plat-E cells [8] (*see* **Note 1**).
2. Retroviral construct containing Bcr-Abl cDNA (*see* **Note 2**).
3. Cell culture medium for Plat-E cells, Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % FBS, 1 % penicillin/streptomycin.
4. Trypsin.
5. Stock solution 10 mg/ml puromycin.
6. Stock solution 10 mg/ml blasticidin.
7. 2 M $CaCl_2$ solution. Weigh 22.198 g of calcium chloride. Add water to a volume of 100 ml. Filter the solution using 0.22 μm sterile filter.
8. 2× HBS Puffer: 274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 15 mM D-Glucose, and 42 mM HEPES (acid free). Weigh 8.0 g NaCl, 0.375 g KCl, 0.0995 g Na_2HPO_4, 0.1350 g D-Glucose, and 5.0 g HEPES (acid free). Fill up with ultrapure water to 450 ml. Adjust pH with NaOH to 7.05–7.06 (*see* **Note 3**). Fill up to a final volume of 500 ml using ultrapure water. Sterile filter the solution using 0.22 μm filter. Produce aliquots and store at 4 °C or at −20 °C for a longer storage period. Avoid multiple freeze/thaw cycles (*see* **Notes 4** and **5**).
9. PBS, cell culture grade.
10. Filters (sterile, 0.22 μm).
11. Plasticware (cell culture dishes 10 cm/6 cm, 15 and 50 ml tubes).
12. Ultrapure water, sterile.
13. Vortexer.
14. Cell counter (e.g., Thoma chamber or automatic cell counting).

2.2 Transduction

1. BM cells from 5-fluorouracil (FU)-treated tumor suppressor knockout and wild-type mice.
2. Noncoated 6-well plates.
3. 15 ml tubes.
4. Sterile ultrapure water.
5. 2 % HSA: e.g., dilute 333 μl 30 % HSA solution with 4.6 ml sterile ultrapure water.
6. 1× HBSS buffer: e.g., dilute 10× HBSS buffer with sterile ultrapure water.
7. Retronectin solution: dilute retronectin (Takara Bio Inc.) 1:12.5 using PBS. Mix, e.g., 400 μl of retronectin with 5 ml sterile PBS (*see* **Note 6**).
8. Retroviral supernatant.
9. Cytokines: recombinant murine IL-3 (stock 100 ng/μl), recombinant murine IL-6 (stock 100 ng/μl), and recombinant murine stem cell factor (SCF, stock 100 ng/μl).
10. 5-Fluorouracil (5-FU, Sigma-Aldrich, ≥99 % HPLC, powder; prepare 10 mg/ml stock solution and use aluminum foil to protect from light).
11. Syringe and needle (20G 1 ½ 0.9 × 40 mm).
12. Medium for infection: BIT9500 (5×, Stemcell Technologies) diluted with IMDM to a working concentration of 1×, supplemented with 1 % penicillin/streptomycin, 10 ng/ml, IL-3, 5 ng/ml, IL-6, and 50 ng/ml SCF (*see* **Note 7**).
13. FACS (fluorescent activated cell sorting) device for detection of GFP-positive cells.

2.3 Transplantation

1. Irradiation facility.
2. Antibiotic (e.g., 96 mg/ml cotrimoxazole stock solution).

2.4 FACS Analysis

1. For LSK (lin^-;$Sca1^+$;$c\text{-}kit^+$) staining, use the following antibodies: PE-Cy5-labeled Gr1 (Invitrogen, RM3006), CD11b (BioLegend, M1/70), B220 (Life Technologies, RM2606), Ter119 (BioLegend, 116210), CD8a (life technologies, 5H1), CD4 (BD Pharmingen, RM4-5) and CD3 (BD Pharmingen, 17A2), combined with Sca-1 (Biotin Ly-6A/E E13-161.7 and BD Pharmingen PE-Cy7 Streptavidin 557598), and c-kit-APC-Cy7 (BD Pharmingen 2B8). To differentiate LT and ST HSC, combine LSK with CD150-APC (BioLegend TC15-12F12.2) and CD48-PB (BioLegend HM48-1) antibodies. For megakaryocytes, use CD41 (eBioscience eBioMWRReg30).

3 Methods

First, consider if your controls are appropriate and comprehensive. For example, if you want to analyze the effect of a tumor suppressor knockdown on CML development, you may want to compare Bcr-Abl-transduced knockout cells with Bcr-Abl-transduced wild-type cells that are derived from the identical genetic background as well as age- and gender matched. In order to rule out any effect that is due to the knockout alone, you may also want to include empty vector control retrovirus that will be transduced into knockout and wild-type BM. Using a retroviral vector that contains a fluorescing marker is highly recommended and will allow you to calculate the number of infected cells that have to be transplanted as well as facilitate tracking of these cells in vivo. The schematic is shown in Fig. 1.

3.1 Production of Bcr-Abl and Empty Vector Control Retrovirus

1. Grow Plat-E cells in DMEM containing 10 % FBS and 1 % penicillin/streptomycin. Two weeks prior to transfection, use puromycin and blasticidin at a final concentration of 1 μg/ ml and 10 μg/ml, respectively, to ensure optimal selection of cells expressing viral proteins.
2. Split cells 5× to 7× every 2–3 days when the culture reaches 80–90 % confluence. Therefore, wash cells once using PBS and completely remove excessive liquids. Subsequently add 3 ml 0.05 % trypsin (dilute in PBS) to a 10-cm dish. Incubate for 2–5 min at 37 °C. Transfer 10 ml of culture medium to a 15-ml

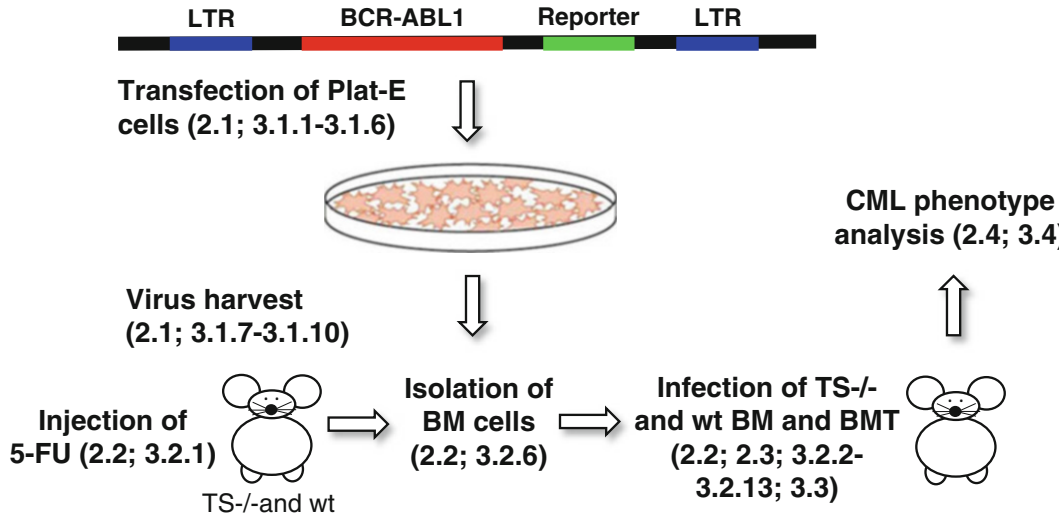

Fig. 1 Schematic overview showing the sequence of techniques that has to be applied for testing of tumor suppressor function in CML in vivo. *TS−/−* tumor suppressor knockout, *wt* wild type, *LTR* long terminal repeats, *BMT* bone marrow transplantation

tube. Using the same pipette, resuspend detached cells and transfer to the 15-ml tube-containing medium. Centrifuge for 5 min at (314 × *g*) and room temperature. Discard the supernatant and wash the cells once with PBS. Resuspend the cell pellet using 5 ml of culture medium and dilute as required by initial cell density. Add puromycin and blasticidin as described.

3. For transfection of Plat-E cells, seed 1.5×10^6 cells/6-cm dish in 5-ml culture medium without puromycin and blasticidin selection (*first day*). Avoid cell clumps and make sure that the cells are equally distributed in the cell culture dish, as this would otherwise reduce your transfection efficiency.
4. Change culture medium 24 h after cell seeding (*second day*). Washing with PBS is not required. Be careful as cells detach easily. Medium should be changed 1–2 h prior to transfection.
5. Prepare plasmid-$CaCl_2$ master mix. Therefore, mix ultrapure water with 2 M $CaCl_2$ and 8 μg of your empty vector control or Bcr-Abl retroviral construct in a 50-ml tube (*see* **Note 8**). The master mix needs to be vortexed rigorously. While vortexing, add dropwise the indicated amount of 2× HBS buffer. This will form the plasmid calcium phosphate-DNA precipitates. Immediately after adding 2× HBS, the mixture needs to be added dropwise to the Plat-E cells (1 ml per 6-cm dish). Make sure that the mixture is distributed homogenously throughout the whole dish.
6. Approximately 18 h after transfection, replace the medium containing the calcium-phosphate-DNA mixture with 5 ml of fresh medium (*third day*) (*see* **Note 9**).
7. Collect the retroviral supernatant on the *fourth day* (24 h after changing the medium) (*see* **Note 10**).
8. Viral supernatants need to be sterile-filtered before storage or further use.
9. It would be ideal to use the virus shortly after production, but storing at 4 °C for up to 2 weeks or at −80 °C is also possible.
10. Test virus with the appropriate method to evaluate the correct titer (*see* **Note 11**).

3.2 Retroviral Transduction of Murine Knockout Cells Using Retronectin

1. Inject 4 mg/20 g body weight 5-FU i.v. into mice harboring a genetic depletion for the potential tumor suppressor gene 4 days prior to bone marrow harvest (*see* **Note 12**).
2. On the day of bone marrow harvest, first add 1.5 ml of diluted (1:12.5) retronectin solution to noncoated 6-well plates. Incubate at room temperature for at least 2 h. Alternatively, coating can be performed overnight at 4 °C.

3. Remove retronectin from each well and block using 2 % HSA for 30 min at room temperature (*see* **Note 13**).
4. Remove the HSA solution and wash each well using 1 ml 1× HBSS puffer.
5. Add 3 ml of retrovirus to each well and centrifuge at (1363 × *g*) for 45 min at 4 °C. Remove viral supernatant and repeat the procedure twice.
6. Apply up to 2 × 10^6 lymphocytes per 6-well plate. Therefore, isolate cells by flushing the tibia and femur using 4 °C PBS/2 % FCS, a syringe, and 20G 1 ½ needle. Centrifuge the cells for 5 min at (314 × *g*), 4 °C, and resuspend them in IMDM-diluted BIT9500 medium for infection containing 10 ng/ml IL-3, 5 ng/ml IL-6, and 15 ng/ml SCF. Apply cells onto the well.
7. Centrifuge the 6-well plate for 5 min at (314 × *g*) and room temperature to spin down cells and promote direct cell-virus interaction.
8. Incubate cells overnight at 37 °C, 5 % CO_2.
9. Repeat **steps 2–5** on the second day.
10. Resuspend cells by carefully pipetting up and down. Transfer the cells onto the new virus-coated 6-well plate and repeat **step 7**.
11. Repeat **steps 2–7** (excluding 6) on the third day.
12. Evaluate transduction efficiency, e.g., by FACS for fluorescent marker on the evening of that day.
13. Calculate numbers of transfected cells for subsequent equal transplantation of infected cell numbers and inject approximately 1200 Bcr-Abl-infected cells for disease development within 2–3 weeks (*see* **Note 14**).

3.3 Transplantation

1. Prior to transplantation, irradiate recipients lethally to facilitate long-term engraftment of donor cells. Dose of irradiation is depending on the mouse genetic background but also affected by the respective radiation source. Use genetically matched donor/recipient mice. For transplantation, resuspend the number of cells that have to be injected per mouse i.v. in 100–200 µl PBS per recipient. As infection prophylaxes, add an antibiotic (e.g., cotrimoxazole 1:1000) to the drinking water that should be changed twice a week until 2 weeks after transplantation.

3.4 FACS Analysis

1. Peripheral blood can be analyzed to confirm engraftment and disease development by FACS analysis for fluorescing cells.
2. Analyses of BM and spleen cells can be performed by FACS using antibodies for specific markers (e.g., $Gr1^+$;$CD11b^+$ for myeloid cells, $CD3^+$ for T cells, $B220^+$ for B cells, $CD41^+$ for

megakaryocytes, Ter119$^+$ for erythrocytes, lin$^-$;Sca-1$^+$;c-kit$^+$ for LSK cells, LSK$^+$;CD150$^+$;CD48$^-$ for long-term repopulating hematopoietic stem cells, LSK$^+$;CD150$^-$,CD48$^-$ for short-term repopulating hematopoietic stem cells, and LSK, CD150+,CD48+ for multipotent progenitors, combined with the vector-confirmed expression of the fluorescing marker to identify leukemic cells). Moreover, spleen weight and histology of the spleen and BM as well as level of Bcr-Abl expression will allow for identification of an altered phenotype due to inactivation of the potential tumor suppressor gene. To assess for self-renewal capacity, serial transplantations should be performed.

4 Notes

1. Splitting Plat-E cells one the day before seeding increases the rate of transfected cells. Prevent that Plat-E cells exceed a confluence of 90 %.
2. The transfection efficiency increases with DNA purity and concentration, and this often results in higher virus titer.
3. Calibrate pH meter before use. It is critical to adjust the exact pH value. Otherwise, transfection efficiency is decreasing drastically.
4. Test the produced 2× HBS prior to use for large transfection approaches.
5. The ability to form $CaPO_4$ DNA complexes of 2× HBS buffer is reduced after 6 months, even upon storing at −20 °C.
6. It is recommended to aliquot retronectin prior to dilution to avoid repeated thaw/freezing cycles. These aliquots need to be stored at −20 °C.
7. Prepare culture media, always fresh before use. Store media at 4 °C and use it up within 1 week otherwise cytokines can lose their activity.
8. Up to four transfection reactions can be mixed together in one 50-ml tube.
9. If your vector confers expression of a fluorescing marker, you can check the success of your transfection using a fluorescent microscope.
10. Isolation of protein lysates of transfected Plat-E cells will allow you to confirm expression of Bcr-Abl via western blotting in transfected cells.
11. For subsequent experiments, it is important to match virus titer of Bcr-Abl retrovirus by, e.g., combining different batches before transduction. If you want to compare the effect of knockout in Bcr-Abl-positive cells versus Bcr-Abl-negative

cells side by side, you should match virus titer of Bcr-Abl retrovirus and empty vector control in addition.

12. Prepare 5-FU solution freshly on the day of injection and rotate for approximately 3 h at room temperature before use. Protect from light.
13. Retronectin can be reused for up to five times. Therefore, reuse retronectin within 3 weeks and store at 4 °C.
14. Do not exceed 3 days of cultivation as the cells undergo differentiation and this reduces the potency to induce CML phenotype in recipient mice. If you used matched titers for Bcr-Abl transduction of knockout and wild-type cells, you can inject the same number of total cells, after confirming same transduction efficiency by FACS. If you transplant 1×10^6 cells, rescue BM is not required. However, if you reduce the number of total injected cells due to high transduction efficiency, you should add 1×10^5 rescue BM or spleen cells.

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Chapter 9

Detecting Autophagy and Autophagy Flux in Chronic Myeloid Leukemia Cells Using a Cyto-ID Fluorescence Spectrophotometric Assay

Sujuan Guo, Kevin J. Pridham, and Zhi Sheng

Abstract

Autophagy is a catabolic process whereby cellular components are degraded to fuel cells for longer survival during stress. Hence, autophagy plays a vital role in determining cell fate and is central for homeostasis and pathogenesis of many human diseases including chronic myeloid leukemia (CML). It has been well established that autophagy is important for the leukemogenesis as well as drug resistance in CML. Thus, autophagy is an intriguing therapeutic target. However, current approaches that detect autophagy lack reliability and often fail to provide quantitative measurements. To overcome this hurdle and facilitate the development of autophagy-related therapies, we have recently developed an autophagy assay termed as the Cyto-ID fluorescence spectrophotometric assay. This method uses a cationic fluorescence dye, Cyto-ID, which specifically labels autophagic compartments and is detected by a spectrophotometer to permit a large-scale and quantitative analysis. As such, it allows rapid, reliable, and quantitative detection of autophagy and estimation of autophagy flux. In this chapter, we further provide technical details of this method and step-by-step protocols for measuring autophagy or autophagy flux in CML cell lines as well as primary hematopoietic cells.

Key words Autophagy, Chronic myeloid leukemia, Cyto-ID fluorescence spectrophotometric assay, Cyto-ID autophagy assay

1 Introduction

Macroautophagy (referred to as autophagy hereafter) is a degradation pathway utilized by cells under stressful conditions such as nutrient deprivation or drug treatment to support extended survival. During this process, cellular components are broken down to macromolecules to be re-assimilated for consumption to sustain cell survival [1–4]. With autophagy serving an important role between life and death for a cell, it has implications in not only maintaining homeostasis of normal cells but also the growth and survival of malignant cells [5, 6]. Therefore, it plays a central role in

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the pathogenesis as well as drug resistance of cancer and other human diseases such as diabetes, heart failure, infectious disease, and neurodegenerative disease along with many more [5, 7].

Chronic myeloid leukemia (CML) is a hematopoietic malignancy arising from myeloid progenitor cells that manifests from the accumulation of malignant granulocytes in the bone marrow, blood, and spleen. This cancer was first described by Peter C. Nowell and David Hungerford more than 50 years ago [8]. Its molecular signature—the Philadelphia (Ph) chromosome—was then discovered in the 1970s [9] and verified in the 1980s [10–12]. The Ph chromosome results from the translocation of the long arms of chromosomes 9 and 22, which leads to the fusion between two genes, *c-ABL* (human homologue of the Abelson murine leukemia virus) and *BCR* (breakpoint cluster region). This fusion results in an oncoprotein, BCR-ABL, which is a constitutively active tyrosine kinase that aberrantly regulates molecular pathways important for cell survival/death including autophagy [13, 14]. The first line of treatment for CML is imatinib, a tyrosine kinase inhibitor that selectively targets BCR-ABL [15]. However, CML patients eventually relapse and become resistant to imatinib due to BCR-ABL mutations or activation of other BCR-ABL-independent cellular survival pathways [16–19]. In this regard, autophagy has emerged as a key factor in BCR-ABL-independent drug resistance. Targeting autophagy has been extensively investigated in antagonizing such resistance [20–28]. However, a major hurdle that prevents the development of effective autophagy-related treatments is the lack of an accurate quantitative method to measure autophagy in cells and tissues.

To address this problem, we recently developed a novel autophagy detecting method dubbed the Cyto-ID fluorescence spectrophotometric assay (abbreviated as the Cyto-ID autophagy assay) [29]. This assay uses the Cyto-ID cationic amphiphilic tracer dye that labels autophagic compartments such as autophagosomes, amphisomes, and autolysosomes, with minimal staining of lysosomes [29, 30]. This Cyto-ID autophagy assay is significantly more sensitive, reliable, and time saving over other classic approaches such as LC3B immunoblotting or monodansylcadaverine staining [29]. Autophagy is a dynamic process composed of multiple steps. The entire operation is therefore called autophagy flux. Measuring autophagy flux is important because it indicates the completion of the autophagy process and whether autophagy cargos are successfully degraded. However, monitoring autophagy flux is difficult [31–33]. The Cyto-ID autophagy assay permits a quantitative and reliable estimation of autophagy flux [29]. In this chapter, we provide technical details describing the quantitative measurement of autophagy and estimation of autophagy flux in CML cell lines as well as primary hematopoietic cells using the Cyto-ID autophagy assay.

2 Materials

2.1 Cells

1. K562: K562 is a human CML cell line purchased from the American Type Culture Collection. Cells are maintained in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (*see* **Note 1**).
2. 32D/BCR-ABL: 32D/BCR-ABL cells (kindly provided by Dr. Michael Green of the University of Massachusetts Medical School) are mouse myeloid 32D cells transformed with the BCR-ABL oncogene. Cells are maintained in RPMI-1640 medium supplemented with 10 % FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (*see* **Note 1**).
3. Mouse peripheral white blood cells: Peripheral blood is collected freshly from C57BL/6 mice. Red blood cells are removed using the approach described below (*see* Subheading 3.3, **step 2**). The resulting white blood cells are immediately used for autophagy assays.
4. Mouse primary bone marrow cells: Primary bone marrow cells are harvested freshly from femurs and tibiae of SCID beige mice. Red blood cells are removed from the bone marrow using the approach described in Subheading 3.4, **step 4**. Isolated bone marrow cells are immediately used for autophagy assays.

2.2 Reagents

1. Cyto-ID fluorescence dye: The Cyto-ID fluorescence dye is purchased from Enzo Life Sciences. After receiving the dye, prepare 5-μl aliquots in amber microcentrifuge tubes or in tubes wrapped with aluminum foil and store aliquots at −20 °C (*see* **Note 2**). Prepare the working solution (1:1000 dilution) freshly when needed (Subheading 3.1, **step 4**).
2. CellTiter 96® Aqueous MTS Reagent (MTS) (*see* **Note 3**): The MTS reagent powder is purchased from Promega. To prepare the stock solution, dissolve 42 mg of MTS powder in 21 ml DPBS (0.2 g/l KCl, 8.0 g/l NaCl, 0.2 g/l KH_2PO_4 1.15 g/l Na_2HPO_4 100 mg/l $MgCl_2{\cdot}6H_2O$, and 133 mg/l $CaCl_2{\cdot}2H_2O$). Adjust the pH of MTS/DPBS solution to 6.5 using 1 N HCl. Sterilize the solution by passing it through a 0.2-μm filter. Keep sterilized solution in the dark. Prepare 0.92 mg/ml phenazine methosulfate (PMS) (Sigma) using DPBS. Sterilize the solution using the above approach and keep it in the dark. Mix 1 ml of PMS solution and 20 ml of MTS/DPBS solution. Aliquot 1 ml of the resulting MTS reagent into 1.5-ml amber microcentrifuge tubes. Store aliquots at −20 °C. Freshly prepare working solution as described in Subheading 3.1, **step 7**.

3. CellTiter-Blue® Cell Viability Assay kit (*see* **Note 3**): The CellTiter-Blue® Cell Viability Assay kit is purchased from Promega. After receiving the reagent, prepare 1-ml aliquots using 1.5-ml amber microcentrifuge tubes. Store aliquots at −20 °C. Freshly prepare working solution (Subheading 3.1, **step 7**).

2.3 Chemicals

1. Chemicals for cultured cells: Chloroquine and imatinib are used for either blockade of autophagy flux or autophagy induction, respectively (*see* **Note 4**) [20, 34]. Prepare stock solutions for cultured cells as follows. Dissolve 25 mg of chloroquine in 0.969 ml of sterile water to make 50 mM stock solution. Dissolve 25 mg of imatinib in 0.848 ml of DMSO to make 50 mM stock solution. Prepare 50-μl aliquots. Store aliquots at −20 °C. When needed, prepare 2× working solutions using cell culture media.
2. Chemicals for autophagy assay in mice: Prepare stock solutions of chloroquine and imatinib used for mice as follows. Dissolve 30 mg of PP242 in 1 ml DMSO to prepare a 5× stock solution, 5 mg of chloroquine, or 10 mg of imatinib in 1 ml of sterile water to make a 10× stock solution (*see* **Note 5**). Prepare 100-μl aliquots. Store the aliquots at −20 °C. When needed, prepare 1× working solutions using sterile water.

2.4 Animals

6–8-week-old C57BL/6 and SCID/beige mice are purchased from Charles River Laboratories. In the results presented previously [29], mice were housed in the animal facility located at the Virginia Tech Carilion Research Institute, and all the experiments using these animals were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech. It is critical to house the animals in a proper facility and the protocol for using animals in your experiments should be approved by the IACUC of your institution before your experiments start.

3 Methods

3.1 Autophagy Detection in K562 Cells

1. When K562 cells reach 50–80 % confluence, shake the flasks to break any cell clumps and count cells using a hemocytometer. Inoculate cells in a T-75 flask at a cell density of 5×10^4 cells per ml (*see* **Note 6**). Incubate cells at 37 °C with 5 % CO_2 overnight.
2. Count cells again and plate 7.5×10^4 cells in 0.75 ml culture medium per well in a 24-well plate (*see* **Notes 7** and **8**). Prepare 2× working solutions of imatinib (4 μM) and chloroquine (10 μM) in 0.75 ml culture media from the stock solutions described in Subheading 2.3, **item 1**. Add drug dilutions to cells directly. The final concentrations of imatinib and

chloroquine are 2 and 5 μM, respectively. The final cell density is 5×10^4 per ml. Use equal volume of DMSO or sterile water as the vehicle controls.

3. Incubate cells at 37 °C with 5 % CO_2 for 4–8 h. Perform the rest of the steps at room temperature. Spin cells down at 400 × *g* for 5 min. This centrifugation setting is used for collecting K562 cells thereafter. Carefully remove supernatant by aspiration. Warm 10× assay buffer to room temperature. Prepare fresh 1× assay buffer using sterile deionized water and 1× assay buffer supplemented with 5 % FBS (*see* **Note 9**). Add 1 ml of 1× assay buffer supplemented with 5 % FBS and gently pipette up and down to resuspend cells (*see* **Note 10**). Collect cells by centrifugation and carefully aspirate the supernatant.
4. Stain cells with Cyto-ID according to the manufacturer's instructions with modifications. Thaw Cyto-ID dye solution (Subheading 2.2, **item 1**) at room temperature. Protect the solution from light by wrapping the tube with aluminum foil (*see* **Note 11**). Prepare Cyto-ID working solution by mixing 1 μl Cyto-ID dye with 1 ml of 1× assay buffer supplemented with 5 % FBS (*see* **Note 12**). Resuspend cell pellets in 100 μl of Cyto-ID working solution (*see* **Note 13**). Break cell clumps by gentle pipetting. Incubate cells at 37 °C with 5 % CO_2 for 30 min. Protect cells from light.
5. Perform cell wash as follows. Spin cells down. Add 1.4 ml of 1× assay buffer to the cell suspension. Resuspend cells by pipetting up and down gently. Collect cells by centrifugation. Carefully aspirate the supernatant without disturbing the cell pellets. Repeat this cell wash step once. Collect cells by centrifugation and aspirate the supernatant. Resuspend cell pellets in 500 μl of 1× assay buffer. Mix well by gentle pipetting.
6. Add 75 μl of cell suspension per well (three wells in total) to a black round-bottom 96-well plate (Fisher Scientific) (*see* **Note 14**). Include blank controls by adding 75 μl per well (three wells in total) of 1× assay buffer with no cells (*see* **Note 15**). Shake the plate for 60 s in a FilterMax F3 microplate reader (Molecular Devices) using a high-speed orbital shaking mode. Wrap the plate with aluminum foil and leave the plate at room temperature for 5–10 min (*see* **Note 16**). Measure the Cyto-ID fluorescence at excitation 480 nM and emission 530 nM using the FilterMax F3 microplate reader.
7. Add 75 μl of cell suspension per well (three wells in total) in a 96-well cell culture plate (not the black round-bottom plate) (*see* **Note 17**). Include blank controls by adding 75 μl of 1× assay buffer with no cells per well (three wells in total). Prepare 2× MTS working solution by diluting stock solutions (Subheading 2.2, **item 2**) 1:10 using RPMI-1640 medium

supplemented with 20 % FBS. Add 75 μl of 2× MTS working solution to each well. Mix by gentle pipetting and incubate at 37 °C with 5 % CO_2 in the dark for 1–4 h. Record the MTS absorbance at 490 nm using the FilterMax F3 microplate reader. The manufacturer recommends using Hoechst 33342 to determine the cell number. However, our results suggest that Hoechst 33342 staining is not appropriate for determining cell number particularly when cells are treated with growth inhibitors (*see* **Note 18**).

8. Alternatively, cell number can be determined by the CellTiter-Blue viability assay (*see* **Note 19**). To carry out this assay, resuspend cell pellets in 250 μl of 1× assay buffer and measure the Cyto-ID fluorescence as described in Subheading 3.1, **step 6**. Prepare 2× CellTiter-Blue working solution by diluting stock solutions (Subheading 2.2, **item 3**) 1:10 using RPMI-1640 medium supplemented with 20 % FBS. Add 75 μl of 2× CellTiter-Blue working solution to each well. Mix by gentle pipetting and incubate at 37 °C with 5 % CO_2 in the dark for 1–4 h. Record CellTiter-Blue florescence at excitation 560 nm and emission 590 nm using the FilterMax F3 microplate reader.
9. Analyze data as follows. Subtract the readings of MTS absorbance (or CellTiter-Blue fluorescence readings depending upon which method is used) or Cyto-ID fluorescence with the average readings of corresponding blank controls. Divide the readings of Cyto-ID fluorescence by those of MTS absorbance (or CellTiter-Blue fluorescence) yielding the relative Cyto-ID fluorescence intensities. Divide the relative Cyto-ID fluorescence intensities of treated groups (i.e., imatinib or chloroquine) with those of untreated groups (DMSO or water). The resulting fold changes represent the difference in the amount of autophagic compartments between untreated and treated groups.

3.2 Autophagy Flux Detection in K562 Cells

1. Prepare K562 cells as described in Subheading 3.1, **step 1**. Prepare 4× imatinib (4 μM) and chloroquine (10 μM) using cell culture medium from stock solutions (Subheading 2.3, **item 1**). Set up four treatment groups as follows: (1) DMSO, (2) 1 μM imatinib, (3) 2.5 μM chloroquine, and (4) imatinib + chloroquine. Incubate cells at 37 °C with 5 % CO_2 for 6 h.
2. Collect cells and perform the Cyto-ID autophagy assay as described in Subheading 3.1, **steps 2–9**.
3. Calculate the fold changes of Cyto-ID fluorescence by normalizing the Cyto-ID intensities of group 2–4 with that of group 1 (*see* **Note 20**). Please refer to the results presented in Fig. 5a in reference 29.

3.3 Autophagy Detection in Peripheral Blood Cells Harvested from Mice

1. Prepare working solutions of PP242 (6 mg/ml) and chloroquine (5 mg/ml) freshly (Subheading 2.3, **item 2**). Administer 60 mg/kg of PP242 or 50 mg/kg of chloroquine into C57BL/6 mice (Subheading 2.4) in a BSL-2 biosafety cabinet through intraperitoneal injection using an insulin syringe with a 28-gauge, ½-inch long needle (0.36 mm × 13 mm). Use equal volume of DMSO or water as the control. After 0, 2, 4, and 8 h, cut the tail vein using a small blade and collect 30 μl of blood from each animal using a capillary pipette with ethylenediaminetetraacetic acid (EDTA) (*see* **Note 21**). Place the capillary pipettes into a 1.5-ml microcentrifuge tube filled with 1 ml of ice-cold 1× PBS (0.201 g/l KCl, 8.006 g/l NaCl, 0.272 g/l KH_2PO_4, and 1.420 g/l Na_2HPO_4). Keep the tubes on ice.
2. Flush blood from the capillary pipettes using 1× PBS. Spin cells down at 400 × *g* for 15 min at 4 °C (*see* **Note 22**). This centrifugation setting is used for collecting primary peripheral blood or bone marrow cells thereafter. Carefully decant the supernatant without disturbing the cell pellets. Add 750 μl of red blood cell lysis buffer containing 8.3 g/l ammonium chloride and 0.01 M Tris-HCl (pH 7.5). Gently mix for 4 min on a rotary shaker. Transfer the cell suspension into a 15-ml Falcon tube and add 14 ml of 1× PBS supplemented with 5 % FBS. Spin the white blood cells down at 4 °C. Aspirate the supernatant carefully without disturbing the cell pellets.
3. Resuspend cell pellets in 100 μl of the Cyto-ID working solution (per 10^5 cells) (Subheading 3.1, **step 4**). Transfer cell suspension into a new 1.5-ml microcentrifuge tube. Perform the Cyto-ID staining described in Subheading 3.1, **step 4**.
4. Wash cells twice as described in Subheading 3.1, **step 5**, except that cells are spun at 400 × *g* for 15 min, and the washed cell pellets are resuspended in 50 μl of 1× assay buffer before reading fluorescence (*see* **Note 23**).
5. Add all the 50 μl of cell suspension to a black round-bottom 96-well plate. Measure the Cyto-ID fluorescence as described in Subheading 3.1, **step 6**. After recording, add 50 μl of 2× - CellTiter-Blue reagent. Follow steps described in Subheading 3.1, **step 8**, to determine the cell number using the CellTiter-Blue viability assay.
6. Calculate the fold changes of Cyto-ID fluorescence using the approach described in Subheading 3.1, **step 9**. Please refer to the results shown in Fig. 10a in reference 29.

3.4 Autophagy Detection in Bone Marrow Cells Isolated from Leukemic Mice

1. Spin 32D/BCR-ABL cells at 400 × *g* for 5 min and resuspend cells in RPMI-1640 medium at 10^7 cells/ml. Keep cells on ice when transferring them from cell culture room to animal facility. Anesthetize SCID/beige mice using isoflurane inhalation.

Dilate tail veins using a Mouse Tail Illuminator Restrainer (Braintree Scientific, Inc.). Inject 150 μl of cell suspension (equivalent to 1.5×10^6 cells per injection) into a SCID/beige mouse through the dilated tail vein using an insulin syringe with a 28-gauge, ½-inch long needle.

2. 6 days after injection of 32D/BCR-ABL cells, SCID/beige mice are treated as follows. Prepare working solutions of imatinib (10 mg/ml) and chloroquine (5 mg/ml) from stock solutions (Subheading 2.3, **item 2**). Feed mice daily with 10 μl of imatinib per gram of mouse body weight (equivalent to 100 mg/kg/day) through gavage using an 18-gauge gavage-feeding needle. Inject mice daily with 10 μl of chloroquine per gram of mouse body weight (equivalent to 50 mg/kg/day) through intraperitoneal injection using an insulin syringe with a 28-gauge, ½-inch long needle. Repeat the treatments daily for 5 days.
3. 5 days after treatment, euthanize the mice. Position the mouse on a dissection board and wipe the skin with 70 % ethanol. Expose the femurs and tibiae by removing skin and muscle using a razor blade. Cut both ends of the bones. Withdraw 3 ml sterile 1× PBS into a 3-ml syringe with a 26-gauge needle. Insert the needle into one end of the bone. Flush out the bone marrows into a 50-ml Falcon tube.
4. Spin cells down at 400 × *g* for 10 min at 4 °C. This centrifugation setting is used for collecting bone marrow cells thereafter. Carefully aspirate the supernatant without disturbing the cell pellets. Resuspend cell pellets in 0.5 ml of 1× PBS. Add 5 ml of red blood cell lysis buffer described in Subheading 3.3, **step 2**. Gently mix for 5 min in a rotary shaker. Stop the reaction by adding 14 ml of 1× PBS supplemented with 5 % FBS. Spin cells down. Aspirate the supernatant carefully without disturbing cell pellets.
5. Resuspend cell pellets at a cell density of 10^6 cells/ml using 1× assay buffer supplemented with 5 % FBS. Dispense 100 μl of cell suspension to a 1.5-ml microcentrifuge tube and spin down cells. Resuspend cell pellets in 100 μl Cyto-ID staining solution (Subheading 2.2, **item 1**). Perform the Cyto-ID autophagy assay following Subheading 3.1, **steps 4–9**. Please refer to the results shown in Fig. 10b in reference 29.

4 Notes

1. K562 cells proliferate rapidly with a doubling time of approximately 21 h. Thus, these cells are easily overgrown and have a higher level of basal autophagy. Such a high basal-level autophagy often interferes with the detection of induced or inhibited

autophagy. It is therefore recommended that K562 cells be maintained at a cell density of 10^5–10^6 cells/ml. This caution should also be applied to 32D/BCR-ABL and other fast-growing cancer cells.

2. We recommend that the Cyto-ID dye avoid frequent freeze/thaw cycles and not be exposed to light. This step helps preserve the dye for long-term use.
3. To minimize the difference of cell numbers among each measurement of Cyto-ID fluorescence, we recommend using CellTiter 96® Aqueous MTS Reagent or CellTiter-Blue® reagent (*see* **Note 17** for details).
4. Chloroquine is a lysosome inhibitor that blocks the formation and function of autolysosomes thereby impairing autophagy flux [34]. Imatinib activates autophagy in K562 cells [20]. These two drugs are used as the positive controls to monitor the inhibition of autophagy flux or induction of autophagy.
5. Imatinib methylate is water soluble and suitable for gavage feeding. Imatinib tablets (for human patients) form turbid liquid in water and this type of liquid is also suitable for gavage feeding in animals. We have successfully fed mice with imatinib tablets dissolved in water in our previous reports [20, 35].
6. As described above, basal-level autophagy increases in overgrown K562 cells. To address this, we recommend seeding K562 cells at a cell density of 5×10^4 cells/ml and incubating overnight before performing autophagy assays.
7. It is less likely that overnight incubation changes cell number significantly as K562 cells have a doubling time of approximately 21 h. However, we recommend recounting K562 cells and plating an equal number of cells for each treatment.
8. We showed that K562 cells at a high cell density exhibited increased levels of Cyto-ID fluorescence (Fig. 1), which indicate a high basal-level autophagy. To further determine the effect of cell density on autophagy induction, we treated K562 cells with imatinib at the dose that induces autophagy [20]. Our results show that imatinib induced a >fivefold increase of Cyto-ID in K562 cells with a cell density at 5×10^4 cells/ml, whereas only twofold increase was observed in imatinib-treated K562 cells with a cell density at 10^5 cells/ml (Fig. 2). These results suggest that K562 cells plated at 10^5 cells/ml or higher yield high background levels of Cyto-ID, which significantly compromises the detection of induced autophagy. Thus, the optimized cell density for the Cyto-ID autophagy assay in K562 cells is approximately 5×10^4 cells/ml. If using different types of cells, we strongly recommend performing the Cyto-ID autophagy assay at different cell densities to determine the optimal cell density that gives rise to a

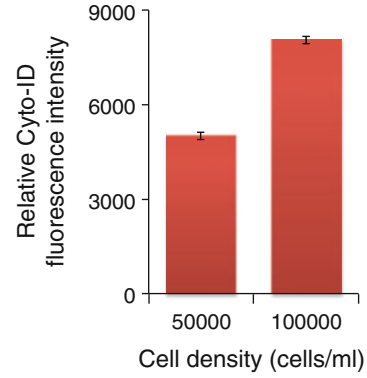

Fig. 1 The relative Cyto-ID levels in K562 cells at different cell densities. K562 cells at the cell densities as indicated were stained with Cyto-ID. The relative Cyto-ID fluorescence intensities were determined using the approach described in Subheading 3.1. Error bars represent standard deviations from three independent experiments

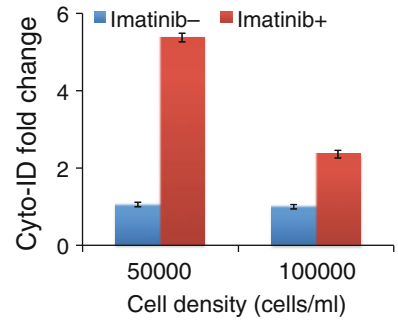

Fig. 2 Imatinib-induced autophagy in K562 cells at different cell densities. K562 cells seeded at the cell densities as indicated were treated with either vehicle (imatinib−) or 2 μM imatinib (imatinib+) for 4 h. The Cyto-ID fold changes were determined using the approach described in Subheading 3.1. Error bars represent standard deviations from three independent experiments

lower background level of Cyto-ID and does not compromise the detection of induced autophagy.

9. 5 % FBS helps maintain cell viability and reduces basal-level autophagy. In this protocol, 1× PBS may substitute 1× assay buffer.
10. Cell clumps interfere with effective staining. Repeatedly pipetting helps achieve single cell suspension. However, pipetting should be as gentle as possible.
11. The Cyto-ID fluorescence dye is sensitive to light based on manufacturer's instructions. It is recommended that this dye be kept in the dark.

12. The actual amount of concentrated Cyto-ID dye needed for each experiment depends on cell number. We recommend that 1 μl Cyto-ID dye be used for staining 10^6 cells.
13. The volume of Cyto-ID working solution used for cell staining is recommended at 100 μl per 10^5 cells. Using less volume of Cyto-ID working solution to label more cells causes ineffective staining and significantly reduces fluorescence signals.
14. Black plates are recommended by the manufacturer to detect the Cyto-ID fluorescence. The plate reader only detects Cyto-ID fluorescence from the center point of the well. Thus, to ensure accurate reading of Cyto-ID fluorescence using a microplate reader, we recommend using black 96-well plates with a round bottom. Suspension cells often accumulate at the center of the round-bottom well. To detect autophagy in cells grown adherently, it is also recommended that cells be in suspension during Cyto-ID staining and subsequent fluorescence detection.
15. It is important to include blank controls to increase the accuracy of fluorescence detection. The blank controls should also be included when measuring cell number.
16. We recommend shaking the plate for 60 s followed by 5–10 min incubation before reading the Cyto-ID fluorescence. This step is essential for cells to accumulate at the center of round-bottom wells.
17. The MTS viability assay detects viable cells by measuring absorbance of the product generated by viable cells. The manufacturer recommends using a transparent 96-well cell culture plate but not a black plate to give rise to an accurate MTS measurement.
18. To determine whether Hoechst 33342 was appropriate for measuring cell number, we compared this approach with the MTS or CellTiter-Blue viability assay used in our previous reports [20, 35, 36]. The linear regression coefficients for Hoechst 33342, MTS, and CellTiter-Blue were 0.96356, 0.99414, and 0.99605, respectively (Fig. 3a–c). We then compared Hoechst 33342 staining with the MTS viability assay in K562 cells treated with imatinib at the dose that inhibits cell growth and induces autophagy [20]. Surprisingly, cell numbers measured by Hoechst 33342 staining in imatinib-treated groups (imatinib+) were significantly higher than those in untreated groups (imatinib–) (Fig. 3a). By contrast, cell numbers measured by MTS in imatinib + groups were relatively lower than those in imatinib– groups, consistent with our expectation that a low dose of imatinib slows down cell division (Fig. 3b). Furthermore, the Cyto-ID fold changes normalized by Hoechst 33342 measurements failed to show imatinib-induced

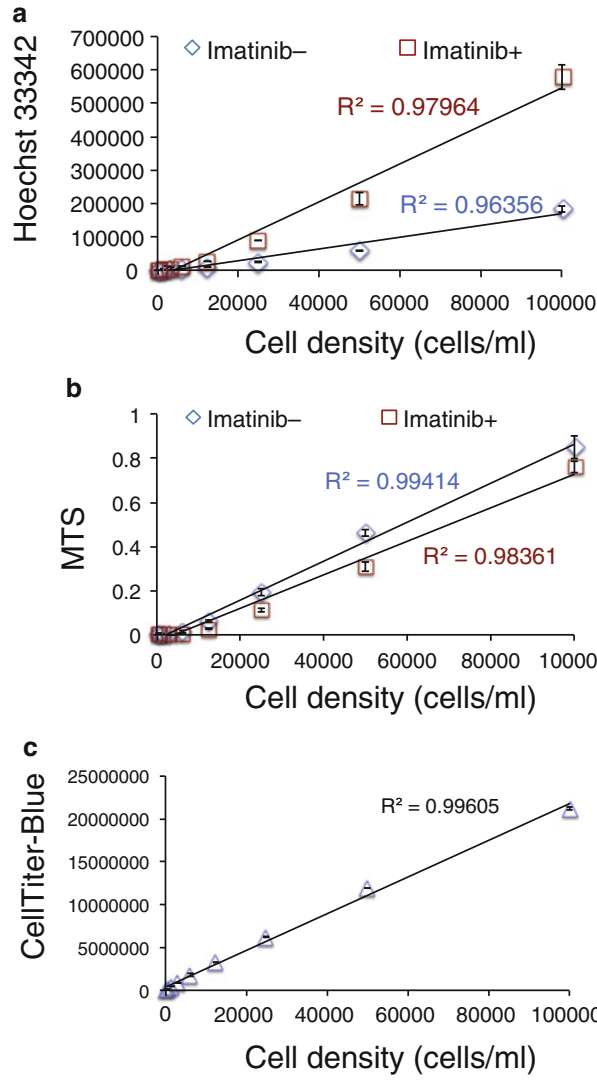

Fig. 3 Cell number determination using Hoechst 33342 staining, MTS viability assay, or CellTiter-Blue viability assay. K562 cells were plated at different cell densities as indicated. Cells were treated with either vehicle (imatinib−) or 2 μM imatinib (imatinib+) for 4 h. The cell number was determined by Hoechst 33342 staining (**a**), MTS viability assay (**b**), and CellTiter-Blue viability assay (**c**). Error bars represent standard deviations from three independent experiments

autophagy, whereas a robust increase of Cyto-ID fluorescence was observed in imatinib-treated K562 cells when the Cyto-ID intensities were normalized by the MTS readings (Fig. 4). Collectively, Hoechst 33342 staining is not suitable for monitoring cell number, particularly when cells are treated with growth inhibitors. We therefore strongly recommend using the MTS or CellTiter-Blue viability assay to determine cell number.

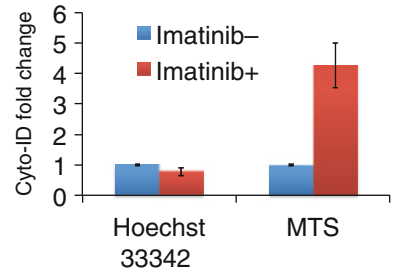

Fig. 4 Determination of imatinib-induced autophagy. K562 cells were plated at a cell density of 5×10^4 cells/ml. Cells were treated with either vehicle (imatinib−) or 2 μM imatinib (imatinib+) for 4 h. The cell number was determined by the Hoechst 33342 staining or MTS assay, respectively. The Cyto-ID fold changes were determined using the approach described in Subheading 3.1. Error bars represent standard deviations from three independent experiments

19. The CellTiter-Blue viability assay can be performed in the same well of the black round-bottom plate because this assay detects a fluorescence product generated by metabolically viable cells.
20. Imatinib inhibits the activity of MTOR and induces formation of autophagic compartments [20]. By contrast, chloroquine blocks autophagy flux, which in turn results in an accumulation of autophagosomes [34]. Thus, the consequence of imatinib and chloroquine combination is a synergistic increase of autophagosomes. Please refer to the results shown in Fig. 5a in reference 29.
21. EDTA prevents blood from clogging.
22. Mouse primary peripheral blood cells are much smaller than K562 cells. We therefore recommend performing the centrifugation at 400 × *g* for 15 min. This setting should also apply to primary bone marrow cells.
23. The number of mouse primary white blood cells from 30 μl of whole blood is limited. We recommend measuring the cell number in the same well using the CellTiter-Blue or MTS reagent after recording Cyto-ID fluorescence.

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Chapter 10

DREAM: A Simple Method for DNA Methylation Profiling by High-throughput Sequencing

Jaroslav Jelinek and Jozef Madzo

Abstract

The digital restriction enzyme analysis of methylation (DREAM) is a simple method for DNA methylation analysis at tens of thousands of CpG sites across the genome. The method creates specific signatures at unmethylated and methylated CpG sites by sequential digests of genomic DNA with restriction endonucleases *Sma*I and *Xma*I, respectively. Both enzymes have the same CCCGGG recognition site; however, they differ in their sensitivity to CpG methylation and their cutting pattern. *Sma*I cuts only unmethylated sites leaving blunt 5′-GGG ends. *Xma*I cuts remaining methylated CC^{mc}CGG sites leaving 5′-CCGGG ends. Restriction fragments with distinct signatures at their ends are ligated to Illumina sequencing adaptors with sample-specific barcodes. High-throughput sequencing of pooled libraries follows. Sequencing reads are mapped to the restriction sites in the reference genome, and signatures corresponding to methylation status of individual DNA molecules are resolved. Methylation levels at target CpG sites are calculated as the proportion of sequencing reads with the methylated signature to the total number of reads mapping to the particular restriction site. Aligning the reads to the reference genome of any species is straightforward, since the method does not rely on bisulfite conversion of DNA. Sequencing of 25 million reads per human DNA library yields over 50,000 unique CpG sites with high coverage enabling accurate determination of DNA methylation levels. DREAM has a background less than 1 % making it suitable for accurate detection of low methylation levels. In summary, the method is simple, robust, highly reproducible, and cost-effective.

Key words DNA methylation, CpG sites, Restriction endonuclease, *Sma*I, *Xma*I, High-throughput sequencing, DREAM

1 Introduction

DNA methylation at CpG sites is an important epigenetic mechanism determining chromatin configuration and accessibility of genes for expression in mammalian cells [1]. Aging is associated with erosion of the epigenomic integrity that can be detected as discrete methylation changes [2, 3]. The disruption of epigenome is frequently accentuated in cancer leading to vast disorganization of DNA methylation patterns [4]. Aberrant DNA methylation

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interacts with genetic mutations in cancer development and progression [5–7]. Cancer-specific DNA methylation changes are used as biomarkers [8–10]. Next-generation or high-throughput sequencing made possible to assess DNA methylation profiles genomewide. Affinity enrichment for methylcytosine, bisulfite conversion of unmethylated cytosines, and restriction enzymes distinguishing methylated and unmethylated CpG sites are three main approaches used in DNA methylation analyses [11, 12].

Here we present a simple method that can accurately measure DNA methylation levels at approximately 50,000–100,000 CpG sites across the genome [13]. The method is based on methylation-specific signatures created by restriction enzymes and deciphered by high-throughput sequencing. *Sma*I and *Xma*I are a unique pair of restriction endonucleases that target the same recognition site, CCCGGG, but differ in their sensitivity to CpG methylation and cutting pattern. *Sma*I cuts only unmethylated sites leaving blunt ends. The enzyme is completely blocked by CpG methylation. *Xma*I can cut both unmethylated and CpG-methylated sites ($CC^{me}CGGG$) leaving 5′CCGG overhangs. We utilize the enzymes sequentially. First, genomic DNA is exposed to *Sma*I that cuts all unmethylated sites, leaving all $CC^{me}CGGG$ sites intact (Fig. 1a). Having digested all available unmethylated sites, we continue the cleavage by adding *Xma*I. The enzyme cuts the remaining sites that have been protected from *Sma*I by CpG methylation (Fig. 1b). The unmethylated sites thus have the GGG signature whereas the methylated sites have the CCGGG signature at the 5′ ends of the restriction fragments (Fig. 1c). Next, we stabilize the methylated signatures by filling the 3′ recesses using the exonuclease-deficient Klenow fragment DNA polymerase. This enzyme adds 3′-dA overhangs to all fragments for subsequent ligation of the sequencing

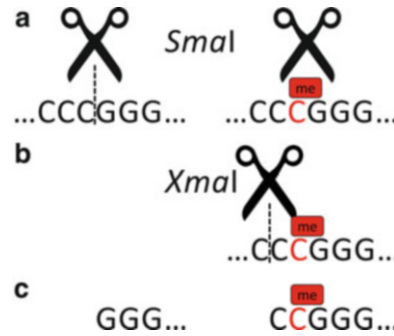

Fig. 1 Principle of the DREAM DNA methylation analysis. (**a**) *Sma*I restriction endonuclease cleaves CCCGGG sites in the middle. Only sites with unmethylated cytosines are cut, since CpG methylation completely blocks the enzyme from cutting. (**b**) *Xma*I restriction endonuclease added in the next step cleaves the remaining sites with methylated CpG cutting after the first cytosine. (**c**) Distinct GGG or CCGGG signatures are created at the 5′ ends of restriction fragments reflecting unmethylated or methylated CpG sites, respectively

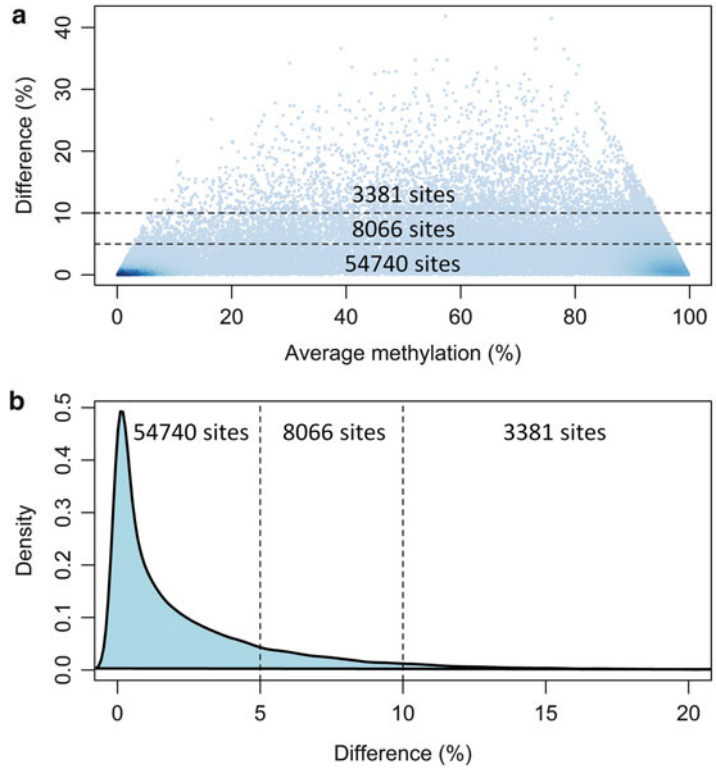

Fig. 2 Reproducibility of DREAM. Methylation differences at 66187 CpG sites covered with the minimum sequencing depth of 20 reads were evaluated in technical replicates. (**a**) Density plot shows bimodal distribution of methylation by the highest data density close to 0 % and 100 %. (**b**) Kernel density plot of methylation differences between technical replicates. *Dotted lines*: methylation differences smaller than 5 % were observed at 54740 CpG sites (83 % of total). Methylation differences 5–10 % were observed at 8066 CpG sites (12 % of total). Differences greater than 10 % were observed at 3381 CpG sites (5 % of total analyzed)

adaptors. Standard procedures for making libraries and high-throughput sequencing follow. The sequencing reads are mapped to the *Sma*I/*Xma*I restriction sites in the reference genome. Methylation levels at individual CpG sites are calculated based on the counts of reads with methylated and unmethylated signatures mapping to the site. Paired end sequencing and 40 base read length is sufficient to map up to 200,000 unique CpG sites in the human genome. Using 25 million sequencing reads per sample typically yields more than 50,000 CpG sites covered with 20 and more reads. The assay is highly reproducible. Technical replicates show minimum differences and high correlation of methylation values at individual CpG sites (Figs. 2 and 3). Capture of consistent CpG sites with the high-sequencing depth makes the method suitable for analysis of large sample sets. Although the targets are restricted to accessible CCCGGG sites in the genome, the method has several

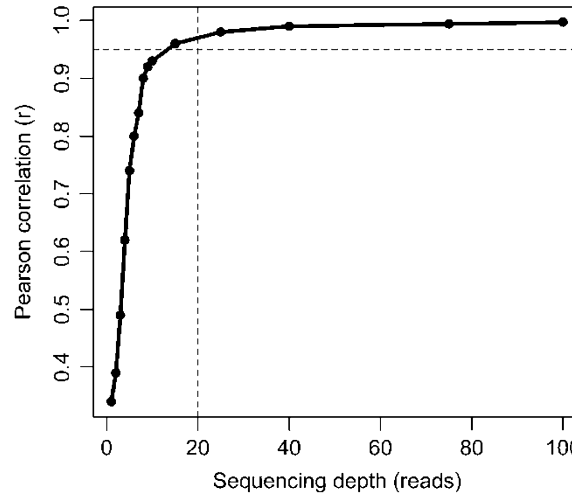

Fig. 3 High correlation of methylation values in technical replicates. Methylation levels at CpG sites sequenced with 20 reads showed Pearson correlation $r = 0.97$ between technical replicates. This correlation was 0.997 for CpG sites sequenced with 100 reads

advantages over bisulfite-based approaches. The background is below 1 %, since *Sma*I, the enzyme specific for unmethylated signature, is completely blocked by CpG methylation. Additionally, mapping the reads to the unconverted reference genome is computationally straightforward. The accuracy and reproducibility of DREAM is further increased by spiking the samples with standards of known methylation levels before enzymatic processing [13, 14]. These standards are used for fine adjustment of raw methylation levels and can be used for mitigating potential batch effects. We and others have successfully used DREAM as a robust, highly reproducible, and cost-effective method for global DNA methylation analysis of more than 1000 samples of human [3, 10, 13, 15–19], mouse [20], and zebrafish [21] cells and tissues.

2 Materials

2.1 Making Methylation Standards

1. *E. coli* genomic DNA, unsheared (Affymetrix, USB Cat# 14380).
2. Lambda bacteriophage DNA (New England Biolabs).
3. *Sma*I restriction endonuclease (New England Biolabs).
4. *Hpa*II CpG methyltransferase (New England Biolabs).
5. *Hpa*II restriction endonuclease (New England Biolabs).
6. *S*-adenosylmethionine (SAM) (New England Biolabs).
7. Oligonucleotide primers (Sigma, IDT or other suppliers).

8. Taq DNA polymerase with ThermoPol® Buffer (New England Biolabs).
9. TE buffer (TRIS 10 mM, EDTA 1 mM, pH 8.0).
10. LTE buffer (TRIS 10 mM, EDTA 0.1 mM, pH 8.0).
11. Sodium acetate 3 M, pH 5.0.
12. Isopropanol.
13. Ethanol.
14. Bovine serum albumin (BSA).
15. 96-well PCR plates, not skirted.
16. Adhesive seals for PCR plates.
17. PCR thermal cycler.
18. Agarose gel electrophoresis supplies.
19. QIAquick PCR Purification Kit (Qiagen).

2.2 Construction of Sequencing Libraries

1. *Sma*I restriction endonuclease (New England Biolabs).
2. *Xma*I restriction endonuclease (New England Biolabs).
3. Klenow fragment (3′ → 5′ exo-) (New England Biolabs).
4. T4 DNA ligase (New England Biolabs).
5. dNTP Set (100 mM each A,C,G,T) (GE Healthcare Life Sciences).
6. NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1 and 2) (New England Biolabs).
7. KAPA HiFi HotStart ReadyMix PCR Kit (Kapa Biosystems, Inc.).
8. 96-well PCR plates, not skirted.
9. Adhesive seals for PCR plates.
10. PCR thermal cycler.

2.3 Cleaning, Separation, and Quantitation of Sequencing Libraries

1. Agencourt AMPure XP magnetic beads (Beckman Coulter).
2. DynaMag-96 Side Magnetic Particle Concentrator (Invitrogen Cat. No. 123.31D).
3. Ethanol.
4. Molecular biology grade water.
5. 96-well PCR plates, not skirted.
6. Qubit 2.0 fluorometer, dsDNA BR and HS Assay kits (Life Sciences).
7. NanoDrop UV–vis Spectrophotometer (Thermo Scientific).
8. Agilent 2100 Bioanalyzer (Agilent Technologies).
9. Agilent High Sensitivity DNA Kit or DNA 1000 Kit (Agilent Technologies).

2.4 Next-Generation Sequencing

1. Illumina HiSeq 2500 or a similar instrument for high-throughput sequencing.
2. Linux server and/or bioinformatics support for processing of the sequencing data.
3. Hard disk storage for the sequencing data.

3 Methods

3.1 Making Methylation Standard for Spiking in the Samples

For making an unmethylated standard LA168 as a PCR amplicon, use primers LA248F TCGAAAAAGAGCAGCACAGTGATGCCC and LA248R GTATGCCGCATTGCACTTT with lambda bacteriophage DNA (NEB #N3011) as the template:

1. Mix the following reagents for PCR on ice: 800 ng lambda DNA in 340 μl of water, 40 μl of PCR buffer (final Mg^{2+} concentration 2 mM), 4 μL of 10 μM LA248F primer, 4 μl of 10 μM LA248R primer (final primer concentration 100 nM), 4 μl of 25 mM dNTP mix (final 250 μM), and 8 μl (40 U) of Taq polymerase. Aliquot the reaction mix to 16 wells in a PCR plate.
2. Run the PCR program as follows: Initial denaturation at 94 °C for 3 min; 33 cycles consisting of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s; final extension at 72 °C for 5 min. Pool the PCR products from all aliquots into a single tube.
3. Verify the presence of CCCGGG sites by *Sma*I digestion of 5 μl of the PCR product. Compare the digested and undigested PCR product using 2 % agarose gel electrophoresis. You should obtain a single band of 248 bp from the undigested PCR product and fragments of 168 bp, 53 and 27 bp after *Sma*I digestion. Purify the remaining PCR product using the PCR purification kit (Qiagen, QIAquick PCR Purification Kit #28104) following the manufacturer's protocol. Measure DNA concentration of the unmethylated standard using NanoDrop spectrophotometer and/or Qubit fluorometer.

   Making partially methylated (50 %) standard of *E. coli* genomic DNA

   First, methylate completely *E. coli* genomic DNA with the *Hpa*II methyltransferase at CpG sites in the CCGG recognition sequence. Check the completeness of methylation as the resistance to the *Hpa*II restriction endonuclease. Next, make the 50 % methylated standard by mixing equal amounts of unmethylated and *Hpa*II-methylated *E. coli* DNA (*see* **Note 1**).

4. Methylate *E. coli* genomic DNA. Dissolve 10 mg of *E. coli* genomic DNA (Affymetrix, Part #14380) in 5 ml of TE buffer (10 mM Tris–HCl, EDTA 1 mM, pH 8.0). Set up methylation reaction M1 by pipetting 825 µl of nuclease-free water, 50 µl (100 µg) of *E. coli* gDNA, 100 µl of *Hpa*II methylase buffer, and 2.5 µl of *S*-adenosylmethionine (SAM) 32 mM and 25 µl (100 units) of *Hpa*II methyltransferase (NEB #M0214). SAM and the reaction buffer are provided with the enzyme. Set up a parallel tube M0 for unmethylated DNA: 825 µl of nuclease-free water, 50 µl (100 µg) of *E. coli* gDNA, 100 µl of *Hpa*II methylase buffer, 2.5 µl of *S*-adenosylmethionine (SAM) 32 mM, and 25 µl of water instead of the *Hpa*II methylase. Incubate both tubes overnight in a water bath at 37 °C. Incubate both tubes at 65 °C for 20 min to activate the enzyme.
5. Check the completeness of methylation by *Hpa*II restriction digest. Make the restriction buffer as follows: 360 µl of water, 40 µl of NEB Buffer 1 or NEB CutSmart® Buffer, and 4 µl of $MgCl_2$ 1 M. Mark four microcentrifuge tubes as E1, E2, E3, and E4. Add 40 µl of the restriction buffer to each tube. Add 10 µl of unmethylated *E. coli* DNA from tube M0 to tubes E1 and E2. Add 10 µl of *Hpa*II-methylated *E. coli* reaction from tube M1 to tubes E3 and E4. Add 5 µl (50 units) of *Hpa*II restriction endonuclease (NEB #R0171) in tubes E2 and E4. Incubate all tubes in a water bath at 37 °C for 1 h. Check the restriction digests by running electrophoresis of 20 µl of the reactions and a 100 bp DNA ladder (NEB #N3231) in 2 % agarose. Samples E1 and E3 are undigested controls. Sample E2 shows a smear of digested unmethylated DNA. If methylation of *Hpa*II recognition sites (CCGG) is complete, sample E4 looks like E1 and E3 (Fig. 4).

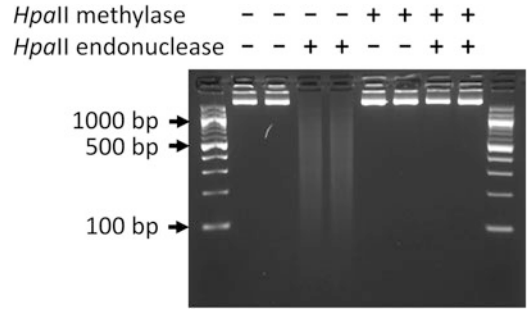

Fig. 4 Methylation of *E. coli* gDNA with *Hpa*II methyltransferase. *E. coli* methylated at CCGG sites with *Hpa*II methyltransferase is protected from cleavage with *Hpa*II restriction endonuclease (*lanes* 7 and 8) while unmethylated DNA (*lanes* 3 and 4) is completely digested. *Lanes* 1, 2, 5, and 6 are controls without the restriction enzyme

6. Clean the DNA in tubes M0 and M1 by alcohol precipitation and dissolve in TE. Add 111 μl of Na-acetate 3 M pH 5.0 to tubes M0 and M1 containing 1000 μl of DNA and mix by vortexing. Split the content of each tube in half by transferring 555 μl of the content to additional tubes M0 and M1. Add 389 μl (0.7 volume) of isopropanol to all tubes. Mix well by inverting and vortexing. Centrifuge the tubes at 12,000 *g* for 15 min. Pour out the supernatant while keeping the DNA pellet attached to the side of tubes. Wash the pellet with 1200 μl of 70 % ethanol two times. Spin briefly the empty tubes with DNA pellets after the second ethanol wash and carefully pipette out all traces of liquid using a fine tip. Add 100 μl of TE to each tube and dissolve the DNA pellets by incubation at 50 °C for 1 h followed by incubation at room temperature overnight. Pool the duplicate M0 and M1 tubes separately making M0 (unmethylated) and M1 (methylated) pools.
7. Measure DNA concentration in M0 and M1 pools using spectrophotometer (NanoDrop) and/or fluorometer (Qubit). Make a stock solution of 50 % methylated *E. coli* standard by pooling of equal amounts of unmethylated (M0) and methylated (M1) DNA. Measure DNA concentration of the pool.

Making working solution of pooled methylation standards

8. Make working solution of the methylation standards by diluting the stock of 50 % methylated *E. coli* solution to the concentration of 2 ng/μl in LTE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0) supplemented with bovine serum albumin (BSA) at 100 μg/ml. Add the unmethylated standard LA168, so that its final concentration is 2 pg/μl (i.e., 1000-fold lower than the concentration of *E. coli* DNA). Make 50 μl aliquots of the working solution of the methylation standards and keep them at −20 °C. Use 1 μl of the methylation standards to spike each sample before making DREAM libraries.

3.2 Preparing DNA Samples by Cleaning and Quantification

The DREAM assay requires unbroken genomic DNA free from traces of phenol, guanidinium salts or other potential contaminants that may interfere with enzymatic reactions. We recommend DNA purification using AMPure XP beads as the initial step before construction of sequencing libraries for all samples. The procedure removes small DNA fragments and traces of contaminants that may affect the activity of restriction enzymes and forming of methylation signatures:

1. Measure DNA concentration in the samples using spectrophotometry (NanoDrop) and/or fluorometry (Qubit). Clean DNA should have OD ratio of 260/230 nm above 2.0 and 260/280 nm ratio in the range of 1.8–2.0. Check the integrity of gDNA by running electrophoresis of 200 ng of gDNA in

1 % agarose with 100 bp and 1 kb markers. Inspect the gel for the smear of fragmented DNA running below 500 bp and estimate the proportion of high molecular weight gDNA migrating above ~ 5 kb.

2. Clean the genomic DNA sample and remove small fragments using AMPure XP beads at 0.5× beads to DNA ratio. Set up a 96-well PCR plate and mark wells for each DNA sample to be purified. We usually process 12 samples at a time. Ensure that AMPure XP beads have reached room temperature and resuspend them well before proceeding.
3. Pipette aliquots of 2.5–10 μg of genomic DNA in the marked wells and adjust the volume to 70 μl with water (*see* **Note 2**). Add 35 μl of AMPure XP beads (0.4× ratio of beads to DNA) into the wells with DNA. Mix well by pipetting up/down. Incubate at room temperature for 15 min (*see* **Note 3**).
4. Transfer the plate to magnetic stand and let it stand for 5 min to completely clear the solution of beads. With the plate on the magnetic stand, carefully remove 95 μl of clear binding buffer without drawing any beads. Save the supernatant containing low molecular weight DNA fragments in separate tubes for potential future use. High molecular weight DNA stays bound to the beads (*see* **Note 4**).
5. Keep the plate on the magnetic stand and wash the beads with freshly prepared 80 % ethanol as follows: Add 200 μl of 80 % ethanol and allow to stand for 30 s. Remove and discard the supernatant. Repeat the wash with fresh 80 % ethanol one more time. Using a P20 pipette with a fine tip, carefully remove the remaining ethanol without disturbing the beads. Allow the beads air dry for 10 min or until fine cracks appear at the bead layer. Inspect each well carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
6. Remove the plate from the magnet. Add 30 μl of room temperature nuclease-free water to the dried beads. Mix thoroughly to ensure all the beads are evenly suspended. Incubate at room temperature for 5 min (*see* **Note 5**).
7. Place the plate on the magnetic stand. Incubate at room temperature for 2–5 min or longer until the supernatant gets completely clear. While keeping the plate on the magnet, carefully collect the eluted DNA, ensuring as few beads as possible are carried over and transfer the purified DNA to a fresh set of tubes.
8. Measure DNA concentration in the samples using spectrophotometry (NanoDrop) and/or fluorometry (Qubit) and calculate the total amount of recovered DNA. One to two micrograms of purified genomic DNA are required for making high complexity DREAM libraries.

9. Spike in 1 μl of methylation standards to each sample. It is important to add the standards in the samples *before* the restriction digests in the following steps.

3.3 Making DREAM Libraries for Illumina High-Throughput Sequencing

1. Digest the DNA sample with spiked in standards with *Sma*I restriction endonuclease. The enzyme cuts all unmethylated CCCGGG sites creating blunt-ended fragments. Set up restriction digests in microcentrifuge tubes as follows: Add 21.5 μl of DNA sample (1–2 μg) spiked with 1 μl of methylation standards, 2.5 μl of CutSmart® Buffer, and 1 μl (20 units) of *Sma*I enzyme in the tube. Mix gently, spin briefly, and incubate in 25 °C water bath for 8 h.
2. Continue the restriction digest by adding the second enzyme, XmaI restriction endonuclease. Add 20.5 μl of water, 2.5 μl of CutSmart® Buffer, and 2 μl (20 units) of the *Xma*I enzyme in each tube with the *Sma*I digest. Mix gently, spin briefly, and incubate in a 37 °C water bath overnight for 16 h.
3. Fill the 3′ recesses left by the *Xma*I digest and add 3′ dA overhangs to all fragments. Add to each tube with *Sma*I/*Xma*I digests 2 μl of the CGA mix (dCTP, dGTP, and dATP, 10 mM each), 3 μl of Klenow fragment (3′ → 5′ exo–) DNA polymerase and mix. Spin briefly and incubate in a water bath at 37 °C for 30 min.

   Purify the DNA fragments by AMPure XP beads (2.0× volume ratio of beads to DNA). Ensure that the beads have reached room temperature and resuspend them well before proceeding. Set up a 96-well PCR plate and mark wells for each sample to be purified.
4. Add 110 μl of AMPure XP beads into the marked wells. Add the contents of the end-filling-A-tailing reactions (55 μl) in the wells with beads. Mix well by pipetting up and down. Incubate at room temperature for 15 min (*see* **Note 3**).
5. Transfer the plate to the magnetic stand, and let it stand for 5 min until the solution becomes completely clear and the beads attached to the wall.
6. Carefully remove 150 μl of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.
7. Keep the plate on the magnetic stand and wash the beads with freshly prepared 80 % ethanol follows: Add 200 μl of 80 % ethanol and allow to stand for 30 s. Remove and discard the supernatant. Repeat the wash with fresh 80 % ethanol one more time. Using a P20 pipette with a fine tip, carefully remove the remaining ethanol without disturbing the beads. Allow the beads air dry for 10 min or until fine cracks appear at the bead layer. Inspect each well carefully to ensure that all the ethanol

has evaporated. It is critical that all residual ethanol be removed prior to continuing.

8. Remove the plate from the magnet. Add 30 µl of room temperature nuclease-free water to the dried beads. Mix thoroughly to ensure all the beads are evenly suspended. Incubate at room temperature for 5 min (*see* **Note 5**).
9. Place the plate on the magnetic stand. Incubate at room temperature for 2–5 min or longer until the supernatant gets completely clear. While keeping the plate on the magnet, carefully collect the eluate with DNA, ensuring as few beads as possible are carried over and transfer the digested dA-tailed DNA to a fresh PCR plate labeled "Ligation."
10. Ligate sequencing adapters to the restriction fragments. Add to each well with DNA the following reagents: 3 µl of 10× ligation buffer (NEB #B0202S), 0.5 µl of NEBNext adaptor for Illumina 10 µM (from NEB kits #E7335 or #E7500), and 2 µl (800 U) of T4 DNA ligase (NEB #M0202). Mix by pipetting up and down. Cover the wells with adhesive seal. Spin briefly. Incubate the ligation reaction at 16 °C overnight.
11. Cleave the hairpin loops in the NEBNext adaptors. Add 3 µl of USER enzyme to each reaction and pipette up and down to mix. Incubate at 37 °C for 15 min.

    Clean up the ligated DNA and perform dual size selection using AMPure XP beads. First, remove large DNA fragments (>450 bp) by binding to beads used at the 0.6× ratio of AMPure beads to sample. At this step, collect the supernatant containing smaller DNA fragments (<450 bp). Next, transfer the supernatant into new wells and add additional beads. Addition of AMPure beads will increase the concentration of PEG and salt and result in binding of DNA fragments larger than 250 bp to the beads. The dual SPRI size selection will isolate target DNA fragments (250–450 bp) and leave behind unligated adaptors, self-ligated adaptor dimers, and large DNA fragments.
12. Add 66.5 µl of water into the wells containing the 33.5 µl of the ligation reaction from the previous step to increase the DNA volume to 100 µl. Add 60 µl of AMPure XP beads (0.6× volume). Mix well by pipetting up and down. Incubate at room temperature for 15 min.
13. Transfer the plate to the magnetic stand and let the beads completely separate from the supernatant for 5 min or more. Carefully collect 155 µl of the supernatant containing short DNA fragments (<450 bp) and transfer to a new set of wells. Note: The beads with large DNA fragments are left behind.

14. Add 20 μl of AMPure XP beads to the 155 μl of clear supernatant in a new set of wells and mix thoroughly by pipetting up/down. Incubate at room temperature for 15 min.
15. Transfer the plate to the magnetic stand and let the beads completely separate from the supernatant for 5 min or more.
16. Carefully remove only 160 μl of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step. DNA fragments larger than 250 bp are attached to the beads. Note: The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact the yield, so ensure beads are not removed with the binding buffer or the wash.
17. With the plate still on the magnet, add 200 μl of freshly prepared 80 % ethanol and allow to stand for 1 min. Remove the ethanol wash using a pipette. Repeat the 80 % ethanol wash one more time, for a total of two washes. Using a fine tip, carefully remove all traces of ethanol from the bottom of each well. Note: It is critical to remove as much of the ethanol as possible after the final wash.
18. Air dry the beads while keeping the plate on the magnet for a minimum of 10 min. Inspect each well carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
19. Remove the plate from magnet. Add 25 μl of room temperature nuclease-free water to the dried beads. Mix thoroughly to ensure that beads in all wells are homogeneously resuspended. Incubate at room temperature for 5 min (*see* **Note 5**).
20. Transfer the plate to the magnet and let it stand for 2 min or until the supernatant becomes clear.
21. While keeping the plate on the magnet, carefully collect 22.5 μl of the eluate, ensuring as few beads as possible are carried over. Transfer the eluted DNA to a new plate labeled "PCR."

    Perform ligation-mediated PCR amplification. Use NEBNext oligos for Illumina (NEB #7335 with barcodes 1–12 and/or NEB #7500 with barcodes 13–27). Make sure to use a unique barcode for each sample.
22. Transfer the PCR plate with DNA on ice. Add to each well 1.25 μl of NEBNext universal primer, 1.25 μl of the NEBNext Index i7 primer with a sample-specific barcode, and 25 μl of KAPA HiFi HotStart ReadyMix 2× (Kapa Biosystems, Inc. #KK2601). Seal the plate, spin briefly, and return on ice.
23. Perform PCR amplification using the initial denaturation temperature of 98 °C for 45 s, followed by 11–12 cycles at 98 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Add the final extension step at 72 °C for 1 min after the cycling (*see* **Note 6**).

Perform a post PCR cleanup with AMPure XP beads (1.2× volume beads to DNA ratio).

24. Add 60 µl of AMPure beads into wells with PCR reactions. Mix well by pipetting up/down. Incubate at room temperature for 15 min.
25. Transfer the plate to the magnet and let it stand for 5 min to completely clear the solution of beads.
26. Carefully remove only 94 µl of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step (*see* **Note 4**). With the plate still on the magnet, add 200 µl of freshly prepared 80 % ethanol and allow to stand for 1 min. Remove the ethanol wash using a pipette. Repeat the 80 % ethanol wash one more time, for a total of two washes. Using a fine tip, carefully remove all traces of ethanol from the bottom of each well (*see* **Note 7**)
27. Air dry the beads on the magnet for a minimum of 10 min. Inspect each well carefully to ensure that all the ethanol has evaporated (*see* **Note 7**).
28. Remove the plate from the magnet. Add 30 µl room temperature nuclease-free water to the dried beads. Mix thoroughly to ensure all the beads are homogeneously resuspended. Incubate at room temperature for 5 min (*see* **Note 5**).
29. Transfer tubes to magnet and let it stand for 2 min or until the supernatant clears out completely.
30. While keeping the plate on the magnet, carefully collect the finished sequencing libraries in 25 µl of the eluate, ensuring as few beads as possible are carried over and transfer to a fresh set of tubes labeled with sample IDs.

3.4 Quality Control

1. Measure DNA concentration in the samples using high sensitivity fluorometry (Qubit HS). Analyze the size distribution and the molarity of the library by Agilent 2100 Bioanalyzer electrophoresis using Agilent High Sensitivity DNA Kit (Fig. 5).
2. Pool 12 samples with unique barcodes for Illumina sequencing using equal amount of femtomoles or nanograms of DNA for each sample (*see* **Note 8**).

3.5 High-Throughput Sequencing and Data Processing

1. Sequence the pooled libraries on the Illumina high-throughput instrument using a paired end setup and 40–50 bases read length (*see* **Note 9**).
2. Obtain the sequences in the fastq format. Align the sequences to the reference genome supplemented with the sequences of the methylation standards (*see* **Note 10**).

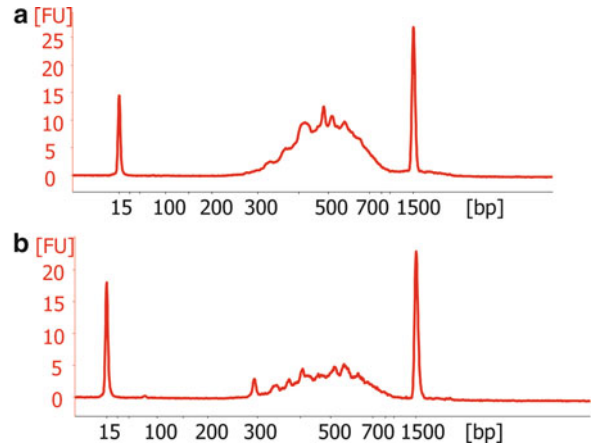

Fig. 5 Size distribution of typical DREAM libraries. Agilent 2100 Bioanalyzer electrophoresis using standard sensitivity DNA 1000 kit. (**a**) DREAM library made from human blood gDNA. (**b**) DREAM library made from mouse liver gDNA

3. Bowtie2 generates files in a generic Sequence Alignment/Map (SAM) format. Next, use SAMtools for conversion of SAM files into sorted indexed binary (BAM) files.
4. Count methylation signatures and compute methylation levels. Use the sorted BAM files as input for the custom Python script which assigns the reads to individual CCCGGG sites positioned in the genome. The script counts the reads with methylated and unmethylated signatures and calculates raw methylation values as the percentage of reads with the methylated signature in the total number of reads. The Python script and annotation tables of target sites for several reference genomes are available at https://github.com/jmadzo/DREAM_project/ and https://github.com/jaroslavj/DREAM_tools.
5. We recommend to normalize the raw methylation values using the observed and expected methylation values of the spiked in methylation standards [13, 14] (*see* **Note 11**).

4 Notes

1. Complete methylation of CpGs in the CCGG recognition sequence is easily achievable by a single treatment of DNA with the *Hpa*II methyltransferase (Fig. 4). All CCGG sites are inside the CCCGGG sites targeted by DREAM. In contrast, multiple enzymatic treatments are needed to achieve complete methylation of all genomic CpG sites using the *M.Sss*I methyltransferase.

2. The amount of DNA used for cleaning should be approximately 2.5–10 μg, depending on availability. The yield of the cleaning procedure depends on the purity and integrity of the DNA sample. One microgram of cleaned high molecular weight gDNA is sufficient for the DREAM assay. The minimum amount for making high complexity libraries is approximately 500 ng.
3. Shaking a sealed plate on a microplate shaker at 1800 rpm may facilitate the binding of DNA to beads. Spin the plate briefly after removing from the shaker.
4. The beads should not disperse; instead, they should stay attached to the side walls of the wells. Significant loss of beads will impact the yield, so ensure beads are not removed with the binding buffer or the wash.
5. Shaking a sealed plate on a microplate shaker at 1800 rpm may facilitate the elution of DNA from the beads. Spin the plate briefly after removing from the shaker.
6. Adjust the optimal number of PCR cycles so that the final concentration of DNA purified after the amplification is in the range of 10–20 ng/μl. Libraries with concentrations lower than 5 ng/μl could be re-amplified by additional 4–6 PCR cycles. Primers for Illumina termini P5 and P7 AATGATACGGCGACCACCGAGATCTACAC and CAAGCAGAAGACGGCATACGAGAT can be used for all libraries, since the sample-specific barcodes were set by the previous PCR (Subheading 3.3, **steps 22–23**).
7. It is critical to remove as much of the ethanol as possible after the final wash.
8. Pooling of 12 DREAM libraries works well for Illumina Rapid Run flow cell. The number of samples to be pooled should be adjusted to obtain approximately 25 million paired end reads per sample.
9. Spike the sequencing pool with 10 % PhiX library to ensure sufficient diversity at first 5 bases. DREAM libraries have either C or G at bases number 1 and 2. Base 3 is invariably G. Spiking in a percentage of PhiX control library increases the nucleotide balance and makes clusters easier for the software to identify.
10. We first build a bowtie2 index combining the reference genome, the *E. coli* genome, and the methylation standards added as extra chromosomes. We align the fastq reads using the Bowtie2 aligner with the sensitive option.
11. We use the spiked in methylation standards to compensate for potential distortions of raw methylation values expressed as the percentage of methylated reads. We compare the expected and observed ratios of methylated to unmethylated reads mapped

to the calibrators and calculate the correction coefficient. We first verify that the background methylation level of the completely unmethylated standard is less than 1 %. Next, we calculate the difference d of log ratios $log\ (M/U)$ and $log\ (m/u)$ for each standard, where M/U is the expected ratio of methylated and unmethylated reads and m and u are observed numbers of methylated and unmethylated reads (a). Correction factor c is expressed as an antilog of the average log difference (expected–observed) from all standards (b). Finally, we compute adjusted methylation values for each CpG site (c). We add 0.5 reads to the number of methylated and unmethylated reads to avoid division by zero.

(a) $d = log\ (M/U) - log\ (m/u)$

(b) $c = exp(mean(d))$

(c) $adjusted\ methylation = c^*(m + 0.5)/[c^*(m + 0.5) + u + 0.5]$

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Chapter 11

ChIP-*seq* Analysis of Human Chronic Myeloid Leukemia Cells

Lars Anders and Zhaodong Li

Abstract

Many transcription factors, chromatin-associated proteins and regulatory DNA elements are genetically and/or epigenetically altered in cancer, including Chronic Myeloid Leukemia (CML). This leads to deregulation of transcription that is often causally linked to the tumorigenic state. Chromatin-immunoprecipitation coupled with massively parallel DNA sequencing (ChIP-*seq*) is the key technology to study transcription as it allows in vivo whole-genome mapping of epigenetic modifications and interactions of proteins with DNA or chromatin. However, numerous DNA/chromatin-binding proteins, including EZH2, remain difficult to "ChIP," thus yielding genome-wide binding maps of only suboptimal quality. Here, we describe a ChIP-*seq* protocol optimized for high-quality protein-genome binding maps that have proven especially useful for studying difficult to 'ChIP' transcription regulatory factors in Chronic Myeloid Leukemia (CML) and related malignancies.

Key words Chronic myeloid leukemia, ChIP-*seq*, Immunoprecipitation, Sonication, Sequencing

1 Introduction

Chronic Myeloid Leukemia (CML) is a myeloproliferative neoplasm that mostly affects adults [1]. The Philadelphia (Ph) chromosome and BCR/ABL fusion gene is the major cytogenetic and molecular marker of CML chronic phase [2, 3]. In addition, mutations or translocations of many transcription factors, including HIF-1α, AML1, C/EBPα, HOX, and GATA family members, have been found to in CML, emphasizing the contribution of aberrant transcription regulation to this malignancy [4–9].

Chromatin-immunoprecipitation coupled with massively parallel DNA sequencing (ChIP-*seq*) has become one of the most powerful technologies to study gene transcription [10]. ChIP-*seq* allows genome-wide mapping of all physical interactions between proteins and DNA, including proteins in direct physical contact with DNA but also those bound indirectly to the genome, that is, via other

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DNA-binding proteins [11]. ChIP-*seq* analysis has allowed unprecedented insights into the function of transcription factors and the transcription machinery [12, 13]. Moreover, it unraveled the histone code—made up by a sheer number of different histone modifications and combinations thereof—and provided us with a clear but continuously evolving picture of epigenetic regulation, including the myriad of proteins that 'write, read and erase' all the modifications with which histones are decorated [14]. This information, in aggregate, has had a major impact on our understanding of the regulation of cell state in health and disease.

The ChIP-*seq* technology is principally based on six key steps: (1) treatment of living cells with formaldehyde to cross-link proteins to DNA; (2) cell lysis and sonication to shear the DNA into fragments; (3) immunoprecipitation and purification of the 'target' DNA sequences bound by the protein of interest; (4) ChIP-*seq* library construction of the obtained sequences; (5) massively parallel DNA sequencing; and (6) alignment of sequences to the genome, peak calling, and analysis.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 M Ω cm at 25 °C) and analytical grade reagents. Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Stock Solutions

1. *1 M HEPES–KOH, pH 7.5 (1 l)*: Weigh 238.3 g HEPES and transfer to a glass beaker. Add water to a volume of 900 ml. Mix and adjust pH to 7.5 with KOH. Make up to 1 l with water. Sterile filter and store at 4 °C.
2. *0.5 M Ethylene DiamineTetraacetic Acid (EDTA), pH 8.0*: Purchased from Westnet Inc, MA (*see* **Note 1**).
3. *0.5 M* Ethylene Glycol Tetraacetic Acid *(EGTA), pH 8.0*: Purchased from Westnet Inc, MA (*see* **Note 1**).
4. *10 % Triton X-100 (200 ml)*: Add 20 ml Triton X-100 to 180 ml water. Thoroughly mix and store at 4 °C.
5. *5 % Na-deoxycholate (500 ml)*: Weigh 25 g Na-deoxycholate and transfer to a glass beaker. Add water to a volume of 500 ml. Stir to completely dissolve Na-deoxycholate. Make up to 500 ml with water. Sterile filter and store at room temperature.
6. *UltraPure 10 % SDS Solution*: Purchased from Life Technologies (*see* **Note 2**).
7. *1 M LiCl solution (1 l)*: Weigh 42.39 g LiCl and transfer to a glass beaker. Add water to a volume of 900 ml. Mix and make up to 1 l with water. Sterile filter and store at room temperature.

8. *10 % NP-40 (200 ml)*: Add 20 ml NP-40 to 180 ml water. Thoroughly mix and store at 4 °C.
9. *5 M NaCl (1 l)*: Weigh 50 g NaCl and Weigh 238.3 g HEPES and transfer to a glass beaker. Add water to a volume of 900 ml. Mix and make up to 1 l with water. Sterile filter and store at room temperature.
10. *1 M Tris–HCl, pH 7.5*: Purchased from Thermo Fisher Scientific (*see* **Note 3**).

2.2 Working Solutions

1. *Dilution Mix Buffer (1406 ml)*: Add 100 ml 1 M HEPES–-KOH, 40 ml 5 M NaCl, 4 ml 0.5 M EDTA, 4 ml 0.5 M EGTA to 1258 ml water. Thoroughly mix and adjust pH to 7.5. Sterile filter and store at 4 °C.
2. *FA Fix Solution*: To prepare fresh FA Fix Solution, mix 37 % formaldehyde (FA) and Dilution Mix Buffer at the ratio of 1:2.3. The FA Fix Solution should always be freshly prepared before use.
3. *Cold PBS, pH 7.4*: Purchased from Life Technologies. Store at 4 °C.
4. *Block Solution (1 l)*: Dissolve 5 g BSA in 1 l PBS. Sterile filter and aliquot in Falcon™ 50 ml conical centrifuge tubes. Store at 4 °C for a few days. For longer time storage, keep at −20 °C and thaw in 37 °C water bath before use.
5. *Sonication Buffer (500 ml)*: Add 25 ml 1 M HEPES–KOH, 14 ml 5 M NaCl, 1 ml 0.5 M EDTA, 1 ml 0.5 M EGTA, 50 ml 10 % Triton X-100, 10 ml 5 % Na-deoxycholate, and 5 ml 10 % SDS to 394 ml water. Thoroughly mix and adjust pH to 7.5. Sterile filter and store at 4 °C. Make sure to add Protease Inhibitor Cocktails before use.
6. *High-Salt Sonication Buffer (500 ml)*: Add 25 ml 1 M HEPES–KOH, 50 ml 5 M NaCl, 1 ml 0.5 M EDTA, 1 ml 0.5 M EGTA, 50 ml 10 % Triton X-100, 10 ml 5 % Na-deoxycholate, and 5 ml 10 % SDS to 358 ml water. Thoroughly mix and adjust pH to 7.5. Sterile filter and store at 4 °C.
7. *LiCl Wash Buffer (500 ml)*: Add 10 ml 1 M Tris–HCl, 1 ml 0.5 M EDTA, 125 ml 1 M LiCl solution, 25 ml % NP-40, and 50 ml 5 % Na-deoxycholate to 289 ml water. Thoroughly mix and adjust pH to 8.0. Sterile filter and store at 4 °C.
8. *Elution Buffer (500 ml)*: Add 25 ml 1 M Tris–HCl, 10 ml 0.5 M EDTA, and 50 ml 10 % SDS to 415 ml water. Thoroughly mix and adjust pH to 8.0. Sterile filter and store at 4 °C.
9. *$CaCl_2$ Solution (100 ml)*: Weigh 3.3 g $CaCl_2$ and dissolve it in 80 ml water. Add 1 ml 1 M Tris–HCl, pH 7.5, and bring volume to 100 ml with water. Sterile filter and store at room temperature.

10. *TE Buffer (500 ml)*: Mix 5 ml 1 M Tris–HCl, pH 7.5, 1 ml 0.5 M EDTA, pH 8.0, and 496 ml water. Sterile filter and store at room temperature.
11. *TE-0.1 % Triton X-100 Buffer (500 ml)*: Mix 5 ml 1 M Tris–HCl, pH 7.5, 0.1 ml 0.5 M EDTA, pH 8.0, 5 ml 10 % Triton X-100, and 490 ml water. Sterile filter and store at room temperature.
12. *Nulcei EZ Lysis Buffer*: Purchased from Sigma. Make sure to add Protease Inhibitor Cocktails before use.

2.3 Other Materials

1. Dynabeads® Protein A or G beads (Thermo Fisher Scientific).
2. Protease Inhibitor Cocktails (Roche Life Science).
3. RNase A (Sigma).
4. Proteinase K (Roche Life Science).
5. Glycogen Solution (Roche Life Science).
6. Phenol:Chloroform:Isoamyl Alcohol (Sigma).
7. Ethanol, Molecular Biology Grade (Thermo Fisher Scientific).
8. Genomic DNA Sample Prep Kit (Illumina, Catalog IDs: FC-102-1001, FC-102-1002, FC-102-1003, FC-102-1004).
9. DynaMag™-5 Magnet (Thermo Fisher Scientific).
10. MaXtractTM High Density 2 ml tubes (Qiagen).
11. LoBindEppendorf 1.5 ml tubes (Eppendorf).
12. Polypropylene Eppendorf 5.0 ml Tube with flip-open cap (Eppendorf).

3 Methods

3.1 Day 1: CML Cells Culture and Antibody-Beads Coupling

1. Seed 100–200 $\times$ 10^6 cells into 15 cm dishes in 20 ml complete culture medium (*see* **Note 4**). Let the cells grow for about 24 h before fixation.
2. Resuspend Dynabeads in the vial by vortex for more than 30 s and, for each sample, add 100 μl Dynabeads® Protein A or G beads to a 1.5 ml Eppendorf tube on ice. Wash beads in 1 ml Block Solution for three times and incubate end over end at 4 °C for 10 min between each wash steps (*see* **Note 5**). Let the tubes stand in the magnet for 1 min before aspiration. Add 250 μl Block Solution to the beads after final wash step. Add 10 μg antibody of your interest and incubate the mixture end over end at 4 °C overnight (*see* **Note 6**).

3.2 Day 2: Cell Fixation, Nuclear Lysate Sonication, and Immunoprecipitation

1. Add 2 ml fresh prepared FA Fix Solution (1/10 of the cell culture medium volume) directly to each dish. Swirl plates thoroughly and incubate at room temperature for 15 min (*see*-**Note 7**). Swirl plates every 3–5 min. Add 5 ml 1 M Tris-Hcl, pH 7.5 to each plate and swirl plates thoroughly. Transfer entire volume to 50 ml Falcon tubes and centrifuge at ($\sim$400 $\times$ *g*) for 5 min at 4 °C. Carefully discard the supernatant and gently suspend cells in 12 ml cold PBS by pipetting up and down. Transfer cell suspension to 15 ml Falcon tubes and centrifuge at ($\sim$400 $\times$ *g*) for 5 min at 4 °C (first wash). Carefully discard the supernatant and repeat the wash in 12 ml cold PBS (second wash). Keep the fixed cells on ice (*see* **Note 8**).
2. Add 10 ml Nulcei EZ Lysis Buffer to the fixed cell pellet and thoroughly suspend cells by pipetting up and down (*see* **Note 9**). Incubate on ice for 5 min, centrifuge at ($\sim$400 $\times$ *g*) for 5 min at 4 °C, and carefully discard the supernatant by vacuum. Repeat the above procedure once in another 10 ml Nulcei EZ Lysis Buffer and carefully discard supernatant by vacuum after centrifuge and keep the nuclear pellet on ice. Add 3 ml Sonication Buffer to each nuclear pellet and thoroughly resuspend the pellets by pipetting up and down using 1 ml pipette. Transfer the nuclear lysate to 5 ml Eppendorf tubes with flip-open cap on ice.
3. Program the sonicator (10 $\times$ 30 s pulses, 5 min in total, 18–21 W of power, 1 min break down cycles, and output level at 4.5 (*see* **Note 10**)) and sequentially sonicate samples on ice. Make sure that no foaming occurs during the sonication (*see* **Note 11**). Dispense sonicated nuclear extracts (SNE) into precooled 1.5 ml Eppendorf tubes on ice (two tubes per SNE) and centrifuge at full speed at 4 °C for 10 min. Collect 3 ml of each cleared SNE into a 15 ml Falcon tube and keep on ice. Transfer 25 μl of each 3 ml cleared SNE to a new 1.5 ml Eppendorf tube and store at −80 °C for the preparation of genomic DNA used as Input Control (*see* **Note 12**).
4. Following the incubation at 4 °C from day 1, wash the antibody-coupled beads for four times in 1 ml Block Solution to clear uncoupled antibodies. Mix the beads thoroughly by inverting tubes between each wash step (*see* **Note 5**). Resuspend the beads in 100 μl Block Solution after final wash; add the 100 μl beads to the cleared SNE in 15 ml Falcon tubes and incubate end over end at 4 °C overnight.

3.3 Day 3: Beads Washing and DNA Elution

1. Keep a magnet on ice. Prechill 1.5 ml Eppendorf tubes on ice and transfer the SNE–beads mixture into the tubes. Let the tubes stand in the magnet for 1 min before aspirating the supernatant and repeat the procedure until all 3 ml SNE–beads mixture is done.

2. To wash the beads, add 1 ml Sonication Buffer, resuspend and mix the beads thoroughly and gently by inverting. Do not vortex. Spin down the liquid on tube caps at ~400 × *g* for 10 s and transfer the tubes to magnet on ice (*see* **Note 5**). Let the tubes stand in the magnet for 1 min before aspiration (first wash). Repeat the washing sequentially using following buffers: Sonication Buffer (second wash), High-salt Sonication Buffer (third wash), LiCl Buffer (fourth wash), and TE-0.1 % Triton X-100‘ Buffer (fifth wash). After final wash, centrifuge at ~5,900 × *g* for 1 min, place tubes in the magnet for 1 min, and aspirate all remaining liquid from the bottom of the tubes.
3. Add 200 µl Elution Buffer to each tube and thoroughly resuspend beads by pipetting up and down. Transfer the suspension to 1.5 ml DNA LoBindEppendorf tubes. Vortex and incubate the beads suspension at 65 °C for 30 min. Vortex every 5–10 min to prevent the beads from forming pellets during the incubation. Following the incubation, centrifuge at full speed for 2 min and transfer the tubes into magnet. After 2 min, transfer all elutes from the bottom of the tubes to new 1.5 ml DNA LoBindEppendorf tubes. Thaw 25 µl Input Control from −80 °C and add 175 µl Elution Buffer. Reverse cross-linking by incubating all elutes and Input Controls at 65 °C overnight.

3.4 Day 4: DNA Purification

1. After reverse cross-linking, add to each elutes and Input Controls 200 µl TE Buffer and 6 µl of 30 mg/ml RNase A. Mix and incubate at 37 °C for 2 h. Then add 7 µl $CaCl_2$ Solution and 6 µl 20 mg/ml Proteinase K, mix and incubate at 55 °C for 30 min. In the meantime, prepare MaXtractTM High Density 2 ml tubes by centrifuging them at full speed for 1 min. Following incubation, add 400 µl Phenol:Chloroform:Isoamyl alcohol to each sample, mix thoroughly by vortex, and transfer all liquid to the MaXtractTM High Density 2 ml tubes. Centrifuge the tubes at full speed for 7 min and transfer the newly formed aqueous layer to new 1.5 ml DNA LoBindEppendorf tubes (*see* **Note 13**).
2. Add to the samples 16 µl 5 M NaCl Solution, 1 µl of 20 µg/µl glycogen solution, and 880 µl ethanol. Mix thoroughly by vortex and cool the mixture at −80 °C for 1 h, followed by 4 °C for 5 min. Centrifuge the mixture at full speed for 20 min at 4 °C to precipitate the DNA. Aspirate the supernatant carefully without disturbing the pellets at the bottom of the tubes (*see* **Note 14**). Spin the tubes at full speed again for 1 min and carefully remove all residual supernatant by pipetting.
3. Add 600 µl 75 % ethanol, mix by vortex, and centrifuge at full speed for 10 min. Aspirate the supernatant carefully without

disturbing the pellets. Spin the tubes at full speed again for 1 min and carefully remove all residual supernatant by pipetting. Air dry the pellets for about 10 min (*see* **Note 15**); add 25 μl TE Buffer to each sample to dissolve the pellets (*see* **Note 16**).

3.5 DNA Library Construction and Sequencing

We follow the Solexa Genomic DNA protocol using Genomic DNA Sample Prep Kit, Illumina. In brief, the immunoprecipitated DNA was end repaired and subjected to 18 cycles of linker-mediated (LM)-PCR using oligos purchased from Illumina. Amplified fragments between 150 and 300 bp were isolated by agarose gel electrophoresis and purified. High-quality samples were confirmed by the appearance of a smooth smear of fragments from 100 to 1000 bp with a peak distribution between 150 and 300 bp. Three nanograms of linker-ligated DNA was applied to the flow cell using the Solexa Cluster Station fluidics device. Samples were then subjected to 26 bases of sequencing.

4 Notes

1. You can also prepare EDTA and EGTA solution using powder purchased from Sigma. To prepare 1 l EDTA or EGTA solution, weigh 146.12 g EDTA or 190.175 g EGTA. Transfer to a glass beaker. Add water to a volume of 800 ml. Stir and adjust pH to 8.0 with NaOH. EDTA and EGTA will not dissolve until the pH value is close to 8.0. Make up to 1 l with water. Sterile filter and store at room temperature.
2. To prepare 500 ml of 10 % SDS solution by yourself, weigh 50 g SDS (Sigma) in 1 l glass beaker. Make sure to wear mouth mask to avoid inhalation of SDS dust. Add 400 ml water and set the magnetic stirrer temperature 68 °C to heat and let SDS dissolve. Make sure to dissolve any residual powder sticking to the glass. Make up to 500 ml with water. Sterile filter and store at room temperature.
3. To prepare 1 l 1 M Tris–HCl, pH 7.5 solution, dissolve 121.14 g Tris in 800 ml water in a glass beaker. Adjust pH to 7.5 with the appropriate volume HCl. Bring final volume to 1 l with water. Sterile filter and store at room temperature.
4. We usually use 100–200 $\times$ 10^6 cells for each ChIP-*seq* assay, especially for proteins that are hard to "ChIP." However, the amount of starting material is dependent on the protein of your interest and the antibody used for immunoprecipitation. For antibodies such as anti-H3K27ac, 1 $\times$ 10^6 cells can produce results of good quality.

5. After inverting the tubes for several times, spin down the liquid on the tube caps at (~400 × *g*) for 10 s before transferring the tubes to magnet on ice. Make sure not to spin down the beads at higher speed. It may damage the beads.
6. ChIP-*seq* highly depends on the antibody used for the immunoprecipitation. If no antibody of your interest protein has been validated for ChIP application, it is helpful to screen a wide variety of available antibodies using small-scale ChIP-PCRs to test for enrichment at a gene-specific level at the known binding sites of your protein of interest before launching a full-scale ChIP-*seq* experiment. Prioritize antibodies that have been validated by immunoprecipitation, immunohistochemistry, or immunocytochemistry. They will be more likely to perform well in ChIP applications as well.
7. In practice, we have found that the fixation time and formaldehyde concentration provided here are generally applicable and work successfully with a variety of proteins in different CML cell lines. But fixation times depend on cell numbers and the proteins to be immunoprecipitated and could theoretically be optimized for each condition. Insufficient fixation can result in the inability to capture protein–DNA contacts. Overfixation can actually denature the protein of interest or cause overaggregation, thus obscuring the epitope. If optimization is needed, we recommend trying different time course while keeping other parameters, such as cell number and formaldehyde concentration.
8. For convenience, the fixed cells pellets can be snap frozen using liquid nitrogen and stored at −80 °C for several weeks, which does not affect the following procedure and the quality of ChIP-*seq* results.
9. If you use the frozen cell pellets, make sure to add Nulcei EZ Lysis Buffer before pellets thaw.
10. Sonication is the most variable step in the process and will vary greatly depending on cell type, cell culture, conditions used, quantity of cells, volume of sonication, degree of cross-linking, and specifics of the sonicator being used. As a result, conditions must usually be optimized for each situation. One way to identify these conditions is to run a time course of sonications. We usually set up with a default power setting but with an extended time of sonication. We then remove small aliquots (100 μl out of 3 ml of material) at discrete time points. After cross-link reversal and purification, as described in the protocol, the DNA can be run on a 2 % agarose gel to estimate the degree of sonication. The optimized sonication should at least produce a quarter of the total DNA that is sized from 200 to 600 bp in size, with less emphasis on the average size or overall spread of fragment sizes.

11. Foaming may cause poorly sheared DNA. To avoid that, keep sonication tip centered and within 0.5 cm of bottom of tube. A little foaming may occur and will not affect the results. But if there is significant foaming, we recommend removing all bubbles by centrifugation followed by gentle resuspension of all material, leaving no foam bubbles.
12. ChIP-*seq* assay is enrichment relative to a reference and not an absolute measurement, thus it is critical to include unenriched, genomic DNA for each cell line used in your experiment as reference sample, or Input Control. If several different antibodies are used in the same CML cell line, we usually only include one Input Control for all of them. You don't need negative controls using "beads only" or beads with an isotype-matched control immunoglobulin for ChIP-*seq*.
13. Hold the tubes steady, transfer the aqueous layer slowly and carefully using 200 μl pipette tips and avoid disturbing lower layers.
14. Be careful when aspirate using vacuum because the pellets may easily detach from the wall of tube. We usually aspirate until about 100–200 μl liquid left at the bottom of the tube and take them out carefully using 200 μl pipette.
15. Although we find that 10 min works well generally, the drying time depends on the size of pellets. Check the pellets every few minutes. The white pellets will become transparent when they are dry.
16. Be careful to handle the tubes. Sometimes we do observe that the pellet jumps in the tube due to static electricity when we are trying to reach the tube, thus loosing the pellets. It helps to spray your gloves with 70 % ethanol. To dissolve the pellets, it usually works well to incubate for 10 min at room temperature, but overnight incubation at 4 °C is recommended. The samples can be stored at −80 °C for weeks.

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Chapter 12

Quantitative Proteomics Analysis of Leukemia Cells

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Abstract

Chronic myeloid leukemia (CML) is driven by the oncogenic fusion kinase Bcr-Abl, which organizes its own signaling network with various proteins. These proteins, their interactions, and their role in relevant signaling pathways can be analyzed by quantitative mass spectrometry (MS) approaches in various models systems, e.g., in cell culture models. In this chapter, we describe in detail immunoprecipitations and quantitative proteomics analysis using stable isotope labeling by amino acids in cell culture (SILAC) of components of the Bcr-Abl signaling pathway in the human CML cell line K562.

Key words Chronic myeloid leukemia, Gab2, Bcr-Abl, Protein complex, Protein dynamics, Mass spectrometry, Proteomics, Posttranslational modification, SILAC, Immunoprecipitation

1 Introduction

Chronic myeloid leukemia (CML) represents about 20 % of all cases of adult leukemia and is caused by a chromosomal translocation between chromosomes 9 and 22 leading to the expression of the fusion kinase Bcr-Abl [1]. This oncogenic tyrosine kinase generates its own signaling network with various components such as kinases Lyn or Jak2 or docking proteins like Gab2 [2–8]. Transformation by Bcr-Abl leads to addiction to constant input signals derived from this kinase [9, 10]. Selective Bcr-Abl tyrosine kinase inhibitors (TKIs), such as Imatinib mesylate (IM), exploit this oncogene addiction and yield unprecedented responses in CML therapy [11]. Despite this great success, about 20 % of CML patients develop TKI resistance, which represents a huge and unsolved problem in the clinic [12–14]. TKI resistance is often, but not exclusively, caused by mutations within the kinase domain of Bcr-Abl and can in many cases be overcome by second-generation TKIs, like dasatinib (DST) or nilotinib (NL) [15–17]. However, about 40 % of resistances are Bcr-Abl mutation independent and still ill defined at the molecular level [18, 19]. For this reason, a deeper knowledge of the Bcr-Abl signaling network is

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essential to identify new components of this network, which may be employed as new drug targets to face the problem of TKI resistance.

Quantitative mass spectrometry (MS)-based proteomics represents an ideal method to analyze the signaling network built up by Bcr-Abl. It allows to determine protein abundance [20], activity [21], as well as protein–protein interactions [22]. For this purpose, there are different mass spectrometry approaches available, which rely on relative protein quantitation to compare different cellular states. This can be realized by the incorporation of isotopically labeled amino acids [23], by chemical labeling [24], or by label-free quantitation [25]. In this chapter, we will concentrate on "**S**table **I**sotope **L**abeling by **A**mino acids in **C**ell culture" (SILAC) which is based on metabolic labeling with arginine and lysine [23, 26]. This technique is widely used in cell culture [21, 27] or animal models such as mouse [28] or *Drosophila* [29]. In the provided protocol, we describe immunoprecipitations of Bcr-Abl signaling components from the human CML cell line K562 (*see* **Note 1**), using liquid chromatography mass spectrometry (LC–MS/MS). This approach allows to analyze the interactome and the posttranslational modifications of your protein of interest under different conditions.

2 Materials

All solutions were prepared using deionized water and analytical grade reagents (unless stated otherwise).

2.1 Cell Lines

1. K562: human CML cell line (*see* **Note 1**).

2.2 Cell Media

1. Culture medium: RPMI 1640, 10 % Fetal Bovine Serum (FBS), 200 mM L-glutamine, 10 mM HEPES.
2. Metabolic labeling medium: SILAC DMEM (deficient in lysine and arginine), 10 % dialyzed Fetal Bovine Serum (FBS), 200 mM L-glutamine, 10 mM HEPES, "light" amino acids (L-arginine, L-lysine, Sigma Aldrich, Hamburg, Germany), "medium-heavy" amino acids (L-arginine-$^{13}C_6$ hydrochloride (Arg6), L-lysine-4,4,5,5-D_4 hydrochloride (Lys4), Silantes, Munich, Germany), "heavy" amino acids (L-arginine-$^{13}C_6$, $^{15}N_4$ hydrochloride (Arg10), L-lysine-$^{13}C_6$, $^{15}N_2$ hydrochloride (Lys8), Silantes, Munich, Germany).

2.3 Cell Harvest, Lysis, and Gel Electrophoresis

1. Normal lysis buffer (NLB): 50 mM Tris–HCl pH 7.4, 1 % Triton-X 100, 137 mM sodium chloride, 1 % glycerin, 1 mM sodium orthovanadate, 0.1 μg/μl aprotinin, 0.01 μg/μl leupeptin, 1 mM AEBSF.
2. Dithiothreitol (DTT): 1 mM.

3. Iodoacetamide (IAA): 5.5 mM.
4. NuPAGE® Novex 4–12 % (Bis-Tris gel system, Invitrogen/Life Technologies): MOPS running buffer.
5. Colloidal Blue Stain (Invitrogen/Life Technologies).
6. Trypsin: Sequence grade modified.
7. Ammonium bicarbonate (ABC) buffer: 50 mM.

2.4 Sample Preparation and LC–MS/MS Analysis

1. Buffer A: 0.5 % acetic acid (high purity, LGC Promochem, Wesel, Germany).
2. Buffer A*: 3 % acetonitrile and 0.3 % TFA.
3. Buffer B: 0.5 % acetic acid in 80 % acetonitrile (ACN) (LC–MS grade, Wako Chemicals GmbH, Neuss, Germany), 20 % water.
4. Empore Discs—C18 material for STAGE tips (3 M, IVA Analysentechnik, Meerbusch, Germany).

2.5 Mass Spectrometry Equipment

1. LTQ Orbitrap XL mass spectrometers (Thermo Fisher Scientific, Bremen, Germany).
2. Agilent 1200 nanoflow–HPLC (Agilent Technologies GmbH, Waldbronn, Germany).
3. NanoLC Ultra (Eksigent, AB Sciex, Radio Rd Redwood City, CA, USA).
4. HPLC–column tips (fused silica): 75 μm inner diameter (New Objective, Woburn, MA, USA) are self-packed with Reprosil–Pur 120 ODS–3 (Dr. Maisch, Ammerbuch, Germany) to a length of 20 cm.

3 Methods

Quantitative proteomics, especially SILAC is often used for the comparison of different cellular states in terms of protein abundance or posttranslational modifications like phosphorylation, ubiquitination, or sumoylation. SILAC can also be combined with classical biochemical approaches as, for example, immunoprecipitations (IP). Here, we specifically provide a protocol for SILAC-based IPs of components of the Bcr-Abl signaling pathway in the human CML cell line K562. However, this experimental approach is generic and the described protocol can be used for other quantitative proteomic studies as well. As outlined in Fig. 1 the workflow starts with the incorporation of the labeled amino acids arginine and lysine. After a labeling period of at least seven cell doublings cells can be expanded. The next step is cell lysis followed by IP of your protein of interest. After IP protein mixtures are combined in a 1:1:1 ratio (*see* **Note 2**). Combined proteins are then separated by SDS-PAGE and enzymatically digested (e.g., trypsin, *see* **Note 3**) in-gel, generating peptides for LC–MS/MS analysis. *See* **Note 4** for an example of such an approach.

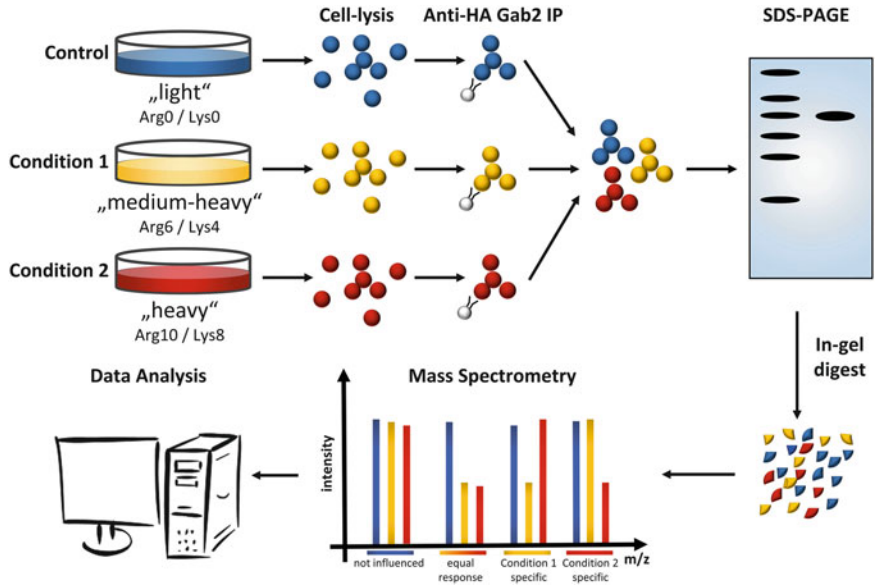

Fig. 1 Workflow. Cells are labeled for each condition with light, medium–heavy, and heavy amino acids. After cell lysis the proteins and their interaction partners are purified by immunoprecipitation and subsequently mixed. The combined lysates are separated by SDS-PAGE and enzymatically digested in-gel for LC–MS/MS analysis

3.1 Cell Culture and Passaging

K562 cells are cultivated in RPMI-1640 and the listed supplements under water vapor-saturated atmosphere at 37 °C and 5 % CO_2. The cells are split every 2 days in a 1:10 ratio. For the metabolic labeling, the cells are switched to SILAC DMEM supplemented with dialyzed FBS and "light" (Arg0/Lys0), "medium-heavy" (Arg6/Lys4), or "heavy" (Arg10/Lys8) amino acids (see Media for details).

3.2 SILAC Cell Culture

1. For a complete incorporation of the labeled amino acids, cells should be cultivated for at least seven doublings in SILAC medium [30]. This can be done in a small format like 6-well plates or small flasks to save SILAC media.
2. For expansion of cells, we recommend to use at least six 15 cm dishes per condition and label (approx. 1.2×10^8 cells).
3. Dialyzed serum with no amino acids has to be used to ensure that only labeled variants of arginine and lysine are present.
4. The arginine to proline conversion [31] (Fig. 2) and the labeling efficiency (Fig. 3) should be analyzed prior to large-scale MS experiments. The content of arginine should be titrated to

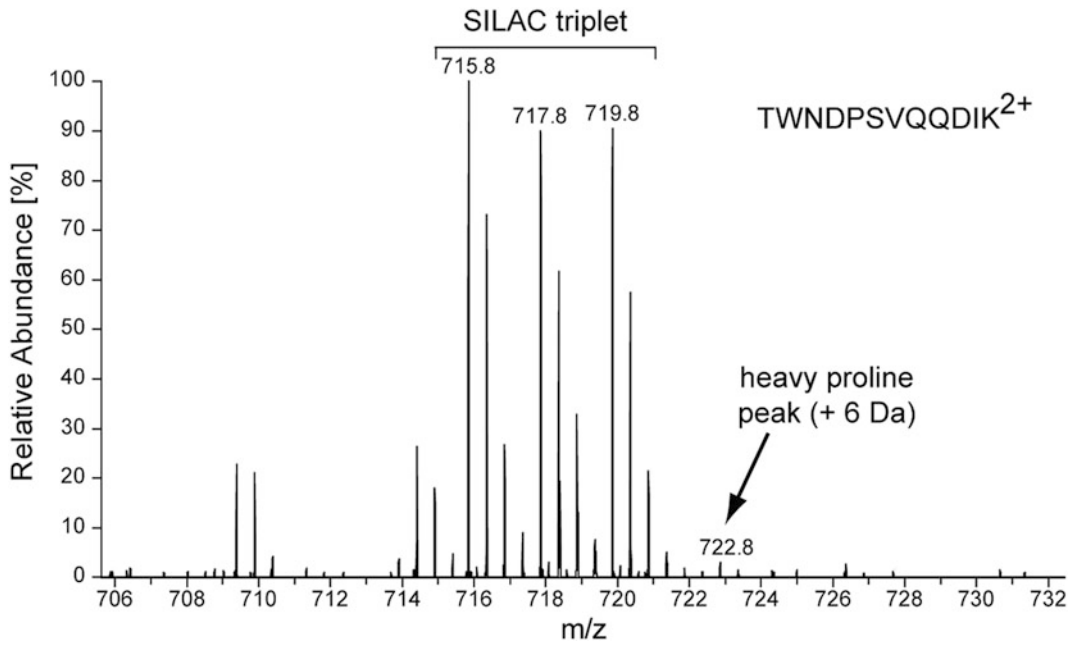

Fig. 2 Proline to Arginine conversion. K562 cells were labeled for 2 weeks using a triple SILAC approach. The SILAC triplet of the doubly charged peptide TWNDPSVQQDIK of HSPA5 is depicted. Heavy arginine (Arg10) can be metabolized to heavy proline (Pro6) leading to quantification errors. Amino acids have to be titrated to yield complete labeling and suppress arginine to proline conversion. As the average relative technical error of MS-based quantifications is around 20 % the aim is to have a proline conversion, which is smaller than that. In the depicted example, 3.2 % of heavy arginine is metabolized to heavy proline

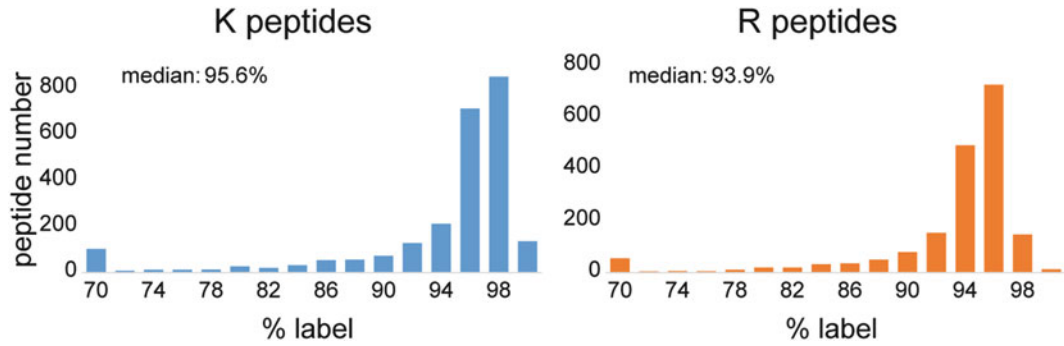

Fig. 3 Label efficiency. Cells were labeled for 2 weeks in SILAC-DMEM medium supplemented with dialyzed FCS, 73 mg/l $^{13}C_6$, $^{15}N_2$-lysine (K8), 42 mg/l $^{13}C_6$, $^{15}N_4$-arginine (R10), and 26.7 mg/l proline. The labeling efficiency was analyzed by MS. For this, the respective dataset was searched using light and heavy SILAC as variable peptide modifications

minimize the conversion to proline. Alternatively, unlabeled proline can be titrated to the medium (*see* **Note 5**).

5. We recommend swapping the labels for a biological replicate to disclose any effects from the label per se.

3.3 Cell Lysis and Immunoprecipitation

1. Harvest cells by centrifugation and collect all cells from the same condition in one tube.
2. Remove supernatant (SN) and resuspend pellet in at least 1 ml normal lysis buffer (NLB) (*see* **Note 6**).
3. Incubate on a nutator or wheel at 4 °C for at least 30 min.
4. Centrifuge the crude lysate at top speed and 4 °C for 10 min.
5. Transfer the supernatant to new tubes already containing the precipitating antibody.
6. Incubate the total cell lysate (TCL) with the precipitating antibody on ice for 1 h.
7. Add 60–100 µl of Protein-G-Sepharose slurry (equilibrated by two washes with lysis buffer) with a cut tip (larger orifice), so that a clearly visible and easy to handle pellet appears after brief centrifugation (for precoupled beads please *see* **Note 7**).
8. Incubate the TCL/antibody/Protein G-Sepharose mix on a nutator or wheel at 4 °C for at least 4 h or overnight.
9. Place the tubes in a cooled centrifuge and spin at 400 × *g* for 1 min. Place the tubes for 1 min on ice or leave them for a 1 min in the cold rotor. This will help to solidify the Sepharose pellet and will facilitate the removal of the SN in the next step.
10. Remove the SN by aspiration using a blue (1000 µl) tip. Be careful not to aspirate the pellet, rather leave a trace of the SN on top of the pellet.
11. Resuspend the pellet in 1 ml of fresh NLB.
12. Repeat **steps 9–11** at least five times.
13. Resuspend the pellet in 100 µl NLB.
14. Combine the pellets of each IP in 1:1:1 ratio in one tube using again a cut tip.
15. Wash the original tubes twice with NLB and collect all in the new tube, to ensure not to lose any beads. If necessary, centrifuge and remove the SN in between the washing steps.
16. Centrifuge the tube with the combined beads and remove residual SN.
17. Resuspend the pellet in 100 µl NLB and proceed with the MS sample preparation or store it at −80 °C.

3.4 MS Sample Preparation by In-Gel Digest

1. Add two times the pellet volume Laemmli buffer supplemented with DTT (1 mM) to reduce disulfide bonds. Incubate for 30 min at 75 °C and vortex or pipette briefly to ensure quantitative lysis.
2. Alkylate thiols by incubation with 5.5 mM iodoacetamide for 30 min at RT in darkness.

3. Separate the protein mixture on a NuPAGE® gel, fix the gel by incubation in 50 % methanol/10 % acetic acid in deionized water for 10 min and stain it using Colloidal Blue to assess the separation quality.
4. Cut each gel lane into ten slices of equal size, cut each slice into approx. 1 mm^3 cubes, transfer cubes into a reaction tube and wash out remaining Colloidal Blue by three alternating 10 min incubations in 50 mM ammonium bicarbonate (ABC) buffer and 100 % ethanol, both 100 µl.
5. To enable the following digest with trypsin remove any ethanol and dry cubes in a speedvac for 5 min [32].
6. Let cubes swell in 50 µl trypsin solution (12.5 µg/ml 50 mM ABC) with 1 µg trypsin per 50 µg protein on ice for 1 h, then add 100 µl ABC buffer (50 mM), and incubate over night at 37 °C.
7. Stop trypsin activity by adding 50 µl of 1 % TFA. Shake gel cubes at 200 g for 20 min at RT, transfer solution to new tube, and repeat step two times with 100–150 µl ethanol to extract peptides. Combine supernatants of respective slices after each step.
8. Concentrate the collected peptide solution to less than 50 µl in a speedvac to remove ethanol and add 200 µl buffer A.
9. Prepare STAGE tips [33] for affinity purification of the peptides to remove salts (*see* **Note 8**): Punch out two 0.5 mm discs from a C18 material, pack them tightly in the tip of a 200 µl pipette tip, and proceed by centrifuging solutions through the discs in the following order: 50 µl methanol to reconstitute the material, 50 µl buffer B to remove impurities, and two times 50 µl buffer A to remove buffer B. Now load the sample, wash one time with 100 µl buffer A, and elute the sample with 50 µl buffer B into a reaction tube.
10. Concentrate eluate to less than 5 µl to remove acetonitrile and add 10 µl buffer A/A* (75/25). The samples are now ready to be loaded on the HPLC.

3.5 LC–MS/MS Analysis

Mass spectrometric measurements are performed on LTQ Orbitrap XL mass spectrometers coupled to an Agilent 1200 nanoflow-HPLC or a NanoLC. HPLC column tips (fused silica) with 75 µm inner diameter are self-packed with Reprosil–Pur 120 ODS–3 to a length of 20 cm. Samples are applied directly onto the column without precolumn. A gradient of Buffer A and Buffer B with increasing organic proportion is used for peptide separation (loading of sample with 2 % B; separation ramp: from 10 to 30 % B within 80 min). The flow rate is 250 nl/min and for sample application 500 nl/min. The mass spectrometer is operated in the data-dependent mode and switched automatically between MS

(max. of 1×10^6 ions) and MS/MS. Each MS scan was followed by a maximum of five MS/MS scans in the linear ion trap using normalized collision energy of 35 % and a target value of 5000. Parent ions with a charge state from $z = 1$ and unassigned charge states are excluded for fragmentation. The mass range for MS was 370–2000 m/z. The resolution is set to 60,000. MS parameters are as follows: spray voltage 2.3 kV; no sheath and auxiliary gas flow; ion transfer tube temperature 125 °C.

3.6 Data Analysis

For the data analysis, we recommend the freely available software Max Quant and Perseus [34, 35].

4 Notes

1. The K562 cell line is the first established human myelogenous leukemia line. The cells were isolated from a 53-year-old female CML patient in blast crisis [36]. The cells are nonadherent and grow in suspension. They harbor the Bcr-Abl oncogene and have proteomic similarities to undifferentiated cells of the granulocytic and erythrocytic lineages [37, 38].
2. Depending on your experimental approach, you can mix the lysates before or after the IP. To catch all interactions (stable and transient) you have to perform separate IPs before combining, otherwise you will lose the transient interactions. For details *see* ref. 22.
3. Depending on your interest, with respect to posttranslational modifications, you can use different enzymes with different cleavage specificities for the digestion of your proteins into peptides [39]. Trypsin is the most commonly used protease and cleaves at the carboxyl side of arginine and lysine if not followed by a proline. But there are also other enzymes available like chymotrypsin, pepsin, Asp-N, Glu-C, Lys-C, or Arg-C.
4. An example for such an approach is described in a publication by Halbach et al. [6]. K562 cells were treated with the TKIs imatinib and dasatinib followed by a immunoprecipitation of the docking protein Gab2. The treatment with the TKIs had a huge impact on the interactome and the phosphorylation levels of Gab2.
5. For K562 cells, we recommend to add proline in a concentration 26 mg/l to avoid conversion of arginine to proline. Concentrations of arginine and lysine are titrated to 42 mg/l and 73 mg/l, respectively (Fig. 2). The labeling efficiency is demonstrated in Fig. 3.
6. Depending on your experimental aim, you can use different lysis buffers. In case of the immunoprecipitations you have

to be careful that your lysis conditions do not destroy protein–protein interactions.

7. If you want to use precoupled beads, you can just mix the TCL with your precoupled beads and proceed with the incubation at 4 °C.
8. You can skip this step if your LC–MS system is equipped with a precolumn.

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Chapter 13

A Convenient Cell Culture Model for CML Acquired Resistance Through BCR-ABL Mutations

Zhiqiang Wang and WenYong Chen

Abstract

Tyrosine kinase inhibitors (TKIs) are the effective treatments for chronic myeloid leukemia (CML). However, clinical resistance to TKIs that leads to patient relapse remains a challenge. Acquisition of BCR-ABL mutations is crucial in the resistance but the underlying molecular mechanisms are poorly understood. Here we describe a cell culture model for CML acquired resistance in which blast crisis CML cells undergo initial apoptosis upon treatment with therapeutically effective doses of TKIs, but the cells regrow quickly with development of resistance through BCR-ABL mutations. This model mimics the clinical process of acquisition of BCR-ABL mutations and will be an important tool to dissect molecular mechanisms of CML drug resistance and to explore strategies to overcome resistance.

Key words Chronic myeloid leukemia, Acquired resistance, BCR-ABL, Mutation, Colony formation assay

1 Introduction

CML is a type of hematological malignancy resulting from BCR-ABL oncogenic transformation of hematopoietic stem cells. The potent ABL tyrosine kinase inhibitors are the effective treatments for the disease, which include imatinib (Gleevec, STI-571), Dasatinib (Sprycel), Nilotinib (Tasigna), Ponatinib (Iclusig), etc. However, clinical resistance to the drugs and toxic side effects associated with newer/more potent drugs limit the power of the drugs, and the relapse particularly in advanced phases of the disease remains a significant challenge. The resistance is primarily mediated by BCR-ABL mutation and/or gene amplification [1]. There are dozens of BCR-ABL mutations identified in CML patients, which cause resistance to TKIs to various degrees [1–3]. Among them, T315I mutation is resistant to most of TKIs in the clinic and patients with the mutation are the most difficult to treat [4–6]. To explore the mechanisms of BCR-ABL mutation acquisition and resistance,

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and to facilitate development of new therapeutic strategies to overcome the resistance, a proper model system is needed.

Traditionally, resistant CML cell lines are generated by exposing cells to gradually increasing concentrations of TKIs. However, the resulting resistant cells harbor only *BCR-ABL* gene amplification but not mutations [7], in contrast to what is seen in many patients. Ectopically expressing mutant BCR-ABL cDNA in non-CML cell lines allows clinically relevant or novel BCR-ABL mutations to render resistance to imatinib in these cells [4–6, 8–13]. However, the mutagenesis process is ignored in such settings. Various BCR-ABL mutations can also occur in primary cultured CML cells in the growth-factor-supplemented medium [14, 15]; however, the preexisting mutant cells in the primary samples cannot be excluded and sometimes are difficult to track.

Here we describe a convenient cell culture model for CML TKI resistance using a blast crisis CML cell line KCL-22 cells [16]. The cells will undergo initial apoptosis upon TKIs treatment but will regrow after 2 weeks with development of resistance through T315I BCR-ABL mutation. This model is simple, tractable, and reproducible. The preexisting BCR-ABL mutations are not required for the resistance in this model.

2 Materials

1. KCL-22 cell line was purchased from German Collection of Cell Cultures (DSMZ), Braunschweig, Germany. It was established from the pleural effusion of a 32-year-old woman with Philadelphia chromosome-positive CML in blast crisis phase. It contains p210 BCR-ABL fusion gene and a p53 mutation (deletion).
2. RPMI cell culture medium.
3. Penicillin Streptomycin (P/S), cell culture grade.
4. Characterized Fetal Bovine Serum (FBS) (HyClone). The quality of this serum is essential to ensure the success and reproducibility of the model. Alternative serum will need to be tested and compared to Hyclone serum (*see* **Note 1**).
5. Trypan blue solution.
6. Imatinib (STI-571), Dasatinib, and Nilotinib: LC Laboratories.
7. Hematocytometer.
8. 100 mm culture dishes, 24- and 6-well plates.
9. 0.005 % Crystal Violet solution prepared in 10 % ethanol.
10. High-fidelity DNA polymerase: PfuUltra HF DNA Polymerase (Agilent).

11. Liquid culture medium: RPMI + 10 % FBS + 1× P/S.
12. 2× RPMI medium: dissolve one package of RPMI powder (Gibco) in 500 mL ddH_2O, add 2 g $NaHCO_3$, adjust PH to 7.05, filter the solution through 0.2 μM filter. Store at 4 °C.
13. 2× soft agar medium: Freshly prepared with 2× RPMI + 20 % FBS + 2× P/S.
14. 2× bottom layer agarose: 1.2 % low gelling temperature agarose (cell culture grade, Sigma) in ddH_2O.
15. 2× top layer agarose: 0.7 % low gelling temperature agarose in ddH_2O. Both agarose solutions should be autoclaved at 120 °C for 20 min and store at 4 °C.
16. RNA Purification Kit, Gel Extraction Kit, and Miniprep Kit are from Qiagen.
17. TA Cloning Kit and CloneJET PCR Cloning Kit are from Life Technologies.

3 Methods

Carry out all procedures in biological safety cabinet unless specified otherwise.

3.1 KCL-22 Cell Culture Maintenance

Freshly thawed cells are seeded at a density of 0.5 million per mL in RPMI culture medium. The cells grow fast and need to be split 1:5 every 2–3 days to maintain the density at 0.2–1 million per mL. It is very important not to overgrow KCL-22 cells (the cell density >2 million/mL) and it is also important to record cell passage number when they are split (*see* **Note 2**).

3.2 Resistance Assay in Liquid Culture

1. Harvest KCL-22 cells from one 100 mm dish and spin down the cells at 300 × *g* for 3 min.
2. Resuspend the cells in 1× RPMI culture medium, count the cells, and adjust the cell density to 0.5 million per mL.
3. Seed 1 mL cell suspension (0.5 million cells) per well in 24-well plates (*see* **Note 3**).
4. Treat the cells with different concentrations of test drug or drug combinations (e.g. STI-571). Each group should have duplicate or triplicate wells.
5. Maintain the cells in culture without changing medium or drugs unless the test drug is not stable in culture.
6. Count viable cells at different time (day) points. At specified time points, the cells are mixed by gently pipetting up and down with a 1 mL pipette for several times and a 10 μL aliquot of cell suspension is removed and mixed with 10 μL Trypan blue solution. Load 10 μL mixed sample onto a

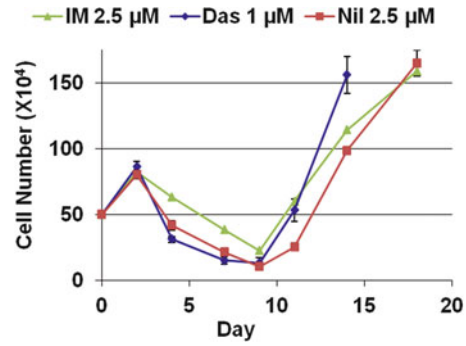

Fig. 1 Representative growth and relapse curves of KCL-22 cells in response to TKIs. IM, imatinib; Das, dasatinib; Nil, nilotinib

hematocytometer and count the viable cells (trypan blue exclusive cells). After 2 weeks' culture, the medium volume would significantly decrease and fresh drug-free medium is supplied to the cells to restore that to the original volume for prolonged culture.

7. Typically the cell number would decrease in the first week and the resistant cells would begin to appear in small clusters in nonagitated wells after a week and regrow back in about 10 days. Once the regrown cell density exceeds 1.0 million per mL, the growth curve can be terminated. The relapsed cells can be collected to extract DNA, RNA, or protein for further analysis. A typical cell growth curve is shown in Fig. 1.

3.3 Resistant Colony Formation Assay in Soft Agar

1. Prepare 2× RPMI soft agar medium freshly as in Material #12. Warm the medium to 37 °C in water bath.
2. Melt 2× agarose solutions completely by heating with a microwave oven. Leave the bottle lid open during heating. Put the agarose solution in 37 °C water bath to cool down to 37 °C for at least half an hour (*see* **Note 4**).
3. Bottom layer soft agar preparation: For each group (3 wells out of a 6-well plate), take 3.5 mL 2× RPMI complete medium into a 15 mL conical tube, add the tested drug (*see* **Note 5**) into the medium to reach 2× desired concentration, and then mix well with 3.5 mL 1.2 % agarose solution at 1:1 ratio. Aliquot 2 mL mixed solution into each well and keep the plate at room temperature until the solution gelatinizes (It would take about half an hour).
4. Top layer soft agar preparation: For each group (3 wells out of a 6-well plate), take 3.5 mL 2× RPMI complete medium into a 15 mL conical tube, add the tested drug into medium to reach 2× desired concentration, and then mix well with 3.5 mL 0.7 %

agarose solution at 1:1 ratio. Keep the mixture in 37 °C water bath until use.

5. Cell preparation: Harvest and count the KCL-22 cells. Put 3.5 million cells into a 15 mL conical tube. Spin down the cells and get rid of the supernatant. Resuspend the cell pellet in 50 μL RPMI culture medium with a pipette and gently mix cells by pipetting up and down for a few times.
6. Add 7 mL top layer soft agar solution (From **step 4**) to the cells and pipette the solution to fully mix them. Gently seed 2 mL suspension solution into each well on top of the bottom layer of agarose. Thus, one million cells per well are seeded.
7. For control wells of colony formation without drugs, 500 KCL-22 cells per well will be similarly mixed with top layer soft agar solution and seeded.
8. Keep the plates at room temperature for at least half hour to allow the top layer gel solidifying.
9. Put the plates back into the incubator and culture for 3 weeks. (To clone or amplify soft agar colonies for further analysis, skip to **step 12**.)
10. Colony staining: 2 mL 0.005 % Crystal Violet solution is added into each well and incubate for 1 h at room temperature. Then the staining solution is removed and the background is destained by ddH_2O rinse for three times.
11. Count colony number with aid of a microscope (*see* **Note 6**).
12. If further analysis of colonies such as DNA sequencing is necessary, individual colonies can be plucked from unstained wells and expanded in liquid culture to enough number of cells for such analysis.

3.4 Sequencing Analysis

3.4.1 cDNA Sequencing

1. Extract mRNA from the relapsed cells and do reverse transcription to obtain cDNA by standard methods.
2. Amplify the ABL kinase domain by PCR with a high-fidelity DNA polymerase using a forward primer 5′-GCGCAACAAGCCCACTGTCTATGG and reverse primer 5′-GCCAGGCTCTCGGGTGCAGTCC that amplify the 579 bp ABL kinase domain (*see* **Note 7**).
3. Clean the PCR product with a PCR purification kit. Alternatively, run the PCR product on an agarose gel and purify the band using a gel extraction kit.
4. Sequence the product to determine the mutation.
5. If the sequencing results indicate overlaying peaks (Fig. 2a), it suggests potential mutations. PCR products will then be cloned into pCR2.1 vector with a TA Cloning Kit or blunt-end ligated into a vector with CloneJET PCR Cloning Kit,

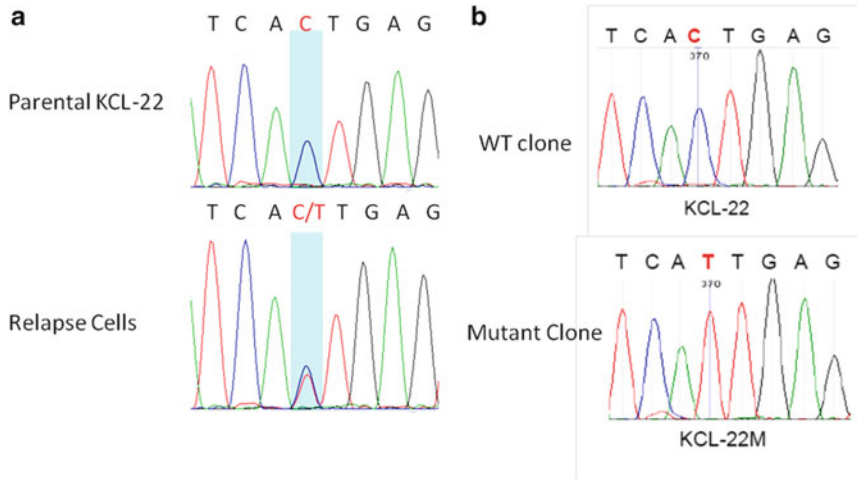

Fig. 2 Sequencing analysis of ABL codons. (**a**) Direct PCR product sequencing showed overlaying C/T peaks in relapsed cells. (**b**) Sequencing of bacterial clones of PCR products

followed by transformation of bacterial competent cells. We typically sequence at least ten bacterial clones for each treatment group to determine the mutation rate in a mixture setting (Fig. 2b) (*see* **Note 8**).

3.4.2 Genomic DNA Sequencing

To further determine the mutations, genomic DNA can also be used for amplification for sequencing.

1. Extract genomic DNA from the relapsed cells.
2. Amplify the ABL kinase domain by PCR using genomic DNA as templates with the intron primers: 5′-GAGCCACGTGTTGAAGTCCT-3′ and 5′-TTTGTAAAAGGCTGCCCGGC-3′ that span ABL exon 6 for T315I mutation, and intron primers 5′-GCCTGTCTCTGTGGGCTGAAG-3′ and 5′-TAATGCCAGCAGACGCCTTG-3′ that span ABL exon 4 for E255K and Y253H mutations.
3. Clean the PCR product as in **step 3** in Subheading 3.4.1. Submit the samples for Sanger sequencing.
4. If the sequence results show overlaying peaks (Fig. 2a), PCR products will be subcloned and sequenced as in **step 5** in Subheading 3.4.1. Typically, about 30–50 % of clones have mutations since KCL-22 cells harbors one wild-type allele of c-ABL gene that is also amplified for sequencing.
5. Primers for other parts of BCR-ABL or other genes: For oligomerization domain, we used forward primer 5′-GAGTGGGCGGGCATTGTTC and reverse primer 5′-GGGACTTTTTGCGCTCCATCT. For sequencing BCR-ABL SH3/2 domain, we used primers described previously [11].

3.5 Extended Mutation Analysis for Ectopically Expressed BCR-ABL cDNA

The BCR-ABL mutagenesis is a locus-dependent process and the mutagenesis potential between the endogenous BCR-ABL locus and exogenously integrated BCR-ABL is quite different [16]. To analyze such difference, we used KCL-22 cells ectopically expressing BCR-ABL by retroviral expression system. When resistant cells develop as in Subheading 3.2 or 3.3, the endogenous BCR-ABL locus and integrated BCR-ABL cDNA can be PCR amplified from genomic templates and distinguished with intron/intron primer pair (as in **step 2** in Subheading 3.4.2) and exon/exon primer pair (as in **step 2** in Subheading 3.4.1), respectively. To amplify the exogenous BCR-ABL template more efficiently, we also use another reverse primer 5′-TAGTCCAGGAGGTTCCCGTAG to pair with the same exon forward primer, which yields a 321 bp PCR product.

4 Notes

1. Serum used for culture is an important factor affecting the assay. While HyClone characterized FBS provides the most consistent results, its cost becomes increasingly high. We typically screened alternative brands and batches of FBS and compared side by side with HyClone characterized FBS. The results can vary significantly from brand to brand and sometimes batch to batch, suggesting that certain growth factors in serum affect the mutagenesis. It is recommended to use the same batch of serum for the experiments if possible. In addition, the quality of FBS should be reexamined if it is stored over a long period (e.g., 1 year).
2. KCL-22 overgrowth may affect resistance assay dramatically and may lead to complete failure of the assay. We also noticed that cell passages affect the assay. Cells with passage later than P30 (P1 is designated for the cells after initial thawing and plating of the frozen cells from the vendor) have less resistant colonies and a delayed relapse. Cells with lower passages give better readouts.
3. We noticed that the wells around the edge of plates sometimes gave less consistent cell relapse results if the culture lasted for longer than 2 weeks. To achieve more consistent results, it is recommended to seed cells in the center wells of a plate and fill the wells at the edge with sterile saline or water. If the edge wells should be used, they should be coupled with the center wells as replicates.
4. Hot medium (>37 °C) would hurt cells and cause failure of the colony formation assay.

5. If single TKI is used, the following concentrations are typically applied: Imatinib 1–10 μM, Dasatinib 0.1–1 μM, Nilotinib 0.5–10 μM. Lower TKI concentrations would be used if they are in combination with other drugs.
6. This cell model can be used to test resistance through other gene mutations, e.g., 6-thioguanine resistance through HPRT mutation [16].
7. The interested regions may be amplified by PCR using different primers and sent for sequencing. The hot mutation spots should be covered.
8. KCL-22 cells carry one copy of wild-type ABL gene in addition to BCR-ABL fusion gene. Therefore, the sequencing results of PCR products usually have mixed signals and need to be examined manually.

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Chapter 14

High-Throughput Screening of Tyrosine Kinase Inhibitor Resistant Genes in CML

Leyuan Ma, Justine Roderick, Michelle A. Kelliher, and Michael R. Green

Abstract

Genome-wide RNA interference (RNAi) screening in mammalian cells has proven to be a powerful tool for identifying new genes and molecular pathways relevant to many cellular processes and diseases. For example, screening for genes that, when inactivated, lead to resistance to cancer therapeutic drugs can reveal new mechanisms for how resistance develops and identify potential targetable strategies to overcome drug resistance. Here, we describe a detailed procedure for performing a high-throughput RNAi screen using a genome-wide human short hairpin RNA (shRNA) library for identifying tyrosine kinase inhibitor (TKI)-resistance genes in a human CML cell line model.

Key words Genome-wide, shRNA screen, RNAi, Tyrosine kinase inhibitor, CML, Imatinib, Drug resistance

1 Introduction

RNA interference (RNAi) is a mechanism by which expression of a specific gene is posttranscriptionally inhibited. RNAi is mediated by a double-stranded RNA, which recognizes and induces sequence-specific degradation of the corresponding mRNA, resulting in silencing or "knocking down" the gene. The development of RNAi as a genetic tool initially held great promise for analyzing loss-of-function phenotypes in mammalian cells using high-throughput, unbiased, function-based genetic screens. However, it was not until the development of RNAi expression libraries about 10 years ago [1–4] that such large-scale screens became feasible. Using these and other RNAi libraries, many studies have uncovered previously unappreciated genes and mechanisms involved in diverse biological processes in mammalian cells, particularly in the context of cancer, such as unknown tumor suppressor genes [5, 6], synthetic lethal interactions [7], and potential drug targets [8, 9].

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Today, a wide variety of RNAi libraries are commercially available. These libraries typically use a lentiviral vector to express a chimeric short hairpin RNA (shRNA), which is subsequently processed into a small interfering RNA (siRNA) to induce target mRNA degradation [10, 11]. Lentiviral-based libraries have high titers and broad trophism, making them suitable for use in a variety of mammalian cell types. Each shRNA in the library contains an easily sequenced barcode, which enable unambiguous identification of the shRNA. The genome-wide screen described in this protocol uses the human lentiviral shRNA library from The RNAi Consortium (TRC), which has ~85,000 shRNA constructs with 3–5 shRNAs per gene, targeting ~22,000 human genes [10, 12]. To facilitate screening such a large number of shRNAs, we have divided the library into "pools," with ~5000 shRNAs per pool for a total of 22 pools. It is worth noting that if other commercially available libraries are used, the protocol below should be modified accordingly (particularly for Subheading 3.5).

BCR-ABL mutation-independent resistance to tyrosine kinase inhibitors (TKIs) frequently occurs in CML [13–15]. Identifying TKI resistance-related genes helps increase our knowledge of how resistance occurs and may lead to the development of better therapeutic strategies for CML. Here we describe a genome-wide screening strategy in a human CML cell line to identify genes involved in resistance to the first-generation TKI, imatinib [16]. Because shRNA-mediated gene knockdown usually takes a few days to lead to the desired biological consequence, CML cells are transduced with the shRNA library and cultured under selective conditions for 1 week. Following an empirically determined lethal dose of imatinib, cells transduced with no shRNA or with an shRNA that does not confer TKI resistance will be killed. Live cells are then collected to identify shRNAs by sequencing, thereby identifying the corresponding genes related to TKI resistance. Here, we describe all the basics for a genome-wide shRNA screen, including shRNA library virus preparation and transduction, drug selection, shRNA identification either using TA cloning and conventional Sanger sequencing or deep sequencing, and essential procedures to validate the target genes.

2 Materials

Prepare all solutions using ultrapure double-distilled water (ddH_2O). Store all commercially obtained reagents according to the manufacturer's instructions.

2.1 Cell Lines and Culture Conditions

1. Cell lines: 293T cells (*see* **Note 1**) and human K562 CML cells (both from American Tissue Culture Collection).
2. Cell culture media: Add 50 mL of fetal bovine serum (FBS) into a 500 mL bottle of DMEM high glucose medium

(for 293T cells) or RPMI-1640 medium (for K562 cells), and add 500 μL of Penicillin–Streptomycin (1000×). Mix well and store at 4 °C. Prior to starting the cell culture experiment, warm the bottle in a 37 °C water bath for about 15 min.

2.2 Virus Preparation and Transduction

1. 6-well clear tissue culture plates.
2. TRC Lentiviral Human Genome shRNA Library (GE Dharmacon) divided into 22 pools (*see* **Note 2**) and a corresponding control nonsilencing (also called nontargeting) shRNA.
3. Lentiviral packaging plasmids pMD2.G (Addgene plasmid #12259) and psPAX2 (Addgene plasmid #12260).
4. Effectene Transfection Reagent kit (QIAGEN), which includes Effectene reagent, Enhancer, and EC buffer.
5. 0.45 μM filters.
6. Polybrene (1 μg/μL) (*see* **Note 3**).
7. Puromycin (5 mg/mL).
8. Phosphate buffered saline (PBS; 10×): 25.6 g $Na_2HPO_4 \cdot 7 H_2O$, 80 g NaCl, 2 g KCl, 2 g KH_2PO_4, ddH_2O to 1 L. Autoclave prior to use. Store at room temperature.
9. Crystal violet colony staining solution (0.05 %): Mix 0.5 g of crystal violet, 27 mL 37 % formaldehyde, 100 mL 10× PBS, 10 mL methanol, 863 mL ddH_2O. Store at room temperature.

2.3 Cell Viability (Imatinib Resistance) Assay

1. T25 tissue culture flasks.
2. Imatinib (10 mM): Weigh 24.68 mg of imatinib free base (MW: 493.60) powder (LC Laboratories) and transfer it into a 1.5 mL microcentrifuge tube. Dissolve with 1 mL of DMSO to reach a 50 mM (5×) stock solution. Dilute the 5× stock solution with DMSO into a 10 mM (1×) working solution. Store at −20 °C.
3. Trypan Blue.
4. Hemocytometer.
5. Dead Cell Removal Kit (Miltenyi Biotec), which contains ready-to-use MicroBeads and 20× Binding Buffer.
6. LS MACS columns and magnetic cell separator (Miltenyi Biotec).
7. Corning® clear bottom 96-well plates.
8. CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega).

2.4 Genomic DNA Preparation for shRNA Identification

2.4.1 Sanger Sequencing

1. Cell lysis buffer: 0.5 % SDS, 200 μg/mL of protease K, 10 mM Tris–HCl, pH 8.0, 10 mM EDTA. Store at room temperature.
2. Phenol:chloroform:isoamyl alcohol 25:24:1 saturated with 10 mM Tris, pH 8.0, 1 mM EDTA. Store at 4 °C.
3. Chloroform. Store at room temperature.
4. Glycogen (20 mg/mL).
5. Sodium acetate (3 M): Dissolve 408.1 g of sodium acetate·$3H_2O$ (MW 136) in 800 mL of ddH_2O. Adjust the pH to 5.2 with glacial acetic acid. Store at room temperature.
6. Ethanol (95 and 70 %).
7. Buffer EB (QIAGEN).
8. Taq PCR buffer (10×): 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris–HCl, pH 8.75, 20 mM $MgSO_4$, 1 % Triton X-100, 0.1 % BSA. Store at −20 °C. Alternatively, it can be purchased.
9. dNTP set (100 mM each A, C, G, T).
10. Primers: Primer1 (MF18; 10 μM), TACGATACAAGGCTGT TAGAGAG; Primer2 (MF19; 10 μM), CGAACCGCAAGG AACCTTC; Sequencing primer (M22; 5 μM), AAACCC AGGGCTGCCTTGGAAAAG.
11. Taq and Pfu DNA polymerases.
12. Dimethyl sulfoxide (DMSO).
13. pGEM®-T Easy Vector Systems (Promega), which includes TA vector (50 ng/μL), T4 DNA ligase (3 U/μL), and 2× rapid ligation buffer.
14. DH5α competent cells. Store at −80 °C.
15. 2× LB broth: Dissolve 20 g of peptone, 10 g of yeast extract, and 5 g of NaCl in 1 L of ddH_2O. Autoclave prior to use. Store at room temperature.
16. Isopropyl-ß-D-thiogalactopyranoside (IPTG; 1 M). Dissolve 1 g of IPTG in 4196 μL ddH_2O. Filter sterilize with syringe and 0.22 μm filter. Store at −20 °C.
17. 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (BCIG or X-gal; 50 mg/mL): Dissolve 50 mg of X-gal in dimethylformamide (DMF). Store at −20 °C, protected from the light.
18. LB Amp plates: Add 15 g of agar to 1 L of 2× LB broth and autoclave for 25 min. Cool down and add ampicillin (100 μg/mL). Pour onto 10 cm dishes, let solidify, and store at 4 °C.

2.4.2 Deep Sequencing

1. DNeasy Blood & Tissue Kit (QIAGEN).
2. TaKaRa Ex Taq™ Polymerase (EMD Millipore), supplied with 10× Ex Taq Reaction Buffer and 2.5 mM dNTP mix.

3. P5-ORF primer mix. Dissolve all 16 P5-ORF primers (*see* Table 1) individually and mix them at an equal molar ratio to reach 5 μM.
4. P7 index primers (5 μM): Primer A, CAAGCAGAAGACGGCATACGAGATCGGTTCAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCTACTATTCTTTCCCCTGCACTGT; Primer B, CAAGCAGAAGACGGCATACGAGATATTGGATTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCTACTATTCTTTCCCCTGCACTGT.
5. QIAquick Gel Extraction Kit (QIAGEN).
6. Bioanalyzer Lab-on-a-chip system (Agilent Technologies).
7. Deep sequencing instrument.

3 Methods

Carry out all experiments in an ultraviolet-sterilized vacuum hood. Incubate cells in a 5 % CO_2 incubator at 37 °C.

3.1 shRNA Lentivirus Preparation

1. Thaw early passage 293T cells (i.e., propagated for less than five passages) and grow them in DMEM high glucose medium for two more passages.
2. On day 1, plate 5×10^5 293T cells in each well of a 6-well plate. Use one well for each of the 22 pools, plus one more for the control nonsilencing (NS) shRNA. Shake the plates well to make sure the cells are evenly spread. Incubate at 37 °C for 24 h.
3. On day 2, aspirate old medium and add 2 mL of prewarmed fresh medium onto the cells (*see* **Note 4**). Incubate the cells at 37 °C until the transfection mixture is added. To make the transfection mixture, mix 1 μg of pooled shRNA plasmids (or control nonsilencing shRNA plasmid), 0.5 μg of pMD2.G (VSV-G envelope expressing plasmid), and 1 μg of psPAX2 (lentiviral packaging plasmid) in 100 μL of EC buffer. Add 3.2 μL of Enhancer, mix well by brief vortexing, and let sit at room temperature for 5 min. Add 10 μL of Effectene, vortex and let sit at room temperature for another 15 min. Dispense 0.5 mL of fresh medium to the transfection mixture and, while holding the plate still, gently dispense the entire mixture evenly on top of the cells.
4. On day 3, aspirate all of the medium and add 2.5 mL of prewarmed fresh medium. Incubate at 37 °C for 48 h.
5. On day 5, collect supernatant with a syringe and dispense it through a 0.45 μm filter to remove cell debris. Aliquot the virus supernatant into 1.5 mL microcentrifuge tubes. Store at −80 °C if not using immediately (*see* **Note 5**).

Table 1
List of primers used to prepare the P5-ORF primer mix

Primer name	Primer sequence (5′ → 3′)
Primer 1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTCTTGTGGAAAGGACGA
Primer 2	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATCTTGTGGAAAGGACGA
Primer 3	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGATCTTGTGGAAAGGACGA
Primer 4	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCGATCTTGTGGAAAGGACGA
Primer 5	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTACGATCTTGTGGAAAGGACGA
Primer 6	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTAGAATCTTGTGGAAAGGACGA
Primer7	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGACGACATCTTGTGGAAAGGACGA
Primer 8	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTGGACACATCTTGTGGAAAGGACGA
Primer 9	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTCTTGTGGAAAGGACGA
Primer 10	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTCTTGTGGAAAGGACGA
Primer 11	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTAGTCTTGTGGAAAGGACGA
Primer 12	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGCCTCTTGTGGAAAGGACGA
Primer 13	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTAAGCTCTTGTGGAAAGGACGA
Primer 14	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGAAACATCTTGTGGAAAGGACGA
Primer 15	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCGAGAAATCTTGTGGAAAGGACGA
Primer 16	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGAAGCATCTTGTGGAAAGGACGA

3.2 Virus Titer Determination

1. Plate 10^4 293T cells in each well of a 6-well plate and incubate at 37 °C for ~16 h. Again, use one well for each of the 22 pools, plus one more for the control nonsilencing shRNA.
2. Thaw the virus supernatant (if previously frozen), and make a series of seven tenfold serial dilutions in DMEM medium. Mix 0.1 mL of diluted virus with 0.9 mL of fresh medium. Add polybrene to a final concentration of 2 μg/mL. Gently dispense the virus mixture on top of the 293T cells and incubate at 37 °C for 2 h. Aspirate the virus supernatant and add 2.5 mL of fresh medium. Incubate at 37 °C for 24 h.
3. Add 1 μg/mL of puromycin to each well and incubate at 37 °C until colonies form (usually about 10 days).
4. Wash colonies once with 1× PBS and stain with crystal violet staining solution at room temperature for 20 min. Wash the colonies multiple times with ddH_2O until the water runs colorless. Air-dry the plate and count the colonies. Calculate the virus titer using the following formula:

   Virus titer = colony number × dilution factor × 10

3.3 Determination of Optimum Imatinib Concentration for the Screen

1. Seed 1×10^6 K562 cells in 5 mL of RPMI-1640 medium in each of six T25 flasks, and add 1 μM, 2 μM, 5 μM, 10 μM, 20 μM, or 50 μM of imatinib to each flask.
2. Monitor cell viability every day for 7 days using a trypan blue assay according to the supplier's instructions. Briefly, mix the cells well by pipetting multiple times, and transfer 10 μL of cell culture to a 1.5 mL microcentrifuge tube and mix with 10 μL of trypan blue. Load the mixture onto the hemocytometer. Determine the cell number per mL using the following formula:

   Number of cells per mL = [number of cells in all four corner squares]/$4 \times 2 \times 10^4$
3. Identify the optimum concentration of imatinib that it is able to eliminate over 99 % of live cells in a week.

3.4 shRNA Library Transduction and Selection

1. Prepare the cell transduction mixture by mixing 2×10^6 K562 cells, 1 μg/mL of polybrene, shRNA virus stock (either a pool or the nonsilencingshRNA) at a multiplicity of infection (MOI) <1 (*see* **Note 5**), and fresh medium up to a total volume of 1 mL (*see* **Note 6**). Transfer the mixture to a well of a 6-well plate, and perform a spin-down infection at 3000 rpm (~2100 × *g*), at room temperature for 90 min in an Allegra X-12 plate centrifuge (Beckman Coulter), or equivalent.
2. Immediately after infection, add 2 mL of fresh medium to each well, and incubate at 37 °C for 48 h. Transfer the cells to T25 flasks, and add 1 μg/mL of puromycin to select for 3 days.

3. Split selected cells into two fractions. Freeze down one fraction in 100 % FBS with 7 % DMSO in liquid nitrogen as a backup. Treat the other fraction of the transduced cells with 20 μM of imatinib for 1 week. Monitor cell viability using a trypan blue assay every day. Stop the drug treatment when >99 % of control (nonsilencing) shRNA-treated cells die.
4. Isolate live cells using the Dead Cell Removal Kit according to the manufacturer's instructions. Briefly, pellet all the cells at 300 × *g* in a tabletop centrifuge. Remove the supernatant completely, and resuspend the cell pellet in 100 μL of MicroBeads per ~10^7 cells. Mix well and incubate at room temperature for 15 min. Meanwhile, place the LS MACS column on the magnetic stand and prepare the column by rinsing with 3 mL of 1× Binding Buffer. Load the cell suspension onto the column and rinse four times with 3 mL of 1× Binding Buffer. Collect the effluent as the live cell fraction.

3.5 Genomic DNA Preparation and shRNA Identification

3.5.1 Method for Sanger Sequencing

1. Pellet 1–5 × 10^6 live cells per pool at (~300 × *g*) for 5 min in a tabletop centrifuge and then resuspend in 500 μL of cell lysis buffer.
2. Incubate the cell lysate at 55 °C for 2 h and then extract with an equal volume of phenol:chloroform:isoamyl alcohol. Transfer the aqueous phase into a new 1.5 mL microcentrifuge tube and extract again with an equal volume of chloroform.
3. Precipitate the DNA by adding 2 μL of glycogen, 0.1 volumes of 3 M sodium acetate, and two volumes of 95 % ethanol. Mix well by vortexing and leave at −80 °C for at least 1 h. Spin in a tabletop centrifuge at top speed at 4 °C for 30 min, and wash the pellet with 1 mL of 70 % ethanol. Aspirate ~900 μL of supernatant and use a pipette to remove the residual 100 μL to avoid losing the pellet. Air-dry the pellet for 3 min, dissolve it in 20 μL of Buffer EB, and measure the DNA concentration (*see* **Note 7**).
4. Assemble the following in a PCR reaction: ~100 ng genomic DNA, 2.5 μL 10× Taq buffer, 1 μL 10 mM dNTP, 1 μL MF18 primer, 1 μL MF19 primer, 1 μL DMSO, 0.5 μL Taq DNA polymerase, 18 μL ddH_2O.
5. Program a PCR machine with following cycling program and run the samples through:

   Step1 94 °C for 2 min

   Step2 94 °C for 30 s

   Step3 55 °C for 45 s

   Step4 72 °C for 1 min

   Step5 Go to **Step 2** for 29 additional cycles

   Step6 72 °C for 5 min

   Step7 4 °C indefinitely

6. To make sure the PCR reaction worked, load 3 μL of the reaction mixture on a 1 % agarose gel. A ~700 bp PCR product should be observed.
7. Ligate the PCR product into the TA cloning vector by setting up a ligation reaction as follows: 3 μL of PCR product, 1 μL of TA vector, 5 μL of 2× rapid ligation buffer, 1 μL of T4 DNA ligase. Incubate the ligation reaction at room temperature for 1 h followed by 4 °C for at least 12 h.
8. The next day, transform the ligation reaction into DH5α competent cells. Thaw cells on ice, and add 10 μL of the ligation reaction mixture into 100 μL of competent cells. Mix well by gently flicking the tube and incubate on ice for 30 min. Heat shock the cells at 42 °C for 1 min, then place on ice for 5 min. Add 900 μL fresh 2× LB medium and incubate at 37 °C for 30 min.
9. Meanwhile, mix 5 μL of 1 M IPTG and 50 μL of 50 mg/mL X-gal and spread on an LB Amp plate. Let sit at room temperature for a few minutes.
10. Spin down the cells in a table stop centrifuge at (~850 × *g*) for 2 min. Aspirate ~900 μL of the medium, and resuspend the cells in the remaining 100 μL medium. Spread on an LB Amp plate (*see* **Note 8**), and incubate at 37 °C for ~16 h.
11. The next day, aliquot 25 μL of ddH_2O into a series of PCR tubes, one for each colony to be picked (*see* **Note 9**). Pick a single white colony from the LB Amp plate using a pipette tip, place the tip in the PCR tube and mix well by pipetting. Remove 20 μL and discard.
12. Prepare a PCR master mix (by multiplying the following recipe by the number of colonies to be screened) and add 15 μL to each tube prepared in **step 11**: 2 μL of 10× Taq Buffer, 1 μL dNTPs, 0.5 μL M18 primer, 0.5 μL M19 primer, 0.125 μL Taq DNA polymerase, 0.125 μL Pfu DNA polymerase, 11 μL ddH_2O.
13. Program a PCR machine with the following cycling program and run the samples through:

    Step1 95 °C for 2 min

    Step2 94 °C for 1 min

    Step3 55 °C for 1 min

    Step4 72 °C for 3 min

    Step5 Go to **Step 2** for 34 additional cycles

    Step6 72 °C for 5 min

    Step7 4 °C indefinitely

14. To make sure the PCR reaction worked, load 3 μL of the reaction mixture on a 1 % agarose gel. Again, a ~700 bp PCR product should be observed.
15. Add 80 μL of ddH_2O to each tube and mix well by pipetting. Mix 2 μL of the diluted PCR product and 2 μL of M22 sequencing primer. Send for sequencing.
16. To identify shRNAs from the sequencing results, search for the sequence TTCAAAAA to find the beginning of the shRNA, CTCGAG to define the loop, and CCGGTG to define the end. Then map the shRNA sequence with the TRC shRNA library database (https://www.broadinstitute.org/rnai/trc/lib) to find the corresponding gene.

3.5.2 Method for Deep Sequencing

1. Pellet 1–5 × 10^6 live cells per pool at (~300 × *g*) for 5 min in a tabletop centrifuge. Isolate genomic DNA using a DNeasy Blood and Tissue Kit according to the manufacturer's protocol for cultured cells. Determine the genomic DNA concentration (*see* **Note 7**).
2. Assemble the following in a PCR reaction: 3.3 μg DNA from each pool (*see* **Note 10**), 10 μL Ex Taq Reaction Buffer, 8 μL dNTPs, 10 μL P5-ORF primer mix (*see* **Note 11**), 10 μL P7 primer (A or B) (*see* **Note 12**), 1.5 μL TaKaRa Ex Taq™ polymerase, and ddH_2O to a total volume of 100 μL.
3. Program a PCR machine with the following cycling program and run the samples through:

   Step1 95 °C for 1 min

   Step2 95 °C for 30 s

   Step3 53 °C for 30 s

   Step4 72 °C for 30 s

   Step5 Go to **Step 2** for 27 additional cycles

   Step6 72 °C for 10 min

   Step7 4 °C indefinitely
4. To make sure the PCR reaction worked, load 10 μL of the reaction mixture on a 2 % agarose gel. The PCR product, including the shRNA region and Illumina sequences, should be ~298 bp.
5. Run the remaining 90 μL of PCR product on a 2 % agarose gel and purify the band using a QIAquick Gel Extraction Kit following the manufacturer's protocol with the following exception: Prior to the PE wash step, wash the column with one volume of PB buffer. Measure the concentration of the isolated PCR product (*see* **Note 7**).
6. Run each PCR product on a Bioanalyzer Lab-on-chip system as a quality control.

7. Combine an equivalent amount of PCR product from each of the 22 reactions as the final library and perform deep sequencing with single end 50 bp reads (*see* **Note 13**).
8. To identify the shRNAs from the screen, assess the quality of the sequence libraries using FastQC, a free software quality control tool for high-throughput sequence data [17]. Prior to alignment of the sequence reads, use a custom Perl script to identify the first four bases flanking the informative sequence at the 5′ end. Extract the core 16–21 bp sequences and map them to the human genome(hg19), allowing up to two mismatches, using Bowtie1, a software program designed to align short DNA sequences [18]. Do not perform further analysis on reads that do not contain the first four bases of the 5′ sequence.

3.6 Validating Candidate Genes

1. Prepare individual virus supernatants for each shRNA clone identified from the screen, as described earlier in Subheading 3.1.
2. Infect 2×10^5 K562 cells with 0.5 mL of virus supernatant and 2 μg/mL of polybrene. Add 3 mL of fresh medium and culture for 24 h, then add 1 μg/mL of puromycin to select for 5 days. Cells may be divided and half frozen at −80 °C as a backup (*see* **Note 14**).
3. To quantify imatinib resistance, plate 2500 knockdown cells (counted by trypan blue assay) per well in triplicate in 96-well plates. Culture the cells in plain medium (200 μL total volume) and add either 10 μM imatinib or DMSO (control) for 4 days (*see* **Note 15**). Add 20 μL of CellTiter 96® Aqueous One Solution Cell Proliferation Assay MTT reagent to each well (*see* **Note 16**), mix by pipetting, and incubate at 37 °C for at least 1 h (*see* **Note 17**). Record the absorbance at 490 nm (A490) using a Victor3 or SpectroMaxM5 plate reader (*see* **Note 18**).
4. Determine the resistance to imatinib by calculating the ratio of relative cell viability of candidate gene knockdown cells to that of control cells (*see* **Notes 19** and **20**).

   Resistance = ([A_{490} Candidate shRNA Imatinib]/[A_{490} Candidate shRNA DMSO])/([A_{490} NS shRNA Imatinib]/[A_{490} NS shRNA DMSO]).
5. RNAi-mediated gene knockdown is known to have off-target effects [19, 20]. Therefore, for candidates that test positive, it is imperative to pick a second or third shRNA for the same candidate gene and test for knockdown efficiency using qRT-PCR and imatinib resistance. Any gene with two or more effective shRNAs that confer imatinib resistance can be considered a validated candidate for follow-up studies.

4 Notes

1. 293T cells are used to prepare the shRNA viruses, as they transfect easily and support high-level expression of viral proteins.
2. To facilitate screening the genome-wide shRNA library, we generated 22 pools of ~5000 shRNAs each. To derive the pools, we grew bacterial stocks from 50 96-well plates and pooled aliquots of them together prior to plasmid preparation.
3. Polybrene improves overall transduction efficiency by enhancing receptor-independent virus absorption through the cell membrane. Usually, the more polybrene that is added, the higher the transduction efficiency. However, too much polybrene can be deleterious to target cells and may lead to increased cell size, reduced proliferation, or even cell death. Therefore, a preliminary experiment should be done to find the optimum tolerated amount of polybrene. Test a range of concentrations, from 1 to 10 μg/mL, and monitor cells for size, proliferation, and viability.
4. Before changing the medium, make sure cells have reached ~70 % confluency. Cells with low confluency will be more easily disturbed, which can cause a low virus titer.
5. Virus titer may drop significantly over time when frozen at −80 °C. Therefore, if the viral stocks are frozen, the titer of the virus needs to be retested before using for library screening.
6. With suspension cells, such as K562 cells, it is advisable to test transduction efficiency before performing an shRNA library infection. Empirically determine parameters such as the highest tolerated amount of polybrene and minimal amount of virus required to reach 50 % infection.
7. We use a NanoDrop spectrophotometer (Thermo Scientific) to measure DNA concentrations in solutions of precipitated DNA and PCR reactions.
8. On occasion, there might be too many colonies growing on the plate due to high competency of bacteria. To avoid this, plate two or three tenfold serial dilutions of the bacteria on different LB Amp plates.
9. The number of colonies to be picked for Sanger sequencing is estimated based on the percentage of live cells after drug treatment. As a starting point, pick 10–20 colonies per pool. Stop sequencing when the same shRNA identified more than once, indicating you have reached saturation.
10. We amplify genomic DNA from individual viral pools separately, so that if a pool fails to amplify it can be easily detected

and repeated. The amount of DNA used for each PCR reaction should be empirically determined based on the percentage of live cells after infection and drug selection. A typical mammalian diploid genomic DNA mass is ~6.6 pg. A representation of ~200 cells per shRNA with 5000 shRNAs per pool will require ~10^6 cells, and the total amount of genomic DNA required for PCR will be 6.6 μg. With drug selection killing >50 % of cells in majority of pools, we use 3.3 μg of DNA for each PCR reaction.

11. P5-ORF primer mix is a mixture of 16 different P5 primers with unique barcodes. Usually, at least 12 unique barcodes are required to aid in the cluster calling to sequence one sample.
12. Perform two separate PCR reactions for all 22 pools, one with P7 primer A and one with P7 primer B. Each primer has a unique barcode to serve as technical replicates.
13. For our deep sequencing we used a MiSeq Sequencing System (Illumina) and prepared samples using MiSeq® Reagent Kit v3 (Illumina) according to the manufacturer's instructions.
14. Try to freeze down the shRNA knockdown cells as early as possible (within a week of transduction) to prevent the effect of shRNA knockdown from getting lost over time.
15. MTT assays usually have a high background reading. Therefore, make sure to add control wells that contain only medium and DMSO.
16. Open the MTT reagent bottle in the cell culture hood to prevent contamination.
17. After adding the MTT reagent, test a series of incubation times (e.g., 30 min, 1 h, 2 h, 4 h) to determine an optimal time so that the color signal will not be saturated. Ideally, the reading should be in the signal range from 0.0 to 2.0. However, it is best to keep all the readings within the linear range and keep the highest absorbance close to 1.
18. Before loading the plate for reading, check if there are brown clumps clustering on the side of the well, which will compromise the accuracy of the absorbance reading and therefore the determination of cell viability. If present, try to break them up by pipetting a few times using multichannel pipette, then gently remove the bubbles.
19. Always use the same batch of cells to test the nonsilencing shRNA control as that used to test the candidate shRNA.
20. If there are greater than 50 % false-positive shRNA, it is likely that dead cells were not completely removed in Subheading 3.4 **step 4**. As a result, any contamination of shRNA-containing genomic DNA would lead to identification of contaminating shRNAs. If this is the case, thaw the backup cells stored in

Subheading 3.4 **step 3**, and perform the experiment again, this time using fluorescence-activated cell sorting (FACS) to directly sort live cells into lysis buffer, prior to DNA extraction.

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Chapter 15

Biological Analysis of Human CML Stem Cells; Xenograft Model of Chronic Phase Human Chronic Myeloid Leukemia

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Abstract

Xenograft mouse models have been instrumental in expanding our knowledge of hematopoiesis and can provide a functional description of stem cells that possess engrafting potential. Here we describe methodology outlining one way of analyzing human malignant cells that are able to engraft immune compromised mice. Using models such as these will allow researchers to gain valuable insight into the primitive leukemic subtypes that evade current therapy regimes and are critical to understand, in order to eradicate malignancy.

Key words Chronic myeloid leukemia, Immune compromised, NSG, Xenograft, Engraftment, Mouse

1 Introduction

Although in vitro techniques have been important for investigating leukemic stem cells, these approaches are limited when investigating microenvironment cellular interactions, oxygen sensitivities, and how drugs affect leukemic cell viability on a pharmacokinetic and pharmacodynamic level. Immune deficient mice, however, can provide more comprehensive environmental conditions to study both hematopoietic and leukemic stem cell biology [1–4]. Multiple strains of mice are currently available with various genetic differences and the individual researcher will need to determine what strain will be optimal as a readout for the question being investigated. NOD.Cg-Prkdc^{scid} IL2rg^{tm1Wjl}/SzJ (NSG) and NOD.Cg-PRKdc^{scid}/IL2rg^{tmlSug}/Jic (NOG) mice are commonly integrated into xenograft models to elucidate stem cell function [5].

Chronic myeloid leukemia (CML) is a clonal disorder that results in the overproduction of myeloid cells in peripheral blood [6]. This disease arises from the reciprocal translocation of chromosomes 9 and 22, resulting in the Philadelphia chromosome (Ph$^+$). The resulting chimeric oncogene Bcr-Abl encodes a constitutively

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active tyrosine kinase, which is directly responsible for the malignancy that develops. Xenograft models of chronic myeloid leukemia offer valuable insight; however, also possess particular challenges, as CML patient samples contain normal as well as malignant stem cells. Importantly during the expansion of transplanted leukemic clones, CML xenograft mice lack clinical signs of disease (elevated white cell counts, splenomegaly, hepatomegaly, and weight loss) [6], as observed with other leukemic models such as AML [7]. Individual CML patient samples also possess engrafting potential unique to the sample, highlighting (1) interpatient heterogeneity and (2) interspecies barriers that still remain despite large improvements in the development of immunodeficient mice. Engraftment of patient cells does not indicate the confirmation of leukemic cells due to contaminating normal cells that reside in primary samples; it is therefore imperative to confirm the leukemic (Ph^+) status (e.g., fluorescence in situ hybridization (FISH)) of the resulting cells.

2 Materials

2.1 Solutions and Equipment

1. Serum-free media: (SFM) Iscove modified Dulbecco medium (Sigma-Aldrich) supplemented with (1/5 dilution) BIT9500 (a serum substitute containing bovine serum albumin/insulin/transferrin, StemCell Technologies), 2 mM L-glutamine, 105 units/mL penicillin, 100 mg/mL streptomycin, 0.1 mM 2-mercaptoethanol, and 0.8 μg/mL low-density lipoprotein (all from Sigma-Aldrich). To culture cells, a growth factor cocktail of 0.20 ng/mL recombinant human (rh) stem cell factor, 1 ng/mL rh IL-6, 0.20 ng/mL rh granulocyte colony-stimulating factor (GM-CSF) (Chugai Pharma Europe), 0.05 ng/mL LIF, 0.2 ng/mL MIPα (all from StemCell Technologies except where indicated) is additionally added.
2. Red blood (RBC) Lysis Buffer: A 1× solution can be prepared as follows; dissolve ammonium acetate (8.02 g), potassium bicarbonate (1 g), EDTA (0.02 g) in 1 L of double distilled (dd) water. This solution can be kept at 4 °C. Filter prior to use.
3. 2 % fetal calf serum/phosphate buffered solution (2 % FCS-PBS). Filter prior to use and always keep chilled (on ice).
4. Fc block solution: Dilute rat anti-mouse CD16/32 antibody (1/500) and add 10 μL per reaction.

2.2 For FISH Analyses

1. DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride): A 1000× frozen stock solution can be prepared as follows; 1 mg/mL in double-distilled (dd) water. Store as 10 μL aliquots.

2. 0.75 M KCl stock solution (dilute 1:10 prior to use). Store at 4 °C.
3. Fixative (three parts methanol to one part acetic acid, which is to be prepared on the day of use).
4. Poly-L-lysine stock (dilute 1:10) use to coat multispot slides prior to addition of cells.
5. Multispot slides.
6. Positive and negative control cells (can use K562/HL-60 or mononuclear cells).
7. 20× SSC stock buffer (UltraPure™ SSC, 20×, Catalog number: 15557-036 Invitrogen).
8. Wash 1: 0.4× SSC/0.3 % Tergitol-type NP-40 (NP-40) at pH 7.
9. Wash 2: 2× SSC + 1 % NP-40 buffer at pH 7.
10. 2× SSC at pH 7.
11. FISH probe (Vysis LSI BCR/ABL Dual Color, Dual Fusion Translocation Probe—CE Marked, Part Number: 08L10-001).
12. Rubber cement, slide covers, humidifier box, DAPI and mounting medium, nail polish.
13. Thermo Scientific™ Hybaid OmniSlide Thermal Cycler System or similar instrument capable of heating slides within a controlled humidity chamber.
14. Waterbath.

3 Methods

3.1 Determination of Engraftment Capability and Ph Status of Individual Patient Samples

Our laboratory implements female NOD.Cg-Prkdc^{scid} IL2rg^{tm1Wjl}/SzJ (NSG) mice with success; however, mice with similarly (or more) diminished levels of immunity can be chosen as appropriate. We and others have observed significantly different engrafting levels using males versus females [8].

Individual CML samples will possess differing levels of engraftment within NSG mice and these cells in turn will have varying levels of both Ph$^+$ and Ph$^-$ cells at the end of the 16-week procedure. Prior to the start of the experiment, an assessment of each CML sample to be used must be individually evaluated to establish if the sample is capable of sufficient Ph$^+$ engraftment levels post-treatment (*see* **Note 1**).

3.2 In Vitro or In Vivo Treatment of Human CML Primary Cells

Culture primary cells (fresh or previously frozen) that have been CD34$^+$ selected, in SFM with appropriate growth factors, using a seeding density of ~1 million/mL. Treat cells as per desired

condition (drugs, shRNA constructs, etc.) and culture <48 h, as prolonged culturing time lowers final levels of cell engraftment. Alternatively, NSG mice can be administered drug directly; maximum tolerated doses can be determined on nontransplanted irradiated mice prior to final experiment. Prior to transplant, wash cells twice (centrifuge 300 × *g*/5 min room temperature (RT)) in 2–3 mL 2 % FCS-PBS solution and keep on ice until transplant.

3.3 Irradiation and Transplantation of Primary Cells into NSG Mice

Typically 8-10-week-old mice are used, sublethally irradiated once at 225 cGy (this dosage will need to be empirically determined as irradiators vary from institute to institute). Within 24 h transplant mice via tail vein injection with washed CML cells in a volume of 100 μL using an insulin syringe (1 cc 27G 5/8″). Prepare cells in order to inject approximately two million cells per mouse (*see* **Note 2**).

After transplantation, mice should be maintained under defined sterile conditions developed for NOD/SCID mice experiments. Mice should receive drinking water containing antibiotics such as Baytril (2.5 % solution, which is subsequently diluted 1/250 in water) to inhibit infections as a result of the further dampening of immunity of the NSG mice from radiation. Daily monitoring and health checks are highly recommended during the 2 weeks after transplantation, as the mice are extremely sensitive during this period. If mice are mildly suffering with shortness of breath or appear less active, transfer mice to a heating pad and provide gel food.

3.4 Monitoring Engraftment over 16 Weeks

1. In order to track engraftment over the course of the experiment, peripheral blood (PB) can be collected at 8, 12, and 16 weeks (1 day prior to final sacrifice) (*see* **Note 3**). Approximately 10–50 μL blood samples can be collected from mice via tail vein, collected in EDTA-containing blood tubes (*see* **Note 4**).
2. To prepare blood samples, add 10–50 μL blood to 2 mL RBC lysis buffer (as described earlier) into flow cytometer tubes.
3. Incubate on ice 10 min.
4. Add 1–2 mL PBS to quench reaction.
5. Centrifuge 400 × *g*/5 min/4 °C. Complete lysis is imperative for flow cytometry analyses; if centrifuged pellet remains bright red, add an additional 2 mL of RBC lysis buffer and repeat **step 3–5**.
6. Remove supernatant leaving approximately 50 μL remaining and keep samples on ice. Add 10 μL of Fc block solution (*see* **Note 5**) and incubate for 5 min. Add prepared master mix containing human CD45 and mouse CD45 antibodies and separate samples for isotype and single color controls for flow

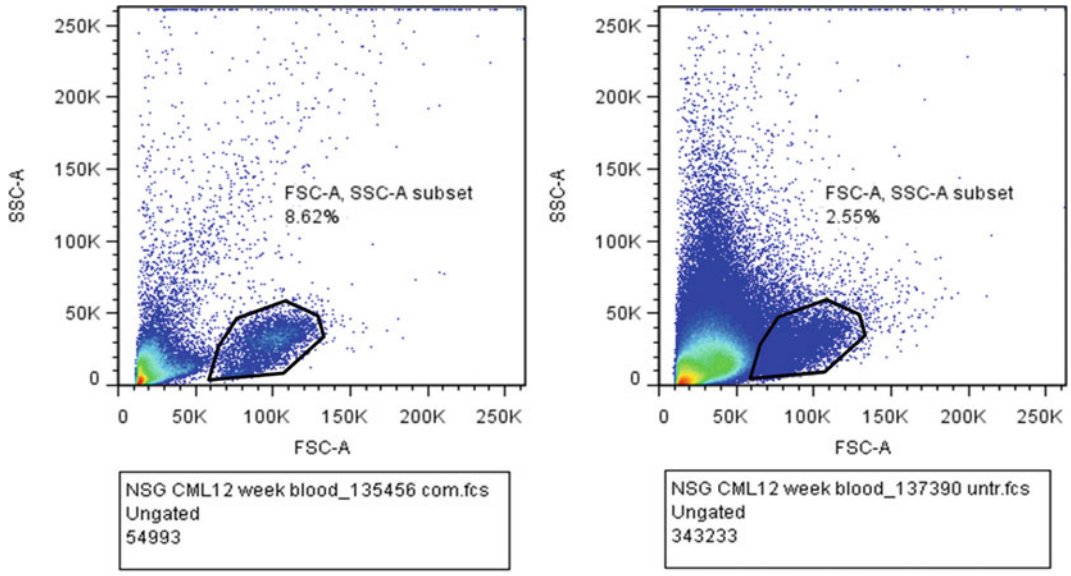

Fig. 1 Flow cytometer dot plot of blood samples taken 12 weeks post engraftment. Gated population indicates human CML cells from a sample with complete RBC lysis (**a**) in contrast to a partially lysed sample (**b**)

cytometry analyses. Incubate 40 min on ice, covered under dark conditions.

7. Dilute DAPI in 2 % FCS-PBS solution, taking care to minimize light exposure. Add 1–2 mL of DAPI-2 % FCS-PBS solution to all samples except controls (include a DAPI-only control, for flow cytometry compensation) (*see* **Note 6**). To all other controls (isotype controls, human CD45, mouse CD45) add 2 % FCS-PBS. Centrifuge 400 × *g*/5 min at 4 °C. Remove supernatant leaving approximately 50 μL remaining in tube. Add approximately 300 μL diluted DAPI-2 % FCS-PBS solution (4 °C) to samples and 2 % FCS-PBS solution to controls. Keep all tubes covered, in dark until analyzed (Fig. 1).

3.5 Sacrificing Mice Harvesting Blood, Bone Marrow, and Spleen

1. To analyze engraftment at the end of 16 weeks, mice are euthanized (with blood sample being taken the day before) and femurs, tibiae, and spleens are harvested and put into PBS until cell extraction.
2. Bone marrow harvest: In a large mortar, pipette 10 mL 2 % FCS-PBS. Clean femurs and tibiae thoroughly removing all connective tissue possible, putting cleaned bones into 2 % FCS-PBS in mortar. Using pestle, crush bones with an up and down motion (avoid vigorous grinding using circular motions) to carefully break bones releasing bone marrow (*see* **Note 7**). Place a sterile 40 μm cell strainer in a 50 mL polypropylene conical bottom tube and pipette liquid contents from mortar through strainer. Re-add 10 mL of 2 % FCS-PBS to bone

remnants and gently crush using an up and down motion again briefly. Pipette remaining contents through strainer, and keep cell suspension on ice.

3. Spleen cell harvest: In a petri dish place 40 μm cell strainer with 10 mL of 2 % FCS-PBS. Gently push spleen through strainer with the rubber end of a 5 mL syringe. Homogenize gently and collect strained cell suspension into a 50 mL polypropylene conical bottom tube and place on ice.
4. Depending on how many events are required to collect, pipette approximately 1–2 mL of bone marrow sample and 0.5 mL of spleen sample into separate flow cytometry tubes. Use untreated samples for all single color controls for flow cytometry compensation and fluorescence minus one (FMO) controls in order to determine gating boundaries. Centrifuge 400 × *g*/5 min/4 °C. Remove supernatant and place samples on ice. Add 10 μL of Fc block solution and incubate 5 min. Prepare antibody master mix with chosen antibodies, reflecting the hemopoietic subsets desired to be stained and analyzed (*see* Table 1* as an example) and add to each sample (amounts will depend on antibody titrations of each). Add single and FMO antibodies mixes accordingly. Incubate 40 min on ice, covered under dark conditions.
5. Dilute DAPI in 2 % FCS-PBS solution taking care to minimize light exposure. Add 1–2 mL to all samples except controls (include a DAPI-only control, for flow cytometry compensation). To all other controls add 2 % FCS-PBS. Centrifuge 400 × *g*/5 min at 4 °C. Remove supernatant leaving approximately 50 μL remaining. Add approximately 300 μL diluted

Table 1
Possible list of hemopoietic markers to use

Antibody	Clone	Fluorochrome	Suggested starting point volumes (μL)
Mouse anti-human CD19	SJ25C1	PE/Cy7	1
Rat anti-mouse CD45	30-F11	PerCP	1
Mouse anti-human CD11b	M1/69	APC/Cy7	1
Mouse anti-human CD14	M5E2	PerCP/Cy5.5	1
Mouse anti-human CD34	581	APC	2
Mouse anti-human CD33	WM53	PE	1
Mouse anti-human CD45	HI30	FITC	5

*Individual antibodies need to be titrated to obtain volumes to be added per experiment
*Choose antibody chromophores that are compatible with DAPI

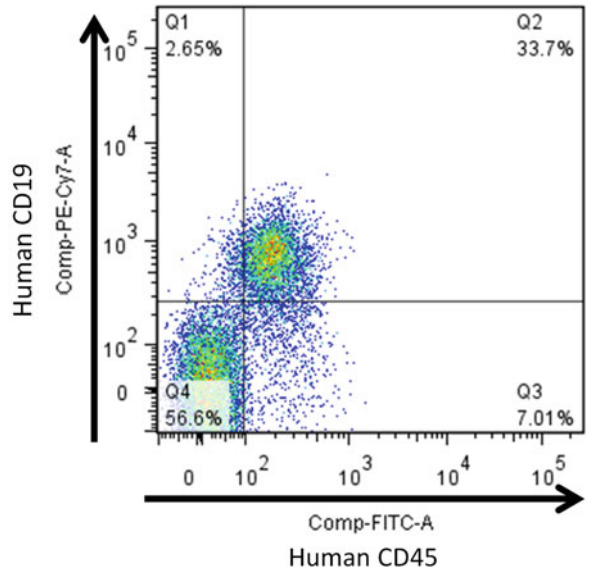

Fig. 2 Representative flow cytometry dot plot of bone marrow sample taken 16 weeks post engraftment stained with human CD45 and CD19 (plot has been gated on the (mouse) $CD45^-$ population)

DAPI solution (4 °C) to samples and 2 % FCS-PBS solution (4 °C) to control samples. Filter samples using a cell strainer prior to reading on flow cytometer (*see* **Note 8**). Keep samples covered in dark, until analyzed (Fig. 2).

3.6 FACS of CD45 Cells for FISH Analysis

1. Pipette approximately 2–3 mL (volume will be dependent on engraftment levels) of bone marrow sample into FACS tubes. Use untreated samples for all single color and isotype controls for flow cytometry compensation. Centrifuge 400 × *g*/5 min at 4 °C. Remove supernatant leaving approximately 100 μL remaining. Add 10 μL of Fc block solution and incubate for 5 min on ice. Add prepared master mix containing human CD45 and mouse CD45 antibodies and prepare controls accordingly. Incubate 40 min on ice, covered under dark conditions. After incubation, add 1–2 mL 2 % FCS-PBS solution and centrifuge 400 × *g*/5 min/4 °C. Remove supernatant leaving approximately 100 μL remaining. Add approximately 200–300 μL 2 % FCS-PBS solution and proceed to cell sort human $CD45^+$ cells and ideally collect between 10^4 and 10^6 cells. Wash by adding 2 % FCS-PBS and centrifuging 400 × *g*/5 min/4 °C. Proceed to fixation.

3.7 FISH Fixation

1. Prepare 1× hypotonic solution (dilute 0.75 M KCl stock 1:10 in dd water). Pre-warm to 37 °C.
2. If there are less than 10^4 cells, it may better to fix cells directly to slides, as subsequent staining and washing steps lead to fewer cell numbers (*see* **Note 9**). Centrifuge cells 400 × *g*/5 min/RT, in a 1.5 mL microcentrifuge tube and discard supernatant.
3. Add 1 mL prewarmed hypotonic solution and incubate for 20 min at 37 °C.
4. Centrifuge 400 × *g*/5 min/RT.
5. Remove supernatant and loosen pellet by gently flicking tube.
6. Add 1 mL freshly made fixative, dropwise while continually vortexing to avoid cell clumping. Incubate at RT for 5 min.
7. Centrifuge 16,000 × *g*/5 min/RT.
8. Remove supernatant and add 1 mL fresh fixative and incubate for a further 5 min.
9. Centrifuge 16,000 × *g*/5 min/RT, repeat **steps 7–9** once more.
10. Finally resuspend cell pellet with 1 mL fixative. Cells can be stored at minus 20 °C until ready to proceed to hybridize cells with FISH probes. Can stop here.
11. Centrifuge cells 16,000 × *g*/2 min/RT. Discard supernatant and resuspend cells in a volume of fresh fixative (volume will depend on total number of cells). Aim to dispense 5 μL per sample.
12. Using multispot slides, gently pipette 5 μL cell suspension onto each spot. Check cell density to ensure there are sufficient cells in field of view without overcrowding, using 10× magnification on a phase contrast microscope. Add or remove cells as required.
13. Apply controls to one slide.
14. Allow to air dry.

3.8 FISH Hybridization

1. Place slides on Hybaid OmniSlide hot-block preset at 65 °C and leave for 10 min.
2. Prepare probe using manufacturer's instructions and hybridization buffer supplied with probe. Dilute 1 μL probe: 10 μL hybridization buffer.
3. To each well, pipette 2–3 μL probe mix and cover with glass coverslip. Seal edges with rubber glue.
4. Place slide on slide hot-block and heat at 72 °C for 2–5 min (to hybridize), followed by 37 °C overnight.

5. Preheat waterbath to 75 °C with a Coplin jar containing Wash 1 (0.4× SSC-0.3 % NP-40). Heat solution to 72 °C and verify directly checking with thermometer.
6. Remove slides from Hybaid OmniSlide and gently remove rubber seal using tweezers, without disturbing coverslip.
7. Place slides in a second Coplin jar containing 2× SSC for 10–20 s and gently remove coverslip. If coverslip cannot be easily removed, repeat dipping slides in 2× SSC until coverslip is easily removable.
8. Place slides without coverslip into Coplin jar with Wash 1 preheated to 72 °C and incubate for 2 min.
9. Place slides in Coplin jar containing Wash 2 (2× SSC -1 % NP-40) for 2 min.
10. Pipette 10–20 μL mounting media containing DAPI onto coverslip. Invert slides over the cover slip to pick it up, and allow mount to spread onto slide; works better if slides are not completely dry.
11. Gently dab sides of slide to remove excess mount and visualize using a fluorescent microscope.

4 Notes

1. Our laboratory has observed engraftment levels of 0–20 % and Ph^+ percentages ranging from 0 to 90 %.
2. Alternatively mice can be transplanted with CML cells intrafemorally; this method requires fewer CML cells.
3. Engraftment levels of progenitor cells have been documented to decrease in percentages to near undetectable levels over 12 weeks, with more primitive subsets increasing or slightly decreasing. At the end of 16 weeks the human cells that remain will be a mixture of the most primitive and progenitors produced from these cells [9].
4. Alternatively engraftment can be monitored by taking bone marrow aspirate samples at the indicated time points.
5. The Fc block solution prevents nonspecific binding of Fc receptor expressing cells, minimizing nonspecific binding of antibodies used for subsequent flow cytometry steps.
6. DAPI is used in this part of the protocol as an exclusion marker for dead cells in flow analyses. Alternative markers for dead cells can be used accordingly.
7. Our laboratory crushes bone marrow as opposed to flushing bones using small needles. There are advantages and

disadvantages to each method and should be decided upon based on desired output of experiment.

8. It is advised to thoroughly clean flow cytometry machine before and after use; mouse cells are extremely adherent and block cytometry tubing. Engraftment percentages of human CML cells are very low and so contaminated, blocked lines may have significant effects on flow cytometry profiles of small subpopulations.
9. Fixing cells directly onto multispot slides:
   (a) Centrifuge $<10^4$ cells in 1.5 mL microfuge tube and resuspend in 20 μL 1× hypotonic solution. Flick tube to mix.
   (b) Incubate cells at 37 °C/15 min.
   (c) To each tube add 5 μL of fresh fixative. Mix well and incubate at RT/5 min.
   (d) Pipette entire contents of each tube onto multispot slides. Check cell density to ensure there are sufficient cells in field of view without overcrowding, using 10× magnification on a phase contrast microscope. Add or remove cells as required.
   (e) Apply controls to one slide.
   (f) Leave cells to adhere to slide for ~20 min (can leave overnight).
   (g) Add 10 μL fresh fixative to each well. Allow to dry out at RT.
   (h) Add an additional 25 μL fixative to wells and allow to dry out at RT.
   (i) Incubate multispot slide in Coplin jar containing fixative for 10 min.
   (j) Decant off fixative and replace with fresh fixative. Repeat.
   (k) Remove slide and allow to air dry.
   (l) Wrap slide in parafilm and store at −20 °C until able to proceed to probe hybridization step.

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Chapter 16

New Mouse Models to Investigate the Efficacy of Drug Combinations in Human Chronic Myeloid Leukemia

Hanyang Lin, Adrian Woolfson, and Xiaoyan Jiang

Abstract

Chronic myeloid leukemia (CML) comprises a simple and effective paradigm for generating new insights into the cellular origin, pathogenesis, and treatment of many types of human cancer. In particular, mouse models of CML have greatly facilitated the understanding of the underlying molecular mechanisms and pathogenesis of this disease and have led to the identification of new drug targets that in some cases offer the possibility of functional cure. There are currently three established CML mouse models: the *BCR-ABL* transgenic model, the *BCR-ABL* retroviral transduction/transplantation model, and the xenotransplant immunodeficient model. Each has its own unique advantages and disadvantages. Depending on the question of interest, some models may be more appropriate than others. In this chapter, we describe a newly developed xenotransplant mouse model to determine the efficacy of novel therapeutic agents, either alone or in combination. The model facilitates the evaluation of the frequency of leukemic stem cells with long-term leukemia-initiating activity, a critical subcellular population that causes disease relapse and progression, through the utilization of primary $CD34^+$ CML stem/progenitor cells obtained from CML patients at diagnosis and prior to drug treatment. We have also investigated the effectiveness of new combination treatment strategies designed to prevent the development of leukemia in vivo using *BCR-ABL*$^+$ blast crisis cells as a model system. These types of in vivo studies are important for the prediction of individual patient responses to drug therapy, and have the potential to facilitate the design of personalized combination therapy strategies.

Key words Chronic myeloid leukemia (CML), $CD34^+$ CML stem/progenitor cells, *BCR-ABL*$^+$ blast crisis cells, Immunodeficient mice, Oral gavage, Tyrosine kinase inhibitor, Intravenous injection, Engraftment, Xenotransplant mouse model, Drug efficacy

1 Introduction

The laboratory mouse has served as a powerful model system for the investigation of human pathology and in particular malignancies due to its closely shared biochemistry and physiology reflecting a high degree of phylogenetic conservation in key genes and genome organization (humans and mice share in the region of 95 % of the same genes and control elements and develop many of the same diseases that result from similar underlying genetic and

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epigenetic abnormalities), and because the mouse genome is readily amenable to manipulation with high precision [1, 2]. There are several reasons to utilize mouse models in the study of human CML. First, it facilitates the study of the pathogenic roles of distinct genetic abnormalities, such as the invariable presence of the *BCR-ABL* fusion gene resulting from a 9;22 chromosomal translocation and its interactions with the bone marrow (BM) niche in the in vivo environment, which contribute to disease development, in ways that would be difficult if not impossible to study using cell lines or primary CML cells in vitro. Second, they provide an appropriate platform for the evaluation of experimental agents such as later generation tyrosine kinase inhibitors (TKIs), and also allow the study of drug combinations and sequencing strategies. CML mouse models have been in use for at least two decades, but the recent trends in translational cancer research have recently led to a renewed interest in the development of improved mouse models to facilitate basic and translational leukemia research.

BCR-ABL transgenic mouse model systems have traditionally utilized constitutively active promoters/enhancers to express the *BCR-ABL* transgene [3–7]. This mouse model was the first to show that the introduction of *BCR-ABL* fusion genes (p190, p210 and p230) is both necessary and sufficient for the induction of the leukemia phenotype in vivo. However, not all mice expressing the chimeric BCR-ABL protein product of the *BCR-ABL* transgene develop a CML-like myeloproliferative disease phenotype, and in the event that the disease phenotype is acquired, the latency is relatively long (8 months of age) [7]. The development recently of an inducible, binary transgene system has led to a significant reduction in transgene toxicity and silencing compared with previous models [8, 9]. Additionally, the transgenic mice invariably develop CML-like illness, including neutrophilia and splenomegaly [8]. This inducible transgenic model also has a shorter disease latency, allowing for the efficient testing of therapeutic drugs to evaluate the effects of therapeutic interventions [10, 11]. This model is not, however, suitable for the evaluation of alternative isoforms and mutations of *BCR-ABL* and related genes, since the generation and maintenance of additional transgenic lines would be time-consuming and costly. Nevertheless, the recent report of an improved transgenic model for blast crisis disease closely mimicking the natural progression of human CML, and that identifies cooperating mutations and therapeutic targets for CML progression using transposon-based insertional mutagenesis, provides a new translational resource for the screening and testing of investigational therapeutic agents targeting the aggressive stage of CML [12].

BCR-ABL retroviral transduction/transplantation models require ex vivo transduction of 5-fluorouracil treated mouse BM followed by transplantation into recipient mice. The demonstration

in 1990 that recipients of *BCR-ABL*-transduced BM developed CML-like disease and other hematologic malignancies was a major breakthrough [13–15]. Much effort has subsequently been made to improve the efficiency of disease induction, through modifications to the retroviral constructs, packaging systems, and optimization of the viral infection conditions. Several groups, including our own, have demonstrated that this improved model is almost 100 % efficient in inducing CML-like disease in mice [16–20]. This model is ideal for studying the biological functions of different isoforms and mutants of *BCR-ABL* as well as other relevant genes, since it is relatively easy to generate retroviral constructs and to make retroviral stocks that can be used to study the biological effects of single genes or the transduction of multiple gene products of interest, as compared to generating new transgenic lines using a transgenic mouse system. Furthermore, the disease latency is much shorter (about 3 weeks), allowing for the evaluation of therapeutic agents in this aggressive CML model system [20–22]. Although this model is perhaps the most frequently applied given that it is relatively straightforward to establish, the principle concern relates to the fact that the *BCR-ABL* expression is driven by a retroviral LTR promoter, such as the murine stem cell virus (MSCV) promoter [16, 18], which is significantly stronger than the endogenous *BCR* promoter that drives the expression of *BCR-ABL* in human CML. As a result, this system may not accurately recapitulate the human CML disease phenotype, and this is especially the case for chronic phase CML (CP-CML), which has a disease latency of 6–7 years.

The most physiologically relevant CML mouse model is the xenotransplant model, in which primitive human CML cells are transplanted into immunodeficient mice. Long-term follow-up and evaluation of mice repopulated with human leukemic cells can then be performed in vivo [23–27]. This model system has a significant advantage over other model systems in that the engrafted human leukemia cells retain a more natural architecture and more closely reflect the heterogeneity and pathology characteristic of primary leukemias. In early attempts, only blast crisis CML (BC-CML) patient samples could be efficiently engrafted into severe combined immunodeficient (SCID) mice [28]. However, CP-CML patient samples were eventually successfully engrafted using higher cell dosages in non-obese diabetic SCID (NOD/SCID) and NOD/SCID-β2 microglobulin–null (NOD/SCID-β2M$^{-/-}$) mice [23–27]. Progressive improvements in the transplant-compliant host animal have resulted in a greatly enhanced utility of these models in basic and translational leukemia research. For instance, this new model system identifies specific subpopulations of primary CML cells, including leukemic stem cells. These cells are capable of engrafting in mice and have long-term leukemia-initiating activity [23, 26, 29]. Moreover, given the

different engraftment efficiencies of chronic, accelerated, and blast crisis cells, this model may provide useful prognostic information that may facilitate the design of more effective and personalized therapeutic strategies. Similar to the *BCR-ABL* retroviral transduction/transplantation model, the xenotransplant mouse model also allows for the investigation of biological functions of *BCR-ABL* isoforms or mutants incorporated along with other related genes in normal human $CD34^+$ cord blood cells through the lentiviral or retroviral transduction of *BCR-ABL* or/and other cooperative oncogene or tumor suppressor constructs. The transduced cells may then be injected into NOD/SCID-$\beta2M^{-/-}$ mice for the subsequent analysis of both biological effects and the underlying molecular mechanisms of disease development [30, 31]. Most importantly, this model also enables testing of leukemic stem cell responses to experimental drugs and treatment strategies, since long-term leukemia-initiating cell activity following drug interventions can be evaluated for up to 24 weeks [32, 33, 35]. We have established several CML models in mice using primary CML stem/progenitor cells or other *BCR-ABL*$^+$ primitive human cells with a view to determining the molecular properties of CML stem cells and their responses to later generation of TKIs and other promising investigational agents [20, 23, 26, 30, 31, 33–35]. We have also established a xenotransplant model that exploits the advantages of a long-living, large animal host, the goat, which can be transplanted in a pre-immune state in utero and subsequently followed for several years after birth [36, 37]. Goats that received human *BCR-ABL*-transduced cord blood cells in utero exhibited rapid and long-term deregulated output of their hematopoietic cells, including an excessive production of $CD34^+$ and myeloid cell types in BM and increased white blood cell (WBC) count in the peripheral blood (PB) as compared with control goats (three to five folds). This difference was sustained for approximately 2.5 years, and the cells in this model system are likely to share many properties with those that sustain the CP-CML phenotype in patients [37].

In this chapter, we describe an improved xenotransplant mouse model using NOD/SCID-interleukin 2 receptor γ-chain-deficient (NSG) mice that can be used to determine the efficacy of investigational agents, either alone or in combination, that specifically target CML cancer stem/progenitor cells and assess treatment effects in an aggressive leukemia model system [33, 34]. NSG mice lack mature T-cells, B-cells, and functional natural killer (NK) cells, and are also deficient in cytokine signaling [38]. It has been reported that NSG mice have a much higher frequency of human cell engraftment than NOD/SCID and NOD/SCID-$\beta2M^{-/-}$ mice, which is critical for the evaluation of new monotherapy and combination therapies in vivo. We have investigated the ability of various drug combinations, including TKI inhibitors and JAK2 inhibitors, to eliminate primary CML stem cells with long-term

leukemia-initiating activity by intravenously injecting drug pre-treated human $CD34^+$ CML cells from TKI nonresponders into sublethally irradiated NSG mice (Fig. 1a) [33]. The levels of engraftment of leukemic stem cells, their progeny, and differentiated cells are evaluated to determine the effects on short- and long-term engraftment of total and primitive subsets of CML cells in BM cells in the presence and absence of different drug treatments at various time points through fluorescence-activated cell sorting (FACS) analysis. The levels of *BCR-ABL* transcripts in the BM of each treatment group are then compared to determine treatment effects. This model system is able to determine whether a specific combination treatment is more effective at eliminating CML patient leukemic stem cells, which could in turn be used to predict individual patient responses to select combination regimens and facilitate the design of personalized treatment strategies.

We have additionally utilized *BCR-ABL*$^+$ blast crisis cells (BV173) to investigate new combination treatment strategies for the treatment of aggressive, late-stage disease using the NSG mouse model system (Fig. 1b) [33, 34], as this leads to the more rapid development of the leukemia phenotype than the transplantation of primary $CD34^+$ CML cells, due to their chronic nature. BV173 cells were derived from a blast crisis patient and have been shown to generate a lethal leukemia in mice, thereby comprising an appropriate model system for the determination of drug efficacy for the treatment of a more aggressive leukemia. An oral gavage treatment strategy is applied and changes in survival, palpable splenomegaly, and other physical signs of the disease pre- and post-treatment in various groups are examined. FACS analysis, histological examination of diseased tissues, analysis of mRNA and proteins are applied to the study of the molecular mechanisms underlying drug response, resistance, and disease development. We have in summary identified and developed new treatment strategies for the specific targeting of CML stem/progenitor cells and for the treatment of aggressive disease, indicating that the mouse xenotransplant model comprises a compelling new resource for the identification and modeling of new therapeutic agents and combination treatment strategies for CML and other human leukemias.

2 Materials

2.1 Mice

1. Male NSG mice (maintained in a specific pathogen-free facility in BC Cancer Research Centre Animal Resource Center, Vancouver, Canada), 7–8-week-old at the time of cesium irradiation (*see* **Note 1**).
2. Proper husbandry: food, water, and bedding (Harlan Laboratories, Inc.).
3. Analytical weight balance (Ohaus Corporation, Scout™ Pro).

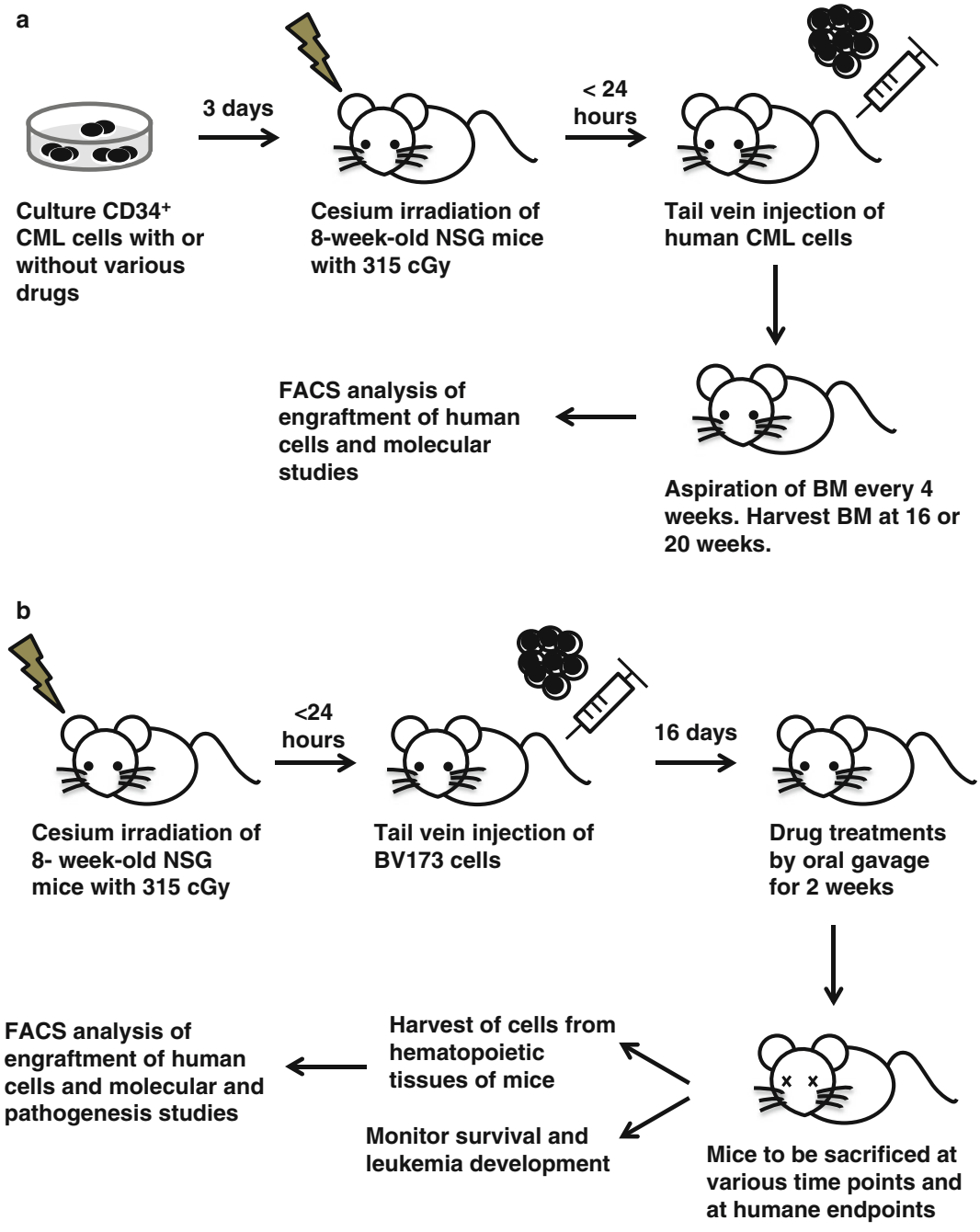

Fig. 1 Xenotransplant mouse model to investigate the efficacy of new drugs in human CML cells using NOD/SCID-interleukin 2 receptor γ-chain-deficient (NSG) mice. (**a**) Schematic of in vivo experiments to assess the efficacy of drug treatments in the eradication of CML leukemic stem cells. Human $CD34^+$ cells from CML patients are treated with various drugs or left untreated for 3 days in vitro, and treated cells are then injected intravenously into 7–8-week-old sublethally cesium-irradiated (315 cGy) NSG mice. Bone marrow (BM) cells are collected every 4 weeks by BM aspiration, and mice are sacrificed and BM cells are harvested at 16 or 20 weeks after transplantation. Engraftment levels of human cells are analyzed by FACS and molecular studies

2.2 Preparations of Human Cells

1. Human $CD34^+$ CML cells purified from patient PB or BM samples (*see* **Note 2**).
2. BV173 cells (Creative Bioarray, Cat# CSC-C0203).
3. Human $CD34^+$ CML culture medium: Iscove's Modified Dulbecco's Medium (IMDM) plus 20 % BIT (bovine serum albumin (BSA), insulin, transferrin, STEMCELL Technologies), 1 % L-glutamine and 10^{-4} M 2-mercaptoethanol supplemented with four growth factors (20 ng/ml IL-3, 20 ng/ml IL-6, 100 ng/ml Flt3-ligand, and 20 ng/ml G-CSF, STEMCELL Technologies).
4. BV173 culture medium: Roswell Park Memorial Institute (RPMI) 1640 media with 10 % fetal bovine serum (FBS) plus 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10^{-4} M 2-mercaptoethanol (STEMCELL Technologies).
5. 5 ml polystyrene round-bottom tube with cell-strainer cap (BD Biosciences).
6. Dulbecco's phosphate buffered saline (PBS, STEMCELL Technologies).
7. Dimethyl sulfoxide (DMSO, Sigma-Aldrich).

2.3 Preparations of Intravenous Injection

1. Cesium irradiator (J.L. Shepherd, Model 81-14R).
2. Ciprofloxacin hydrochloride (Cipro/HCl) water (Novopharm Ltd.).
3. 1/2 cc U-100 insulin syringe needle 28G ½″ (BD Biosciences).
4. Ear punch (Fine Science Tools, Inc.).

2.4 Drug Treatment with Oral Gavage

1. Propylene glycol (Sigma-Aldrich).
2. 1 ml tuberculin slip tip syringe (BD Biosciences).
3. Blunt needle 26G ½″ (SAI Infusion Technologies).
4. Polyethylene tubing (Braintree Scientific, Inc., *see* **Note 3**).
5. Kitten milk powder (Rolf C. Hagen, Inc.).
6. Transgenic dough diet and sunflower seeds (Bio-Serv).

2.5 Tissue Collection

1. Micro-dissecting scissor and micro-dissecting forceps (Sigma-Aldrich).
2. 3 ml Luer-Lok™ tip syringe (BD Biosciences).

Fig. 1 (continued) (**b**) $BCR\text{-}ABL^+$ blast crisis cells (BV173) are injected intravenously into 7–8-week-old sublethally cesium-irradiated (315 cGy) NSG mice. Sixteen days post-injection, mice are treated with single or a combination of drugs once or twice daily for 2 weeks by oral gavage. Mice are sacrificed at desired points or when they reach humane endpoints for comparison of drug effects by collection of peripheral blood (PB), BM, spleen, and liver cells. Engraftment levels of the collected tissues are analyzed, and survival curve and other assays are performed

3. Precision glide needle 23G 1″ (BD Biosciences).
4. 40 μm cell strainer (Corning Inc.).
5. Hanks' Balanced Salt Solution (HBSS) with 10 mM HEPES plus 2 % FBS (STEMCELL Technologies).
6. Digital camera (Canon, PowerShot A560).
7. 10 % neutral buffered formalin (Sigma-Aldrich).
8. Microvette PB collection tube (SARSTEDT).

2.6 Antibodies for Detection of Engraftment of Human Cells by FACS

1. Ammonium chloride (NH_4Cl, STEMCELL Technologies).
2. Blocking buffer: 5 % human serum (Sigma-Aldrich) plus anti-human CD32 antibody (1:200 dilution, STEMCELL Technologies) and anti-mouse CD16/32 antibodies (1:200 dilution, BD Biosciences) in HBSS.
3. Anti-human CD19 PE mouse monoclonal antibody (1:250 dilution, eBioscience).
4. Anti-human CD45 FITC mouse monoclonal antibody (1:100 dilution, BD Biosciences).
5. Anti-human CD45 APC mouse monoclonal antibody (1:100 dilution, BD Biosciences).
6. Anti-human CD34 APC mouse monoclonal antibody (1:100 dilution, BD Biosciences).
7. Anti-human CD19/20 PE mouse monoclonal antibodies (1:50 dilution, eBioscience).
8. Anti-human CD14/15/33/66b FITC mouse monoclonal antibodies (1:50 dilution, BD Biosciences).
9. Anti-human CD3 APC mouse monoclonal antibody (1:100 dilution, BD Biosciences).
10. Anti-human CD19 monoclonal rabbit antibody (1:300 dilution, Abcam, Cat# ab134114).
11. Propidium iodide (PI, Sigma-Aldrich).

3 Methods

3.1 Preparations of Human Leukemic Cells for Intravenous Injection

1. Culture $CD34^+$ primary CML cells (*see* **Note 2**) with IMDM supplemented with 20 % BIT and growth factors. Prior to injection, 2×10^6 $CD34^+$ CML cells (per injection of each mouse) are treated with or without drugs for 3 days in vitro.
2. Culture BV173 cells in 75 cm^2 flasks with 40 ml RPMI complete medium. Each mouse requires 2.5×10^6 cells for intravenous injection (*see* **Note 4**).
3. Cells are washed with PBS, filtered with a cell strainer and collected in FACS tubes to remove cell clumps and FBS fibers.

4. For injection of $CD34^+$ CML cells, recovered cells from each treatment are suspended with 250 μl of PBS for injection into each mouse. For BV173 cells, adjust cell concentration to 10^7 cells/ml with PBS, 250 μl of cell suspension containing 2.5×10^6 cells is injected into each mouse.

3.2 Preparations of Intravenous Injection

1. Prior to irradiation of mice, replace regular water with Cipro/HCl water (*see* **Note 5**).
2. Within 24 h of injection, mice at 7–8 weeks of age are sublethally irradiated with 315 cGy of ^{137}Cs γ-rays.
3. Place an infrared-heating lamp over the cage of mice at least 5 min prior to intravenous injection so the tail vein of the mice can be more easily observed (*see* **Note 6**).
4. Filtered cells are then injected into mice with 28G ½″ insulin syringe needles through the tail vein with 250 μl of cells in PBS.
5. Include mice without injection to serve as no injection controls.
6. Ear notching is performed on each mouse to distinguish mice receiving different drug treatments (*see* **Note 7**).
7. Mouse weight should be recorded at the time of injection.

3.3 Drug Treatment with Oral Gavage

Oral gavage is performed for mice injected with BV173 cells, not with mice injected with $CD34^+$ CML cells since $CD34^+$ CML cells are already treated with drugs in vitro prior to injecting mice.

1. Allow mice to recover from irradiation and the initiation of disease for 16 days prior to drug treatment.
2. Formulate drugs according to recommended solvents and concentrations. For example, Imatinib mesylate requires 50–100 mg/kg per mouse, and can be readily dissolved in water. Dasatinib requires 15–30 mg/kg per mouse, and needs to be dissolved in 50 % propylene glycol. For a concentration of 50 mg/kg per mouse, make a 5 mg/ml stock solution so that the volume of the drug will not exceed more than 1 % of mouse body weight. The solvents used to dissolve drugs should be used as vehicle control (*see* **Note 8**).
3. Weigh each mouse right before oral gavage. The volume of the drugs should not exceed more than 1 % of body weight and no more than 280 μl (e.g., a 25 g mouse should not receive more than 250 μl of solution with drugs). For a combination treatment, each drug is prepared separately with a reduced volume (e.g., 125 μl per each drug for a 25 g mouse). Perform oral gavage once or twice daily for 2 weeks. If twice daily is required, a minimum of 6 h is required between treatments (*see* **Note 9**).
4. Perform oral gavage by advancing the gavage needle (26G) through the esophagus smoothly and administer drugs slowly.

During treatment, if a mouse show signs of struggling, including coughing and choking, drugs may be injected into the lungs. Stop and withdraw the needle immediately. Let the mouse rest for a few minutes and re-administer the drugs with great caution. Also, if mice struggle while advancing the needle, this may lead to rupture of the esophagus or pharynx. Mice need to be euthanized if this occurs (*see* **Note 10**).

5. Feed mice with kitten milk powder throughout the 2-week oral gavage process. Supplement the food of all the mice with dough diet and sunflower seeds if reduced weight of some mice is observed.
6. Following oral gavage, monitor and record mouse weight daily. Euthanize mice when they reach humane endpoints. Also monitor and collect data for survival curve and other symptoms, including white blood cell count changes, weight loss, and lethargy.

3.4 Tissue Collection

For mice injected with BV173 cells, depending on the experimental setup, a set of mice across different treatments are usually sacrificed and analyzed at the same time when vehicle control treated mice reach humane endpoints (3–4 weeks post-oral gavage), so that treated and nontreated mice can be analyzed and compared directly (Figs. 2 and 3). Mice can also be sacrificed and analyzed at other time points as needed. For example, if a single drug treatment is to be compared with a combination treatment, mice can be sacrificed when single drug treated mice reach endpoints (Fig. 4) [34]. At endpoints, collect PB samples first, euthanize mice, and then collect other organs, including BM, spleen, and liver (*see* **Note 11**). For mice injected with $CD34^+$ CML cells, BM cells are collected every 4 weeks by BM aspiration to assess short-term and long-term engraftment of leukemic stem cells and their progenitor cells as well as their response to various drug treatments (Fig. 5). Mice are then sacrificed between 16 and 24 weeks by harvesting BM cells for various assays [33].

1. For collection of PB, place a heat lamp over mice that are to be bled.
2. Restrain mice, puncture the tail vein with a scalpel, and collect PB with a collection tube (*see* **Note 12**).
3. Euthanize mice with CO_2, keep them on ice, and collect tissue within 1 h.
4. Take photos of the euthanized mice as necessary.
5. Collect spleen, liver, femurs, and tibias.
6. Wash and clean spleen, liver, femurs, and tibias with HBSS including 2 % FBS.

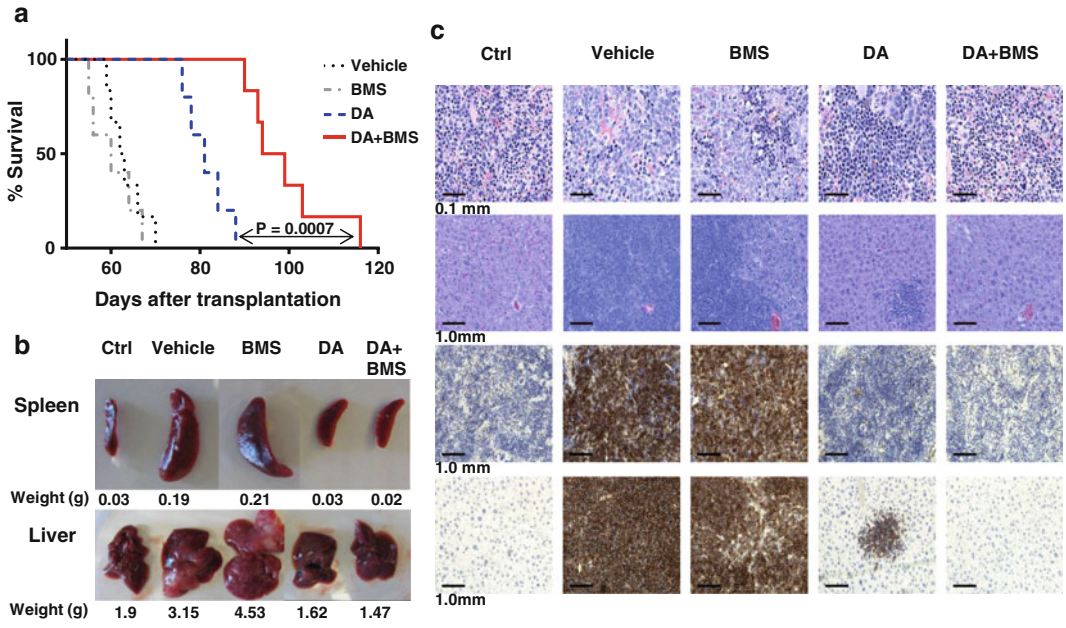

Fig. 2 A combination of a JAK2 inhibitor (BMS-911543) and Dasatinib (DA) significantly enhances survival of leukemic mice. (**a**) BCR-ABL^{+} blast crisis cells (BV173, 2.5 $\times$ 10^{6} per mouse) were intravenously injected into sublethally cesium-irradiated NSG mice. Two weeks after transplantation, oral gavage treatment with or without inhibitors began and continued for 2 weeks. Survival curve is shown for leukemic mice (n = 5–6 mice per group) with vehicle, treated with BMS-911543 (15 mg/kg), DA (15 mg/kg), or DA (15 mg/kg) plus BMS-911543 (15 mg/kg) once a day for 2 weeks. (**b**) At day 54 post-transplant, one mouse per treatment group, including no injection control (Ctrl), vehicle (no treatment), BMS-911543, DA and DA plus BMS-911543, was sacrificed and tissues were analyzed. Spleen (*top panel*) and liver (*bottom panel*) weight of mice from each treatment group. (**c**) Hematoxylin and eosin (H&E) histology staining of spleen and liver from each treatment group (*top two panels*). Immunohistochemical (IHC) staining with CD19 antibody in spleen and liver (*bottom two panels*). This figure is modified from Lin *et al.*, Oncotarget, 2014 published by Impact Journals, LLC under the Creative Commons Attribution License

7. Weigh and take photos of abnormal spleens and livers. An example is shown in Fig. 2b.
8. Take small parts of spleen and liver and fix them in formalin for histopathology studies (Fig. 2c).
9. Mash spleen and liver against 40 μm cell strainer and wash with HBSS.
10. Collect BM cells by flushing out femurs and tibias with 23G 1″ needles and HBSS several times.
11. Cells can now be stored in HBSS at 4 °C for up to 24 h prior to FACS and other analyses (*see* **Note 13**).
12. For BM aspiration, mice are first anesthetized with isoflurane. BM cells are then drawn from the femur with a 22G 1″ needle. Flush the syringe containing BM cells and store them in HBSS at 4 °C for up to 24 h as described above.

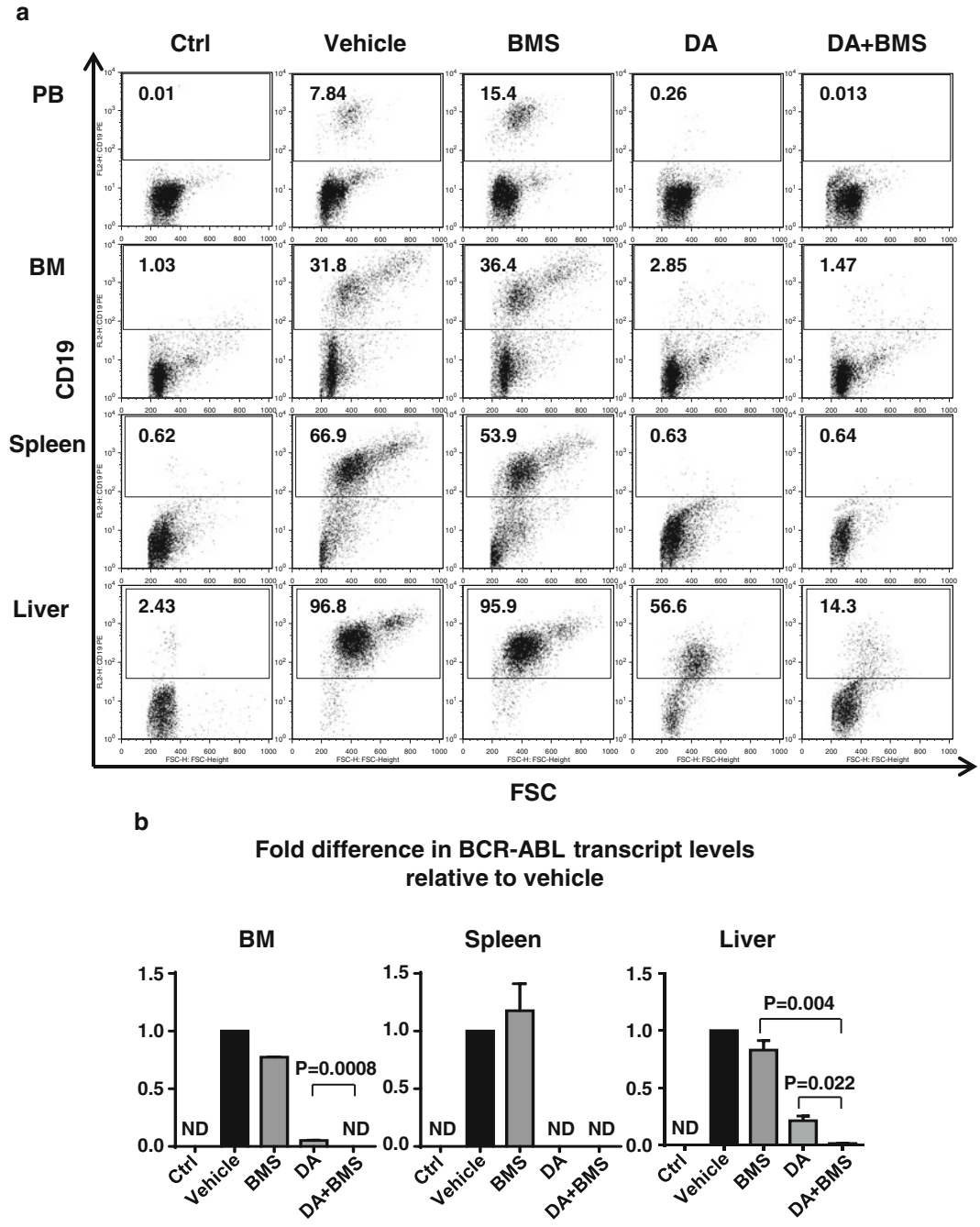

Fig. 3 Effects of oral treatment of JAK2 inhibitor (BMS-911543) in combination with Dasatinib (DA) on the infiltration of leukemic cells into hematopoietic tissues of mice. (**a**) FACS profiles of engrafted human $CD19^+$ cells detected in peripheral blood (PB), bone marrow (BM), spleen, and liver in mice at 54 days post-transplant of BV173 cells treated with various drug treatments, including no injection control (Ctrl), vehicle (no treatment), BMS-911543, DA and DA plus BMS-911543. (**b**) BCR-ABL transcript levels measured by Q-RT-PCR normalized to GAPDH. Ctrl = no BV173 cell injection control; *ND* not detectable. This figure is modified from Lin *et al.*, Oncotarget, 2014 published by Impact Journals, LLC under the Creative Commons Attribution License

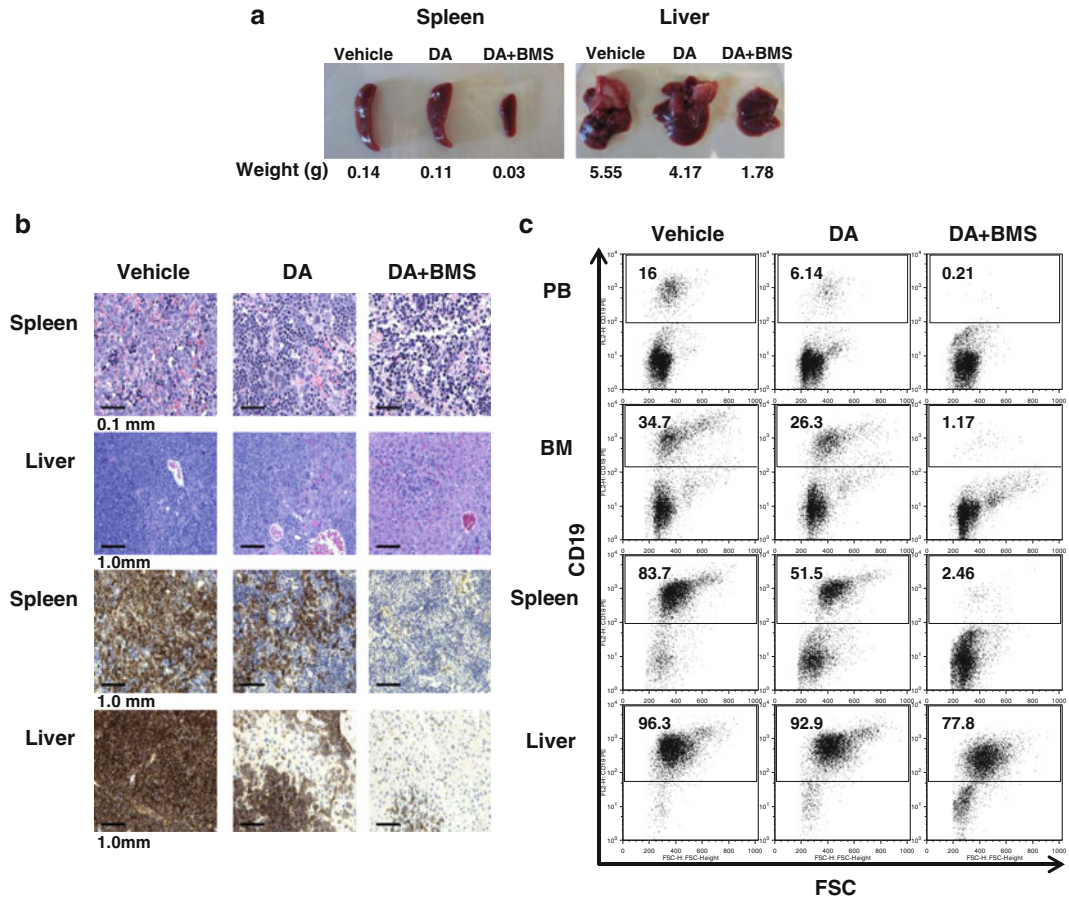

Fig. 4 Oral treatment of JAK2 inhibitor (BMS-911543) in combination with Dasatinib (DA) significantly eliminates infiltrated leukemic cells in hematopoietic tissues 70 days post-transplant. (**a**) At day 70 post-transplant, one mouse per remaining treatment group, including vehicle, DA, and DA plus BMS-911543 was sacrificed and tissues were analyzed. Spleen and liver weights of mice from each treatment group were shown. (**b**) H&E histology staining of spleen and liver from each treatment group (*top two panels*). IHC staining with CD19 antibody in spleen and liver tissues (*bottom two panels*). (**c**) FACS profiles of engrafted human $CD19^+$ cells detected in PB, BM, spleen, and liver. This figure is modified from Lin *et al.*, Oncotarget, 2014 published by Impact Journals, LLC under the Creative Commons Attribution License

3.5 Detection of Engraftment Levels of Human Cells

1. Spin down cells at 1200 × *g*-force for 5 min at 4 °C and discard supernatant.
2. Add at least 1 ml NH_4Cl (0.8 %) each to cells collected from PB, BM, spleen, and liver for removing red blood cells. Vortex and incubate on ice for 10 min.
3. Add same amount of HBSS to cells, and then spin down cells at 1200 × *g*-force for 5 min at 4 °C and discard supernatant.
4. Add 500 μl blocking buffer to each tissue, and incubate on ice for 10 min (*see* **Note 14**).
5. Add 2 ml HBSS directly to wash.

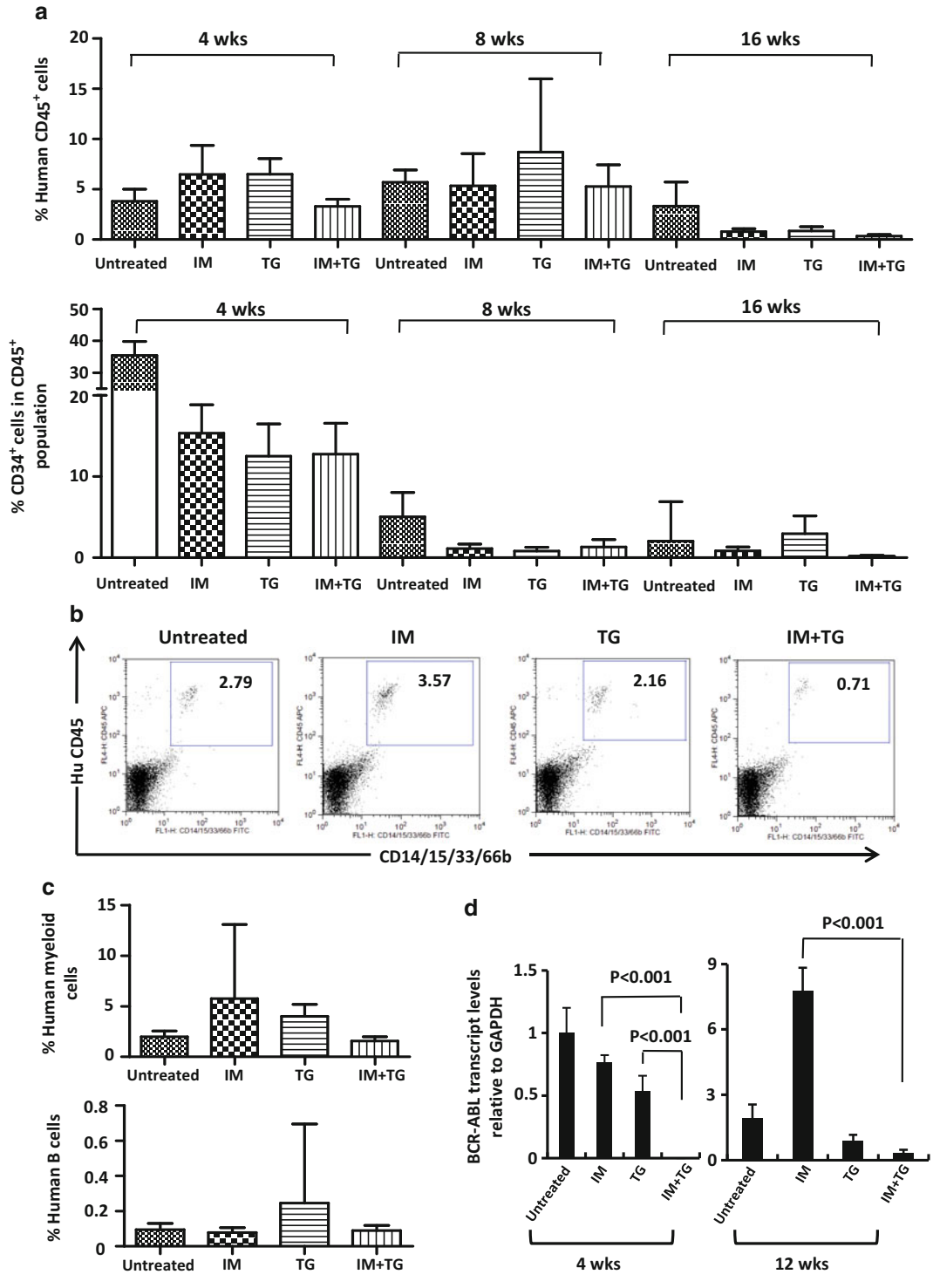

Fig. 5 A combination of a JAK2 inhibitor (TG101209) and Imatinib mesylate (IM) significantly eliminates CML leukemic stem cells in NSG mice. (**a**) $CD34^+$ cells from three CML patients were cultured with 1.0 μM IM, 0.1 μM TG, IM plus TG or without treatment for 3 days. Cells recovered were injected intravenously into NSG

6. Spin down cells at 1200 × *g*-force for 5 min at 4 °C and discard supernatant.
7. Resuspend cells with HBSS: 500 μl each for BM and PB, 2 ml each for spleen and liver.
8. Spin down cells at 1200 × *g*-force for 5 min at 4 °C and discard supernatant (*see* **Note 15**).
9. For cells obtained from mice injected with BV173 cells, stain PB, BM, spleen, and liver cells with anti-human CD19PE antibody (1:250 dilution in HBSS). For cells obtained from mice injected with $CD34^+$ CML cells, stain BM cells with anti-human CD45 FITC antibody (1:100 dilution), CD45 APC antibody (1:100 dilution), CD34 APC antibody (1:100 dilution), CD19/20 PE antibodies (1:50 dilution), CD14/15/33/66b FITC antibodies (1:50 dilution), and CD3 APC antibody (1:100 dilution, *see* **Note 16**).
10. Incubate on ice for 30 min.
11. Add PI (1:1000 dilution of 1 mg/ml).
12. Spin down cells at 1200 × *g*-force for 5 min at 4 °C and resuspend cells in HBSS.
13. Determine the percentage of engrafted human cells and differentiated cells obtained from mice injected with BV173 cells or $CD34^+$ CML cells by FACS analysis as demonstrated in Figs. 3a and 5.
14. For histopathology analysis, spleens and livers previously fixed in formalin are embedded in paraffin, and stained with hematoxylin and eosin (H-&-E), or with anti-human CD19 antibody for immunohistochemical (IHC) staining as demonstrated in Fig. 2c.

4 Notes

1. Male mice are preferred over female mice as the engraftment levels of human cells in male mice are more consistent.

Fig. 5 (continued) mice (three mice per condition per patient sample). After 4, 8, 12, and 16 weeks, bone marrow (BM) aspirates were obtained and the presence of human $CD45^+$ and $CD34^+$ cells measured. (**b**) FACS profiles of regenerated human myeloid cells ($CD45/CD14/15/33/66b^+$) in the BM of representative mice analyzed 12 weeks post-transplant. (**c**) The percentage of human myeloid ($CD45/CD14/15/33/66b^+$) and lymphoid cells ($CD45/CD19/20^+$) in the BM of representative mice analyzed 4 weeks post-transplant. (**d**) BCR-ABL transcript levels measured by Q-RT-PCR in FACS-purified human $CD45^+$ cells obtained at 4 and 12 weeks post-transplant from the BM of mice injected with cells treated with inhibitors and those without. This figure is reproduced from Chen *et al.*, JNCI, 2013 published by Oxford University Press under the Creative Commons Open Access License

2. Obtain Heparin-anticoagulated PB cells from newly diagnosed patients prior to TKI therapy. Isolate mononuclear cells using Ficoll-Hypaque (Sigma-Aldrich) density gradient separation. Enrich $CD34^+$ cells immunomagnetically with an EasySep CD34 positive selection kit (STEMCELL Technologies). Verify purity with CD34 APC antibody (1:100 dilution, BD Biosciences) and FACS analysis [33].
3. Polyethylene tubes are cut into 3–4 cm in length. One end is attached to the blunt needle. The other end is gently melted with a heat block to create a smooth rim to prevent rupture of the esophagus during oral gavage.
4. Make sure cells are under 80 % confluency. Each 75 cm^2 flask can contain about 1.5×10^7 cells at 80 % confluency.
5. Replace water 1 week prior to irradiation so mice get used to the bitterness of Cipro/HCl water. Ciprofloxacin is used to help prevent infection.
6. Place the heat lamp more than 50 cm above the cage. Mice can become easily burned or hyperthermic.
7. When small numbers of mice are to be identified, the code can be as simple as right ear (R), left ear (L), both ears (B), or none (N).
8. Drugs need to be freshly prepared. For example, make enough drugs for 1 week. Sterile filter all solvents with 0.2 μm pore size prior to dissolving drugs. Use Petri dishes to weigh drugs. Add solvents carefully to dish containing drug powder in a class II biological safety cabinet. Avoid exposing drug powder without the lid on in the safety cabinet. If stir bar is used, wash it with ethanol before and after use.
9. Mice also need to rest for 2 days after every 5-day oral gavage treatments or alterative treatment strategies can be used.
10. The key to conducting oral gavage smoothly is good scruffing. Once mice are restrained properly, very little force is needed to pass the gavage needle into the esophagus and toward the stomach. If resistance is encountered, the needle may be entering the trachea and needs to be repositioned.
11. A typical endpoint of mice injected with BV173 cells is characterized by any or a combination of the following: weight loss >15 %, hunched and slow movement, involuntary trembling and quivering, and squinting eyes.
12. The maximum volume of blood that can be drawn is 10 % of the total circulating blood volume, which is around 150–200 μl for a 25 g mouse.
13. Except for PB, which needs to be processed right away with NH_4Cl as it coagulates.

14. At least 100 μl of blocking buffer for 10^7 cells.
15. Aliquots can be made for other analyses including qRT-PCR and Western blots to detect *BCR-ABL* transcript and protein expression and that of other relevant genes/proteins. Usually save at least 100 μl for surface staining, 100 μl for qRT-PCR, and 500 μl for Western blot analysis.
16. In FACS analyses, to determine the percentage of engrafted BV173 cells in hematopoietic organs, first pregate cells with a PI^- population, then look for the percentage of $CD19^+$ cells (Fig. 3a). To determine the percentage of engrafted human $CD34^+$ CML cells, first pregate cells with a PI^- population, then look for the percentage of $CD34^+$ cells in $CD45^+$ population for primitive cells; look for the percentage of $CD14/15/33/66b^+$ cells in $CD45^+$ population for human myeloid cell; and look for the percentage of $CD19/20^+$ cells in $CD45^+$ population for human lymphoid cells (Fig. 5). $CD45^+$ cells can also be purified by FACS sorter to determine *BCR-ABL* transcript and protein expression levels in engrafted human cells.

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Chapter 17

miRNome Analysis of CML Cells

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Abstract

Next-generation sequencing technologies have greatly accelerated the biological and medical progression. As one of the applications, miRNA-Seq is invaluable in detecting and characterizing genome-wide miRNAs of either too high or too low abundance. Besides, it can also be used in detecting novel miRNAs. Here, we describe an ab initio analysis of an example chronic myeloid leukemia miRNA sequencing data set to quantify the global expression of miRNAs, detect differential expression and novel miRNAs, and predict target genes. The run time of this protocol may vary depending on the volume of miRNA sequencing data and available computing resources but takes ~5 h of computing time for typical experiments and less than 1 h of hands-on time.

Key words miRNome, Sequencing, Differential expression, Target

1 Introduction

MicroRNAs (miRNAs) are a family of small, noncoding RNAs that regulate gene expression by binding to the 3′ untranslational region (3′UTR) of target mRNAs. It plays a key role in cell fate determination, proliferation, and cell death [1]. In chronic myeloid leukemia (CML), several miRNAs including miR-203 and miR-17-92 have been found to be related to the expression of BCR-ABL1 fusion gene, which is a major characteristic of CML [2]. It has been demonstrated that ABL1 is directly targeted by miR-203, and that both genetic and epigenetic mechanisms co-ordinately inactivate this miRNA in a T-cell lymphoma model [3] while miR-17-92 cluster is transactivated by BCR-ABL1 fusion protein [4].

Whole-genome miRNA (miRNome) analyses are a powerful approach to explore the function of miRNAs in a full-scale manner and with high sensitivity. Currently, miRNome is yielded mainly through short RNA cloning, microarray techniques, next-generation sequencing [5], etc. Compared comprehensively, next-generation sequencing has unparalleled advantages in fully

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exploring the miRNome profile under a given biological context. Besides revealing the abundance and expression difference of individual known miRNAs, the large amount of data also allowed the identification of novel miRNAs.

The analysis of miRNome data generally includes five steps: (1) read alignment; (2) miRNA quantification; (3) novel miRNA prediction; (4) differential expression, and (5) miRNA target prediction. There has been a lot of software tools developed for miRNA sequencing data analysis, including both standalone and web-based versions [6]. Here we demonstrate a concise pipeline for miRNome analysis of CML cells with miRDeep2 [7] and R packages. Figure 1 shows the software used in this protocol and highlights the main steps of the protocol.

2 Materials

Assuming we are in a proper directory and we will download all needed data and software tools here. You may move some of them to a common place for your convenience. All steps in the protocol are performed in the linux environment. Shell and R script are prefixed with "$" and ">" character, respectively.

2.1 Hardware Setup

The software used in this protocol is intended for operation on a 64-bit UNIX machine. To process miRNA-seq experiments, the machine will need at least 4 GB of RAM.

Downloading and organizing required data:

2.2 Sequencing Data

Requirements vary according to your study goals, and high quality of next-generation sequencing data is recommended. This protocol is illustrated through an example of human leukemia dataset (GSE48059) comparing the miRNA expression profiles in CML and AML cell lines [8]. A single directory named for the example data is strongly recommended, in which you store all raw data and generated analysis data. Here we create a directory named leukemia/.`$ mkdir leukemia$ cd leukemia$ mkdir rawdata$ cd rawdata`

Download example data:`$ curl -o HL60.fq.gz ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR907/SRR907614/SRR907614.fastq.gz$ curl -o K562.fq.gz ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR907/SRR907615/SRR907615.fastq.gz$ curl -o THP1.fq.gz ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR907/SRR907616/SRR909090.fastq.gz`

Decompress example data:`$ gunzip HL60.fq.gz$ gunzip HL60.fq.gz$ gunzip HL60.fq.gz$ cd ../../`

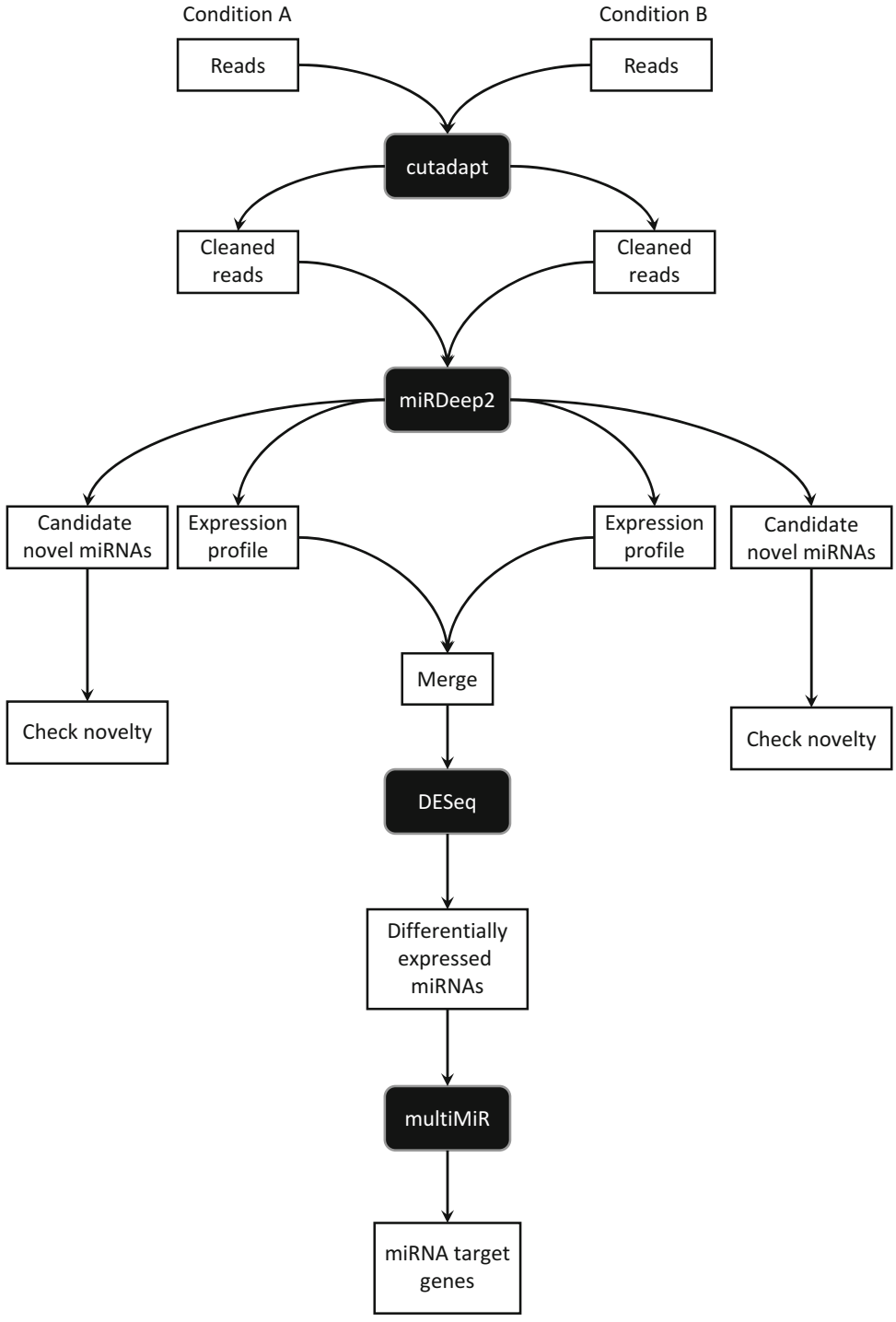

Fig. 1 An overview of the protocol. Sequenced reads are first cleaned with cutadapt. The cleaned reads from different conditions are separately mapped to the genome and then miRNA expressions are quantified with miRDeep2. Novel miRNAs are also predicted with miRDeep2. The expression profiles from different conditions are merged as the input of DESeq for differential expression detection. Then, we could select specific or a group of miRNAs to predict their target genes with multiMiR package

2.3 Reference Data

If it is your first time running this protocol, and you have not aligned any sequencing reads to human reference, please download the human genome reference, miRNA precursor, and mature sequences to your working directory. These datasets are resources that can be reused in future related work and do not need to be redownloaded.

- Human genome reference data, annotation, and bowtie index`$ mkdir Ref$ cd Ref$ wget ftp://igenome:G3nom3-s4u@ussd-ftp.illumina.com/Homo_sapiens/UCSC/hg19/Homo_sapiens_UCSC_hg19.tar.gz$ tar zxvf Homo_sapiens_UCSC_hg19.tar.gz`
- miRNA reference data from miRBase`$ mkdir miRBase$ cd miRBase`

  Download miRNA hairpin sequences:`$ wget ftp://mirbase.org/pub/mirbase/CURRENT/hairpin.fa.gz$ tar zxvf hairpin.fa.gz`

  Get human miRNA hairpin sequences:

  ```
  $ awk 'BEGIN{RS=">"}{if($0~/hsa/)print ">"$0}' hairpin.fa | awk 'length($0)!=0{print $1}' >hsa.precursor.fa
  ```

  Download and decompress mature miRNA sequences:`$ wget ftp://mirbase.org/pub/mirbase/CURRENT/mature.fa.gz$ gunzip mature.fa.gz`

  Get human miRNA mature sequences:

  ```
  $ awk 'BEGIN{RS=">"}{if($0~/hsa/)print ">"$0}' mature.fa | awk 'length($0)!=0{print $1}' >hsa.mature.fa
  ```

  Get homology miRNA, like Pongo pygmaeus:

  ```
  $ awk 'BEGIN{RS=">"}{if($0~/ppy/)print ">"$0}' mature.fa | awk 'length($0)!=0{print $1}' >ppy.mature.fa
  ```

2.4 Software Setup

- Create a directory to store all of the executable programs used in this protocol.`$ cd ../../$ mkdir software/$ cd software/`
- Install cutadapt.

  ```
  $ sudo pip install cutadapt
  ```

- Download and install miRDeep2.`$ wget https://www.mdc-berlin.de/43969303/en/research/research_teams/systems_biology_of_gene_regulatory_elements/projects/miRDeep/mirdeep2_0_0_7.zip$ unzip mirdeep2_0_0_7.zip$cd mirdeep2_0_0_7/`

To install miRDeep2, we start with the provided install.pl script:

```
$ perl install.pl
```

Attach the miRDeep2 executable path to your PATH`$ mirdeepPATH=$(pwd)$ echo 'export PATH=$PATH:$mirdeepPATH' >> ~/.bashrc$ cd ../`

Without the installation script, we can follow the instructions given in the miRDeep2 documentation to install dependencies step by step.

- Install DESeq package.
  Start an R session:`$ RR version 3.1.0 (2014-04-10) -- "Spring Dance"Copyright (C) 2014 The R Foundation for Statistical ComputingPlatform: x86_64-unknown-linux-gnu (64-bit)R is free software and comes with ABSOLUTELY NO WARRANTY.You are welcome to redistribute it under certain conditions.Type 'license()' or 'licence()' for distribution details.R is a collaborative project with many contributors.Type 'contributors()' for more information and'citation()' on how to cite R or R packages in publications.Type 'demo()' for some demos, 'help()' for on-line help, or'help.start()' for an HTML browser interface to help.Type 'q()' to quit R.`
  To install DESeq, Bioconductor provided a ready-to-use package.`>source("http://bioconductor.org/biocLite.R") >biocLite("DESeq")`
- Install multiMiR package.
  To install multiMiR, start an R session and enter:`>install.packages("XML")>install.packages("RCurl")>install.packages("http://multimir.ucdenver.edu/multiMiR_1.0.1.tar.gz", repos=NULL, type="source")`

3 Methods

3.1 Data Organization

To give a full picture of the protocol, the data organization is summarized in Fig. 2.

3.2 Reads Preprocessing

Here we use cutadapt [9] to clip 3′ adapters and remove reads shorter than 14 bases.`$ cd ../leukemia$ mkdir 1-trimming$ mkdir 1-trimming/HL60 1-trimming/K562 1-trimming/THP1$ cutadapt -m 14 -a TCGTATGCCGTCTTCTGCTTG \rawdata/HL60.fastq -o 1-trimming/HL60/trimmed-HL60.fastq$ cutadapt -m 14 -a TCGTATGCCGTCTTCTGCTTG \rawdata/K562.fastq -o 1-trimming/K562/trimmed-K562.fastq$ cutadapt -m 14 -a TCGTATGCCGTCTTCTGCTTG \rawdata/THP1.fastq -o 1-trimming/THP1/trimmed-THP1.fastq`

3.3 File Format Conversion

`$ fastq2fasta.pl 1-trimming/HL60/trimmed-HL60.fastq >1-trimming/HL60/trimmed-HL60.fa$ fastq2fasta.pl 1-trimming/K562/trimmed-K562.fastq >1-trimming/K562/`

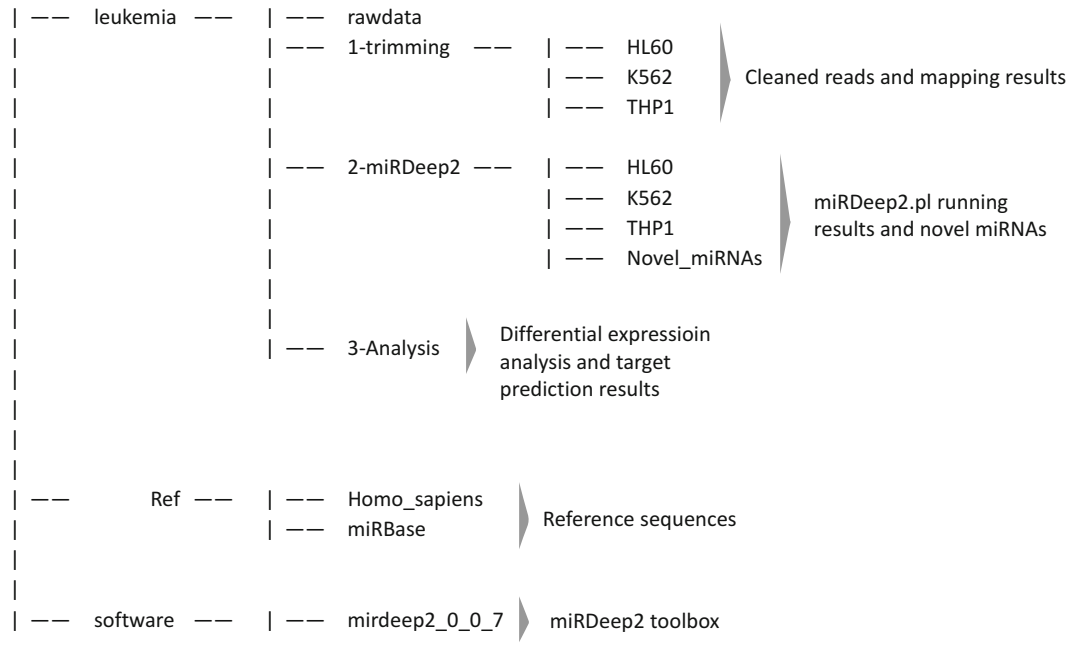

Fig. 2 The whole picture of data organization and a description of corresponding contents

```
trimmed-K562.fa$ fastq2fasta.pl 1-trimming/THP1/
trimmed-THP1.fastq>1-trimming/THP1/trimmed-THP1.fa
```

3.4 Reads Alignment

The mapper module uses bowtie to align cleaned reads to human genome and so bowtie index is needed in this step.

```
$ cd 1-trimming/HL60/$ mapper.pl trimmed-HL60.fa -c -j -m \-k TCGTATGCCGTCTTCTGCTTG -p \../../../Ref/Homo_sapiens/UCSC/hg19/Sequence/BowtieIndex/genome \-s reads_collapsed.fa -t reads_collapsed_vs_genome.arf -v --n$ cd ../K562/$ mapper.pl trimmed-K562.fa -c -j -m \-k TCGTATGCCGTCTTCTGCTTG -p \../../../Ref/Homo_sapiens/UCSC/hg19/Sequence/BowtieIndex/genome \-s reads_collapsed.fa -t reads_collapsed_vs_genome.arf -v --n$ cd ../THP1$ mapper.pl trimmed-THP1.fa -c -j -m \-k TCGTATGCCGTCTTCTGCTTG -p \../../../Ref/Homo_sapiens/UCSC/hg19/Sequence/BowtieIndex/genome \-s reads_collapsed.fa -t reads_collapsed_vs_genome.arf -v --n
```

3.5 miRNA Expression Quantification

```
$ cd ../../$ mkdir 2-miRDeep2$ mkdir 2-miRDeep2/HL60 2-miRDeep2/K562 2-miRDeep2/THP1$ cd HL60$ miRDeep2.pl ../../1-trimming/HL60/reads_collapsed.fa \Ref/Homo_sapiens/ucsc/hg19/Sequence/WholeGenomeFasta/hg19.fa \../../1-trimming/HL60/reads_collapsed_vs_
```

```
genome.arf \../../../Ref/miRBase/21/hsa.mature.fa
\miRBase/21/ppy.mature.fa \../../../Ref/miRBase/
21/hsa.precursor.fa -t Human 2> report.log$ cd ../
K562$ miRDeep2.pl ../../1-trimming/K562/reads_col-
lapsed.fa \../../../Ref/Homo_sapiens/ucsc/hg19/Se-
quence/WholeGenomeFasta/hg19.fa \../../1-trimming/
K562/reads_collapsed_vs_genome.arf \../../../Ref/
miRBase/21/hsa.mature.fa \miRBase/21/ppy.mature.fa
\../../../Ref/miRBase/21/hsa.precursor.fa -t Human
2> report.log$ cd ../THP1$ miRDeep2.pl ../../
1-trimming/ THP1/reads_collapsed.fa \../../../Re/
Homo_sapiens/ucsc/hg19/Sequence/WholeGenomeFasta/
hg19.fa \../../1-trimming/ THP1/reads_collap-
sed_vs_genome.arf \../../../Ref/miRBase/21/hsa.
mature.fa \miRBase/21/ppy.mature.fa \../../../Ref/
miRBase/21/hsa.precursor.fa -t Human 2> report.log
```

3.6 Merge Expression Files from Different Samples

In the last step, we get three miRNA expression file from HL60, K562, and THP1. Here we merge these three files in order for differential expression analysis.

```
$ cd ../../$ mkdir 3-Analysis
$ cd 3-Analysis$ cut -f 1,2 ../2-miRDeep2/HL60/miRNA-
s_expressed_all_samples_04_08_2015_t_15_04_43.csv
> HL60.count$ cut -f 1,2 ../2-miRDeep2/K562/miRNA-
s_expressed_all_samples_04_08_2015_t_17_37_52.csv
> K562.count$ cut -f 1,2 ../2-miRDeep2/THP1/miRNA-
s_expressed_all_samples_04_08_2015_t_15_04_55.csv
> THP1.count$ paste K562.csv HL60.csv THP1.csv \| awk
'{print $1,$2,$4,$6}>' OFS="\t" >merge.count
```

3.7 Sum the Expression of Mature miRNAs from Different Precursors

Some mature miRNAs are generated from multiple precursor miRNAs at different genomic locations; here we sum up the expression value of such mature miRNAs.

```
$ awk '{a[$1]+=$2;b[$1]+=$3;c[$1]+=$4}END{for(i in a)print
i,a[i],b[i],c[i]}' OFS="\t" merge.count >merge.sum.dup.count
```

3.8 Remove miRNAs with Read Count Less Than 2

```
$ awk '{if(!($2<=1 && $3<=1 && $4<=1))print $0}' merge.sum.dup.
count >merge.filtered.count
```

3.9 Differential Analysis with DESeq

DESeq is an R package that provides methods to test for differential expression by use of the negative binomial distribution and a shrinkage estimator for the distribution's variance [10]. It is applicable for data from RNA-Seq or other high-throughput sequencing experiment, in the form of a rectangular table of integer values. According to DESeq's statistical model, the count values must be raw counts of sequencing reads.

Identify differentially expressed miRNAs

- Start an R session:$ RR version 3.1.0 (2014-04-10) -- "Spring Dance"Copyright (C) 2014 The R Foundation for Statistical ComputingPlatform: x86_64-unknown-linux-gnu (64-bit)R is free software and comes with ABSOLUTELY NO WARRANTY.You are welcome to redistribute it under certain conditions.Type 'license()' or 'licence()' for distribution details.R is a collaborative project with many contributors.Type 'contributors()' for more information and'citation()' on how to cite R or R packages in publications.Type 'demo()' for some demos, 'help()' for on-line help, or'help.start()' for an HTML browser interface to help.Type 'q()' to quit R.
- Load the DESeq package into the R environment:
  > library(DESeq)
- Load the raw count data from the miRDeep2 output:> leukemiaCountTable = read.table("merge.filtered.count", header=TRUE, row.names=1) Here, header=-TRUE indicates that the first line contains column names and row.names=1 means that the first column should be used as row names. This leaves us with a data.frame containing integer count values.> head(leukemiaCountTable) K562 HL60 THP1hsa-miR-140-5p 5 397 14hsa-miR-576-3p 5 297 37hsa-miR-1298-5p 0 4 0hsa-miR-548ao-3p 2 0 0hsa-miR-5088-5p 0 10 9hsa-miR-665 122 0 0
- Generate a description of the samples:> condition = c("CML", "AML", "AML")> leukemiaDesign = data.frame(row.names = colnames(leukemiaCountTable), condition = condition)> leukemiaDesign conditionK562 CMLHL60 AMLTHP1 AML
- Instantiate a CountDataSet, which is the central data structure in the DESeq package:
  > cds = newCountDataSet(leukemiaCountTable,condition)
- Normalization
  > cds = estimateSizeFactors(cds)
- Variance estimation:
  > cds = estimateDispersions(cds, fitType = "local")
- Calling differential expression:
  > res = nbinomTest(cds,"CML","AML")
- Plot the log2 fold changes against the mean normalized counts, coloring in red those genes that are significant at 5 % FDR (Fig. 3a):> plotMA(res, col = ifelse(res$pval >= 0.05, "gray32", "red3"))> legend(1e+05, -4, c ("log[2]FC > 10 & p-value < 0.05", "log[2]FC>10 & p-value>=0.05", "log[2]FC<-10 & p-value<0.05",

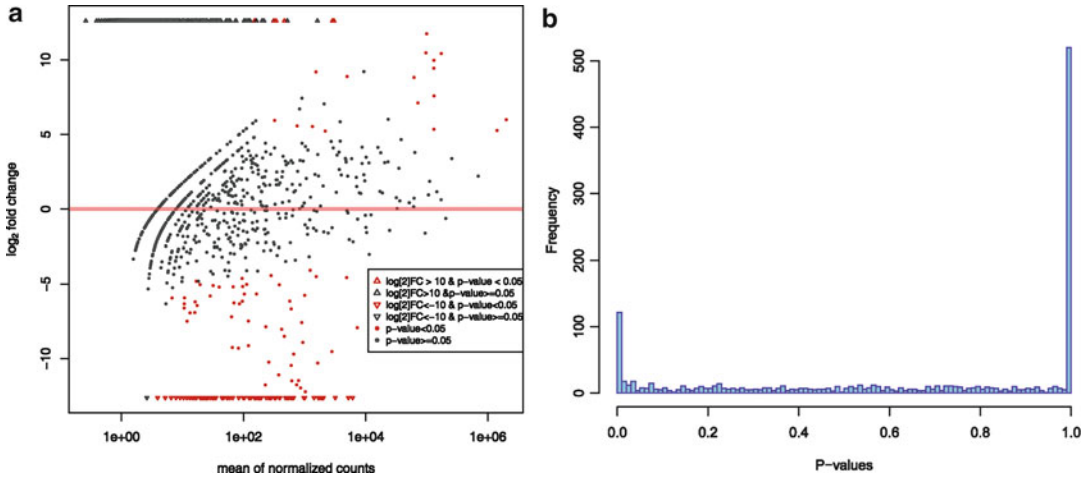

Fig. 3 (**a**) MA plots of miRNAs that differ significantly between CML and AML cell lines. *Red dots* and *triangles* indicate differentially expressed genes with *p*-value $\leq$ 0.05. (**b**) Histogram of *p*-value distribution. Differentially expressed genes with *p*-values smaller than 0.05 were usually considered significant in statistics

```
"log[2]FC<-10 & p-value>=0.05", "p-value<0.05",
"p-value>=0.05"), pch = c( 2, 2, 6, 6, 20, 20 ), col =
c( "red3", "grey32", "red3", "grey32", "red3",
"grey32" ), cex = .5 )
```

- Plot the histogram of p values (Fig. 3b):

```
> hist( res$pval, breaks = 100, col = "skyblue", border = "sla-
teblue", xlab = "P-values", main= "" )
```

- Filter for significant genes, according to some chosen threshold for the false discovery rate (FDR)

```
> resSig = res[ res$padj < 0.05, ]
```

- Save the output to a file:

```
> write.table( res, file = "CML_vs_AML_Result_Table.txt", sep =
"\t", quo = F )
```

3.10 Get the Targets of Differentially Expressed miRNAs

multiMiR [11] is an miRNA-centered R package and database with several features, such as miRNA-target interaction from 14 different databases, including both computationally predicted and experimentally validated ones; the association of disease and miRNA, the drug miRNA response, and miRNA-target binding strength prediction.

Use multiMiR to get the targets of miRNAs.

1. Start an R session:
2. Load the differentially expressed miRNAs:`> miRNAs = read.table( "CML_vs_AML_Result_Table.txt", header = TRUE, sep = "\t", row.names = 1)> miRNAs = miRNAs [miRNAs$padj < 0.1, ]$id`

3. Load the multiMiR package:
   `> library("multiMiR")`
4. Select 5 miRNAs as an example to predict their target genes:
   `> miRNAs = head(miRNAs, 5)`
   `> targets = get.multimir( org = "hsa", table = "predicted", mirna = miRNAs, summary = TRUE )`
5. We can also get the validated target genes by following command:
   `> validatedTargets = get.multimir( org = "hsa", table = "validated", mirna = miRNAs, summary = TRUE )`

3.11 Prediction of Novel miRNAs

The prediction of human novel miRNAs was implemented in the miRDeep2 software. Novel miRNA candidates were selected based on filtering criterion as follows, (1) novel miRNAs and its corresponding reverse sequence (miRNA*) must be detected, (2) miRDeep2 score ≥100.`$ cd ../2-miRDeep2/$ mkdir novel_-miRNAs$ cd novel_miRNAs`

1. Get id and mature sequence of novel miRNA candidates`$ awk '/^provisional id/{getline;print $1,$16;a=1;next}a&&NF<2{a=0}a&&$2>=100{print $1,$16}' ../HL60/result_04_08_2015_t_15_04_43.csv > HL60_novel_candidates$ awk '/^provisional id/{getline;print $1,$16;a=1;next}a&&NF<2{a=0}a&&$2>=100{print $1,$16}' ../K562/result_04_08_2015_t_17_37_52.csv > K562_novel_candidates$ awk '/^provisional id/{getline;print $1,$16;a=1;next}a&&NF<2{a=0}a&&$2>=100{print $1,$16}' ../THP1/result_04_08_2015_t_15_04_55.csv > THP1_novel_candidates`
2. Check the miRNA novelty in miRBase
   Submit novel miRNA candidates to miRBase (http://www.mirbase.org/), click Search > By sequence > paste mature sequences in the blank box or upload the sequence file you wish to use > setting the appropriate parameters (usually as default) and select organisms as human > click Search miRNAs. This search may take a few minutes and then you will be given an alignment result of query to mature miRNAs if it matches to existing miRNAs, or no result if no matches were found for your sequence. This step aims to search for homologs of microRNA sequences by comparing with hairpin precursor sequences and/or mature miRNAs; miRNAs with no homology can be classified as novel miRNAs.

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Chapter 18

An Integrative Analysis of microRNA and mRNA Profiling in CML Stem Cells

Farah J. Nassar, Rabab El Eit, and Rihab Nasr

Abstract

Integrative analysis of microRNA (miRNA) and messenger RNA (mRNA) in Chronic Myeloid leukemia (CML) stem cells is an important technique to study the involvement of miRNA and their targets in CML stem cells self-renewal, maintenance, and therapeutic resistance. Here, we describe a simplified integrative analysis using Ingenuity Pathway Analysis software after performing proper RNA extraction, miRNA and mRNA microarray and data analysis.

Key words CML stem cells, miRNA microarray, mRNA microarray, RNA extraction, miRNA-mRNA integration, Ingenuity pathway analysis

1 Introduction

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell myeloproliferative disorder caused by a balanced chromosomal translocation that results in the formation of Philadelphia chromosome and leads to the fusion of the *bcr* gene on chromosome 22 and the *abl* gene on chromosome 9. This unique bcr-abl fusion gene encodes a constitutively active BCR-ABL tyrosine kinase leading to the activation of multiple signal transduction pathways that enhance cell survival and proliferation [1]. Tyrosine kinase inhibitors (TKI) that target BCR-ABL have become the standard therapy for CML. However, discontinuation of TKI therapy will cause CML to rapidly relapse in most patients, suggesting that CML stem cells are spared and can drive disease progression and even drug resistance [2]. This prompted interest in studying the mechanisms of CML stem cells therapeutic resistance and understanding the molecular signaling pathways of these leukemic stem

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cells governing their persistence. It has been shown that microRNAs (miRNA), a class of small noncoding RNA, regulate multiple mRNA thus affecting multiple pathways. miRNAs were shown to act as key regulators of CML cells maintenance and survival. For instance, miR-486-5p was significantly upregulated in CML stem/progenitor cells as compared with normal CD34+ cells. Interestingly, inhibition of miR-486-5p reduced CML progenitors growth and enhanced apoptosis following imatinib (TKI) treatment [3]. However, the role of miRNA in regulating CML stem cell remains poorly understood. Moreover, in silico tools that predict miRNA targets according to different algorithms might give false negative targets. Hence, the best way to study miRNA along with their respective targets that regulate CML stem cell characteristics is through an integrative analysis of microRNA and mRNA profiling. We have recently demonstrated that the combination of arsenic and interferon alpha triggered a synergistic decrease in myeloid colony formation in cells from CML patients. Moreover, in a murine transplantation model of CML, we showed that their combination sharply diminished transplantation of CML cells in secondary recipients, pointing to exhaustion of CML leukemia initiating cells [4]. We are currently using this type of integrative analysis of miRNA and mRNA to further explore the mechanism of action of arsenic and interferon in CML models and to reveal miRNA whose targets are essential in the specific targeting of CML leukemia initiating cells by this combination.

2 Materials

2.1 Total RNA Extraction

1. 1× Phosphate Buffered Saline (PBS).
2. TRI Reagent® from Sigma Life Science.
3. Chloroform.
4. 100 % isopropanol.
5. RNase-free glycogen.
6. 70–75 % Ethanol: prepared in DEPC treated water from 100 % ethanol.
7. DEPC treated water.
8. Refrigerated centrifuge decontaminated using RNaseZap®.
9. Microcentrifuge decontaminated using RNaseZap®.
10. Micropipettors decontaminated using RNaseZap®.
11. Filtered tips.
12. Vortex mixer decontaminated using RNaseZap®.
13. Non-stick RNase-free tubes.
14. Heat block decontaminated using RNaseZap®.
15. Nanodrop ND-100 Spectrophotometer.
16. RNaseZap®.

2.2 microRNA Microarray

1. FlashTag™ Biotin HSR RNA Labeling Kit (Affymetrix) includes 10× Reaction Buffer, 25 mM $MnCl_2$, ATP Mix, PAP Enzyme, 5× FlashTag Biotin HSR Ligation Mix, T4 DNA Ligase, HSR Stop Solution, RNA Spike Control Oligos, ELOSA Spotting Oligos, ELOSA Positive Control, Nuclease-Free Water and 27.5 % Formamide (*see* **Note 1**).
2. Nuclease-free water.
3. 1 mM Tris-HCl: Dilute 50 µl of 1 M Tris–HCl, pH 8 in 49.5 ml nuclease-free water. Do not take a pH reading. Store at room temperature up to 3 months.
4. Diluted ATP Mix: ATP Mix from FlashTag™ Biotin HSR RNA Labeling Kit 1:500 in 1 mM Tris-HCl (discard any unused diluted ATP).
5. Flat bottom Immobilizer™ Amino—8 well strips.
6. Adhesive plate sealers.
7. 1× PBS (1 l): Dilute 100 ml 10× PBS pH 7.4 in 900 ml nuclease-free water. Store at room temperature up to 3 months.
8. 1× PBS, 0.02 % Tween-20 (1 l): Add water to 100 ml 10× PBS pH 7.4 and 200 µl Tween-20 to have a final volume of 1 l. Store at room temperature up to 3 months.
9. 5× SSC, 0.05 % SDS, 0.005 % BSA (10 ml): 2.5 ml 20× SSC, 50 µl 10 % SDS, 10 µl 5 % BSA in 1× PBS, water to a final volume of 10 ml. Aliquot into ten vials of 1 ml stored at −20 °C, up to 6 months and at 4 °C for 1 week. Do not freeze/thaw each 1 ml aliquot more than four times. Warm at 42 °C to dissolve any precipitate. Use at room temperature.
10. 5 % BSA in 1× PBS (40 ml): 2 g of powdered BSA in 1× PBS to a final volume of 40 ml. Vortex to mix. Aliquot into eight vials of 5 ml to be stored at −20 °C, up to 6 months and at 4 °C for 1 week. Do not freeze/thaw each aliquot more than four times.
11. 25 % dextran sulfate (10 ml): 5 ml of 50 % dextran sulfate in 5 ml nuclease-free water and vortex thoroughly. Store at room temperature up to 3 months.
12. Diluted Streptavidin-HRP: Dilute 1:4000 to 1:8000 SA-HRP (Thermo Scientific/ Pierce) in 5 % BSA in 1× PBS.
13. TMB Substrate Solution (Thermo Scientific/ Pierce).
14. TMB Stop Reagent (Thermo Scientific/ Pierce).
15. Plate reader of reading absorbance at 450 nm.
16. GeneChip® miRNA 3.0 Array (Affymetrix) (*see* **Note 2**).
17. GeneChip® Eukaryotic Hybridization Control Kit (Affymetrix) includes the Control Oligonucleotide B2 and 20× Eukaryotic Hybridization Controls.

18. GeneChip® Hybridization, Wash and Stain Kit (Affymetrix) includes: Hybridization and Stain Modules (Pre-Hybridization Mix, 2× Hybridization Mix, DMSO, Nuclease-free water, Stain Cocktail 1, Stain Cocktail 2, and Array Holding Buffer), Wash Buffer A and Wash Buffer B (*see* **Note 3**).
19. Laser Tough-Spots® 1/2″ diameter (Diversified Biotech).
20. Laser Tough-Spots® 3/8″ diameter (Diversified Biotech).
21. GeneChip® Fluidics Station 450 (Affymetrix).
22. GeneChip® Hybridization Oven 645.
23. GeneChip® Scanner 3000 7G.
24. Affymetrix® GeneChip Command Console® Software (AGCC).
25. Microcentrifuge decontaminated using RNaseZap®.
26. Micropipettors decontaminated using RNaseZap®.
27. Filtered tips.
28. Vortex mixer decontaminated using RNaseZap®.
29. Non-stick RNase-free tubes.
30. RNaseZap®.

2.3 mRNA Microarray

1. GeneChip® 3′ IVT Express Kit (Affymetrix) includes Poly-A Control Stock, Poly-A Control Dilution Buffer, First-Strand Enzyme Mix, First-Strand Buffer Mix, Nuclease-free Water, Second-Strand Buffer Mix, Second-Strand Enzyme Mix, IVT Biotin Label, IVT Labeling Buffer, IVT Enzyme Mix, RNA Binding Beads, aRNA Binding Buffer Concentrate, aRNA Wash Solution Concentrate, (Before use, add 8 ml 100 % ethanol ACS grade) Nuclease-free Water (10 ml), aRNA Elution Solution, 5× Array Fragmentation Buffer, 20× Hybridization Controls, Control Oligo B2, Control RNA (1 mg/ml HeLa total RNA), 8-Strip PCR Tubes & Caps, U-Bottom Plate, and Reservoir (*see* **Note 4**).
2. 100 % ethanol (ACS grade).
3. Ambion magnetic stand-96.
4. Orbital shaker for 96-well plates.
5. Human Genome U133 Plus 2.0 Array chips.
6. GeneChip® Eukaryotic Hybridization Control Kit.
7. GeneChip® Hybridization, Wash, and Stain Kit.
8. Laser Tough-Spots® 1/2″ diameter.
9. Laser Tough-Spots® 3/8″ diameter.
10. Thermal Cycler with heated Lid decontaminated using RNaseZap®.

11. GeneChip® Fluidics Station 450.
12. GeneChip® Hybridization Oven 645.
13. GeneChip® Scanner 3000 7G.
14. Affymetrix® GeneChip Command Console® Software (AGCC).
15. Microcentrifuge decontaminated using RNaseZap®.
16. Micropipettors decontaminated using RNaseZap®.
17. Filtered tips.
18. Vortex mixer decontaminated using RNaseZap®.
19. Non-stick RNase-free tubes.
20. RNaseZap®.

2.4 miRNA-mRNA Integration

1. R program.
2. Ingenuity Pathway Analysis.

3 Methods

3.1 Total RNA Extraction (Fig. 1)

1. Homogenization: Thaw CML cells or CML stem cells previously rinsed with 1× PBS and stored at −80 °C. Under chemical hood, add adequate volume of TRI Reagent® depending on cell count (1 ml for 5–10 × 10^6 cells). Lyse the cells by pipetting up and down multiple times to get a homogenous sample. Incubate the sample at room temperature for 5 min to allow complete dissociation of nucleoprotein complexes.
2. Phase Separation: For 1 ml of sample in TRI Reagent®, add 200 µl of chloroform under chemical hood. Mix all the samples by inversion for 15 s. Centrifuge 15 min at 4 °C at 12,000 × *g* (*see* **Note 5**). Remove the upper aqueous phase (50 % of total volume) by angling the tube at 45 °C and pipetting the solution out without touching the other phases.
3. RNA precipitation: Add 500 µl of 100 % isopropanol to the aqueous phase per 1 ml of TRI Reagent® used for homogenization. Vortex or invert for 2 s and leave on the bench at room temperature for 10 min (*see* **Note 6**). Precipitation could be left overnight at −20 °C for additional precipitation. Centrifuge 15 min at 4 °C at 12,000 × *g* to collect the RNA pellet (*see* **Note 7**). Remove the isopropanol leaving only the pellet.
4. RNA Wash: Wash the pellet with 1 ml of 70–75 % of ethanol per 1 ml of TRI Reagent® used for homogenization. Vortex the sample and centrifuge at 7500 × *g* for 5 min at 4 °C. Remove the ethanol supernatant. Repeat washing steps with

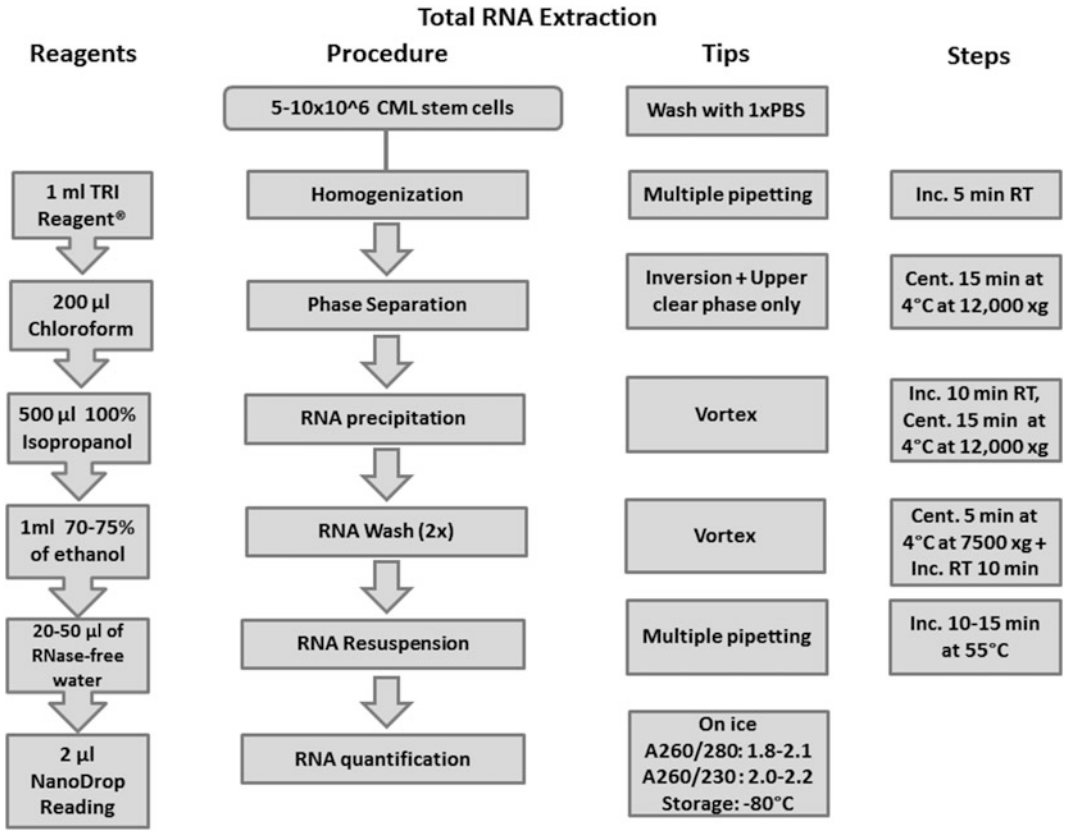

Fig. 1 Total RNA extraction Summary (*Inc.* incubate, *Cent.* centrifuge, *RT* room temperature)

ethanol to make sure the pellet is clean from residual TRI Reagent®. Discard the ethanol supernatant and remove any leftovers by pipette without touching the pellet. Dry the pellet at room temperature for 5–10 min (*see* **Note 8**).

5. RNA Resuspension and quantification: Resuspend the RNA pellet in 20–50 µl of RNase-free water (pass through pipette multiple times) and incubate 10–15 min at 55 °C on heat block. From now on, keep samples on ice. Using NanoDrop® ND-1000 spectrophotometer, read the OD 260 of each sample (2 µl) with DEPC treated water used as Blank. For each sample, check the absorbance ratios, $A_{260/280}$ should be roughly between 1.8 and 2.1 and $A_{260/230}$ should be between 2.0 and 2.2 (*see* **Note 9**). Store the RNA samples at −80 °C.

3.2 microRNA Microarray (Fig. 2) [5–7]

1. Poly A tailing (15 min): On ice, add 2 µl RNA Spike Control Oligos to adjusted input of 8 µl total RNA (*see* **Note 10**). Prepare in a nuclease-free tube a Poly A Tailing Master Mix that includes ~5 % overage to cover pipetting error. Poly A

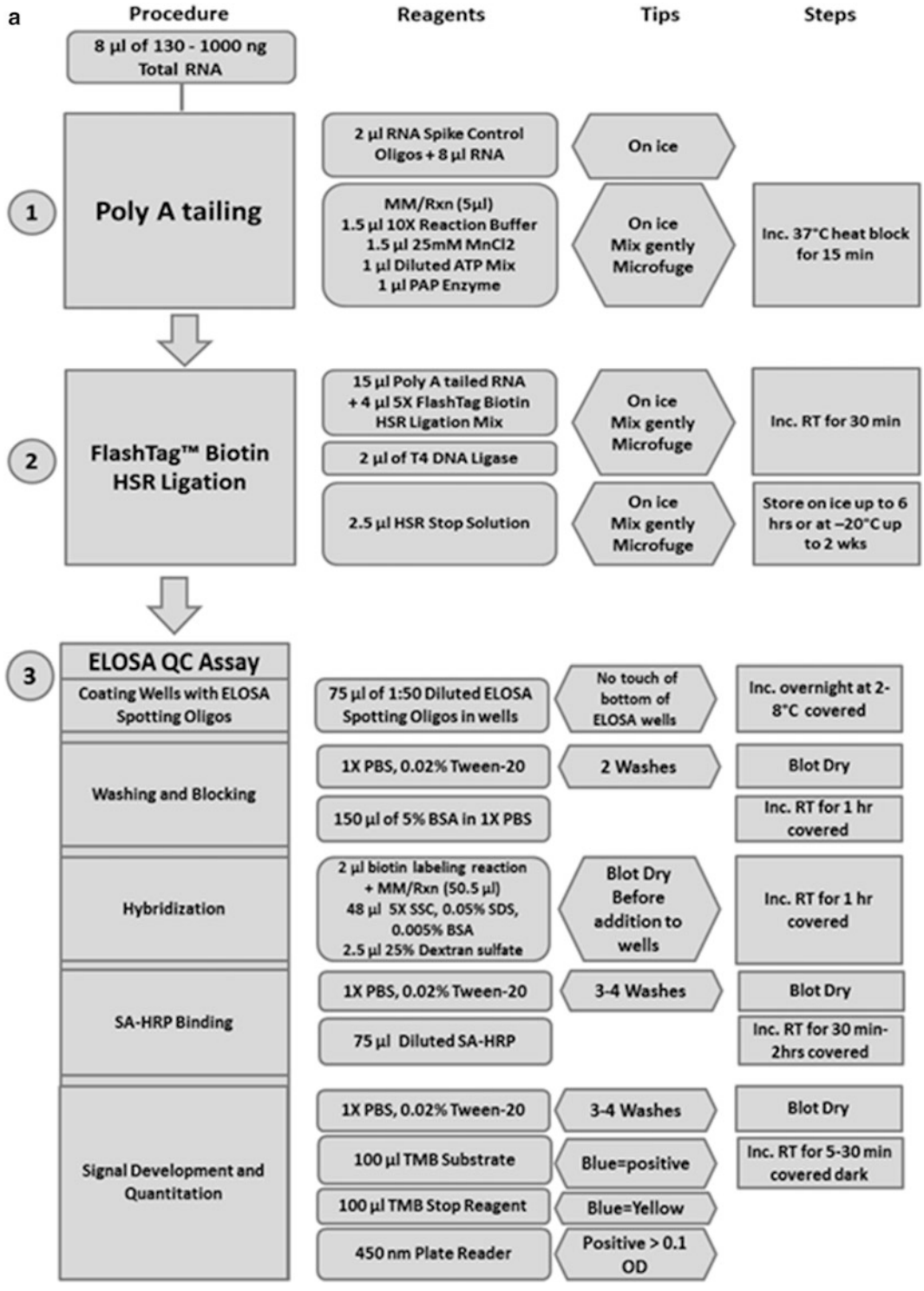

Fig. 2 microRNA microarray Summary. (**a**) Steps 1–3 and (**b**) Steps 4–7 (*Inc.* incubate, *MM* master mix, *Rxn* reaction, *Cent.* centrifuge, *RT* room temperature)

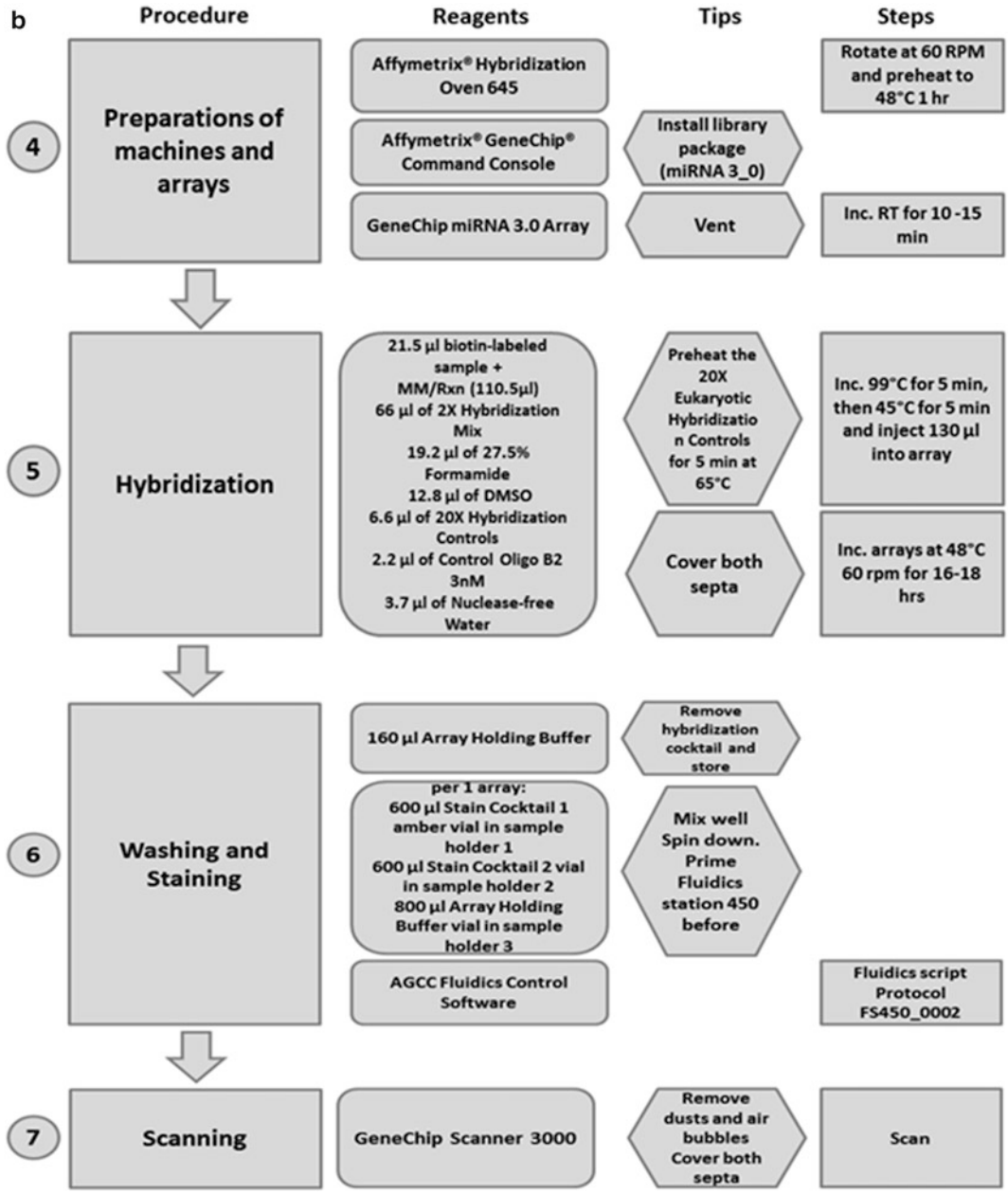

Fig. 2 (continued)

Tailing Master Mix per one reaction contains: 1.5 µl 10× Reaction Buffer, 1.5 µl 25 mM $MnCl_2$, 1 µl Diluted ATP Mix, and 1 µl PAP Enzyme with 10 % overage to cover pipetting errors. Add 5 µl of Master Mix to the 10 µl RNA/Spike Control Oligos. Mix gently without vortexing and microfuge. Incubate in a 37 °C heat block for 15 min.

2. FlashTag™ Biotin HSR Ligation (30 min): Microfuge 15 μl of Poly A tailed RNA briefly. On ice, add 4 μl 5× FlashTag Biotin HSR Ligation Mix followed by 2 μl of T4 DNA Ligase to each sample (*see* **Note 11**). Mix gently without vortexing and microfuge. Incubate at room temperature for 30 min. Add 2.5 μl HSR Stop Solution to stop the reaction. Mix and microfuge the 23.5 μl of ligated sample. Remove 2 μl of the biotin-labeled sample for use with the ELOSA QC Assay (*see* **Note 12**). The remaining 21.5 μl biotin-labeled sample can be stored on ice for up to 6 h, or at −20 °C for up to 2 weeks, before hybridization on Affymetrix GeneChip miRNA Arrays.
3. Enzyme Linked Oligosorbent Assay Quality Control (ELOSA QC) Assay: detect the RNA Spike Control Oligos to check biotin labeling process.

   Coating Wells with ELOSA Spotting Oligos: Dilute the ELOSA Spotting Oligos 1:50 in 1× PBS (for 3 wells: 4.5 μl ELOSA Spotting Oligos in 220.5 μl 1× PBS). Distribute 75 μl of the diluted ELOSA Spotting Oligos to each well of the strip. Do not touch the bottom of the ELOSA wells with the pipette tip. Cover with an adhesive plate sealer and incubate overnight at 2–8 °C (*see* **Note 13**). Washing and Blocking (ELOSA QC): Expel the ELOSA Spotting Oligos liquid into a sink. Wash twice with 1× PBS, 0.02 % Tween-20 and blot dry (*see* **Note 14**). Add 150 μl of 5 % BSA in 1× PBS to each well. Cover the wells with adhesive plate sealer and incubate for 1 h at room temperature. Hybridization (ELOSA QC): Prepare a large Hybridization Master Mix as dextran sulfate is difficult to pipette: 48 μl of 5× SSC, 0.05 % SDS, 0.005 % BSA and 2.5 μl of 25 % Dextran sulfate. Gentle vortexing until the dextran sulfate is in solution. For the positive control, negative control, and samples, add 2 μl of either ELOSA Positive Control, water, or biotin labeling reaction respectively to 50.5 μl of Master Mix for a total volume of 52.5 μl. Expel the BSA blocking solution into a sink and blot dry. Distribute all 52.5 μl of hybridization solution to a well. Cover the wells with adhesive plate sealer and incubate for 1 h at room temperature. SA-HRP Binding (ELOSA QC): Expel the hybridization solution into a sink. Wash 3–4 times with 1× PBS, 0.02 % Tween 20 and blot dry. Add 75 μl of the diluted SA-HRP to each well. Cover the wells with adhesive plate sealer and incubate for 30 min (up to 2 h) at room temperature. Signal Development and Quantitation (ELOSA QC): Expel SA-HRP into a sink. Wash three times with 1× PBS, 0.02 % Tween-20 and blot dry. Add 100 μl of TMB Substrate to each well. Cover the wells with aluminum foil and incubate at room temperature for 5–30 min in the dark. The blue substrate color indicates a positive result. Add 100 μl TMB Stop Reagent

to each well. Blue substrate will convert to a yellow color. Read the absorbance at 450 nm on a plate reader. Readings of greater than 0.1 OD over a negative control should be considered positive.

4. Preparations of machines and arrays: Turn on Affymetrix® Hybridization Oven 645 and allow the oven to rotate at 60 rpm and preheat to 48 °C for at least 1 h. Download and install the miRNA Array library file package (miRNA 3_0) into Affymetrix® GeneChip® Command Console® (AGCC) software using the Command Console Library File Importer tool. The files can be found at: www.affymetrix.com. Upload the sample and array information (sample names, barcode IDs, etc.) into AGCC. Open GeneChip miRNA 3.0 Array chips and leave them on bench top to warm up to room temperature for 10–15 min. Label each array and insert a 20 or 200 µl unfiltered type pipet tip into the upper right septum to allow for proper venting when hybridization cocktail is injected (*see* **Note 15**).
5. Hybridization on Affymetrix GeneChip miRNA Arrays (16–18 h): Thaw the reagents (2× Hybridization Mix, 27.5 % Formamide, 20× Eukaryotic Hybridization Controls, DMSO, Control Oligo B2, and Nuclease-free Water) to room temperature. Heat the 20× Eukaryotic Hybridization Controls (*see* **Note 16**) for 5 min at 65 °C. Prepare in this order the array hybridization cocktail per single reaction of 110.5 µl as total volume: 66 µl of 2× Hybridization Mix, 19.2 µl of 27.5 % Formamide, 12.8 µl of DMSO, 6.6 µl of 20× Hybridization Controls, 2.2 µl of Control Oligo B2, 3 nM and 3.7 µl of Nuclease-free Water. Add the 110.5 µl cocktail to the 21.5 µl biotin-labeled sample (*see* **Note 17**). Incubate at 99 °C for 5 min, then 45 °C for 5 min. Aspirate 130 µl and inject into an array. Remove the pipet tip from the upper right septum of the array. Cover both septa with 1/2″ Tough-Spots® to minimize evaporation and/or prevent leaks. Position the array chips into hybridization oven trays and load them into the hybridization oven. Incubate the arrays at 48 °C and 60 rpm for 16–18 h.
6. Washing and Staining (90 min): After 16–18 h of hybridization, remove the arrays from the oven and remove the Tough-Spots from them. Place a clean pipette tip into one of the septa to vent the arrays and extract the hybridization cocktail with a pipette through the other septum to be reused on another array if necessary. Store the cocktail at −20 °C or at −80 °C for long-term storage. Refill the probe array completely with the 160 µl with Array Holding Buffer (*see* **Note 18**). Mix well Stain Cocktail 1, Stain Cocktail 2, and Array Holding Buffer before aliquoting as follows for one probe array: 600 µl of Stain Cocktail 1 into a 1.5 ml amber microcentrifuge vial (*see* **Note 19**),

600 µl of Stain Cocktail 2 into a 1.5 ml clear microcentrifuge vial, and 800 µl of Array Holding Buffer into a 1.5 ml clear microcentrifuge vial. Spin down all vials to remove any air bubbles. Place vials into sample holders on the Fluidics station 450 (*see* **Note 20**): vial containing Stain Cocktail 1 in sample holder 1, vial containing Stain Cocktail 2 in sample holder 2, and vial containing Array Holding Buffer in sample holder 3. Wash and stain with Fluidics Station 450 using the fluidics script Protocol FS450_0002 (for 100 format array) (*see* **Note 21**): Post Hybridization Wash #1: 10 cycles of 2 mixes/cycle with Wash Buffer A at 30 °C, Post Hybridization Wash #2: 6 cycles of 15 mixes/cycle with Wash Buffer B at 50 °C, first Stain: Stain the probe array for 5 min with Stain Cocktail 1 at 35 °C, Post Stain Wash: Wash 10 cycles of 4 mixes/cycle with Wash Buffer A at 30 °C, second Stain: Stain the probe array for 5 min with Stain Cocktail 2 at 35 °C, third Stain: Stain the probe array for 5 min with Stain Cocktail 1 at 35 °C, Final Wash: 15 cycles of 4 mixes/cycle with Wash Buffer A at 35 °C and Array Holding Buffer: Fill the probe array with Array Holding Buffer.

7. Scanning: Check that the probe array window has no air bubbles and that the array glass surface is free of dust or other particulates. If there are air bubbles, manually fill the array with Array Holding Buffer. If dust is present, carefully wipe the surface with a clean lab wipe. Cover both septa with 3/8″ Tough-Spots and scan on the GeneChip Scanner 3000 (*see* **Notes 22**, **23** and **24**).
8. Array Rehybridization (100 format array): Prepare a 1× Hyb Mix: 25.2 µl Nuclease-Free Water, 66 µl 2× Hybridization Mix, 19.2 µl 27.5 % Formamide, 12.8 µl DMSO, 6.6 µl 20× Eukaryotic Hybridization Controls, and 2.2 µl Control Oligonucleotide B2, 3 nM. Add to the volume of recovered hybridization cocktail at the washing step with 1× Hyb Mix to have a total volume of 132 µl. Follow the hybridization instructions to complete the hybridization process and continue with Washing and Staining.

3.3 mRNA Microarray (Fig. 3) [6–8]

1. Poly-A RNA Control Addition: Serially Dilute Poly-A RNA control stock (*see* **Note 25**) using a volume not less than 2 µl to maintain precision. Mix thoroughly and spin down after each dilution: First dilution (1:20): 2 µl of Poly-A Control Stock in 38 µl of Poly-A Control Dil Buffer, Second Dilution (1:50): 2 µl of the First Dilution in 98 µl of Poly-A Control Dil Buffer, Third Dilution (1:50): 2 µl of the Second Dilution to 98 µl of Poly-A Control Dil Buffer, and Fourth Dilution (1:20, 1:10, 1:4, 1:2 depending on RNA input 50, 100, 250,

or 500 ng respectively): 2 μl of the Third Dilution to 18 μl of Poly-A Control Dil Buffer if 100 ng RNA. Add 2 μl of Fourth Dilution to total RNA sample (*see* **Note 26**).

2. First-Strand cDNA synthesis (2.25 h): All steps done on ice. Thaw First-Strand Enzyme Mix and First-Strand Buffer Mix. Prepare a First-Strand Master Mix in a nuclease-free tube in this sequence that includes ~5 % overage to cover pipetting error (per one reaction): 4 μl First-Strand Buffer Mix and 1 μl

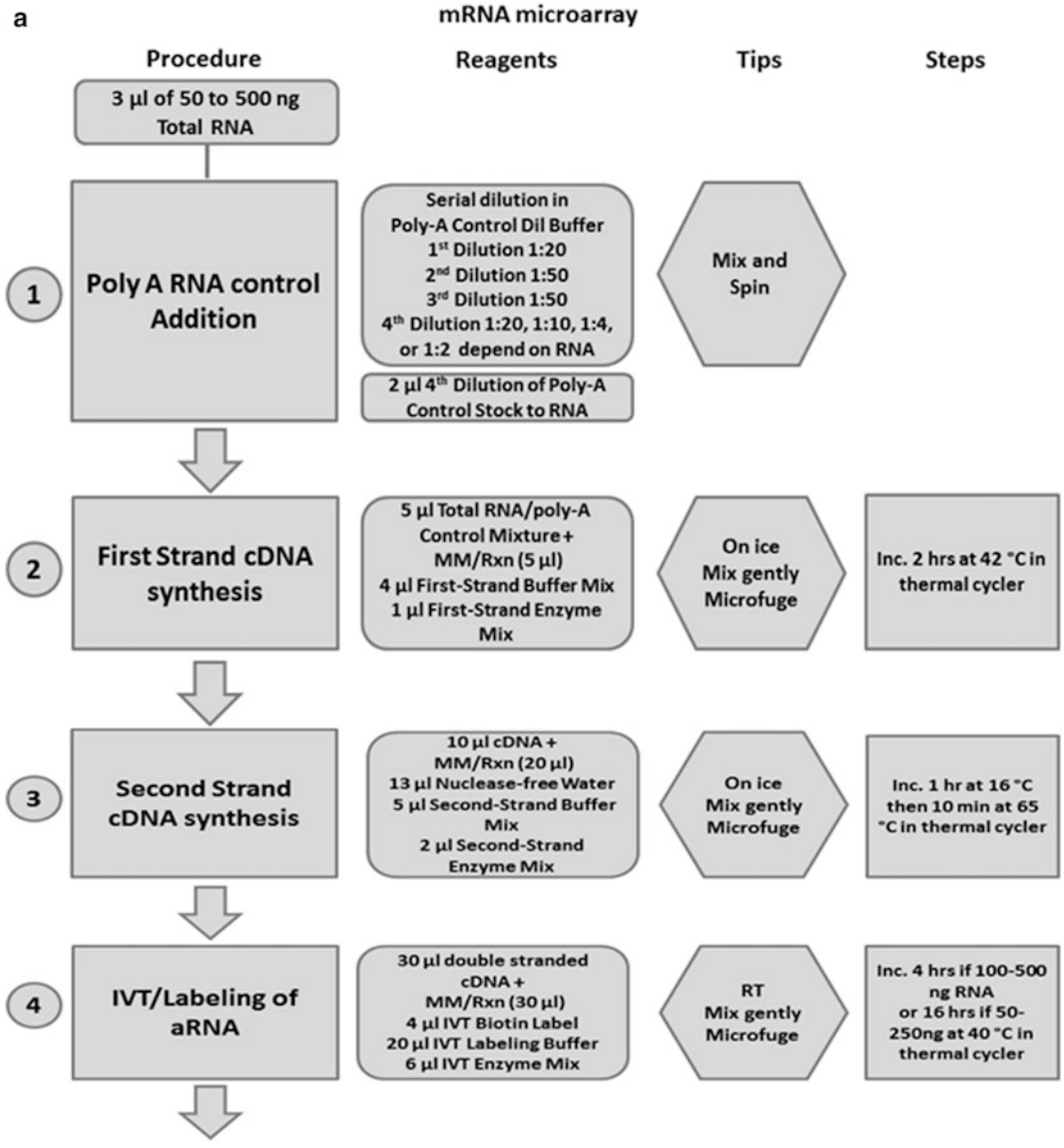

Fig. 3 mRNA microarray Summary. (**a**) Steps 1–4, (**b**) Steps 5–6, and (**c**) Steps 7–10 (*Inc.* incubate, *MM* master mix, *Rxn* reaction, *Cent.* centrifuge, *RT* room temperature)

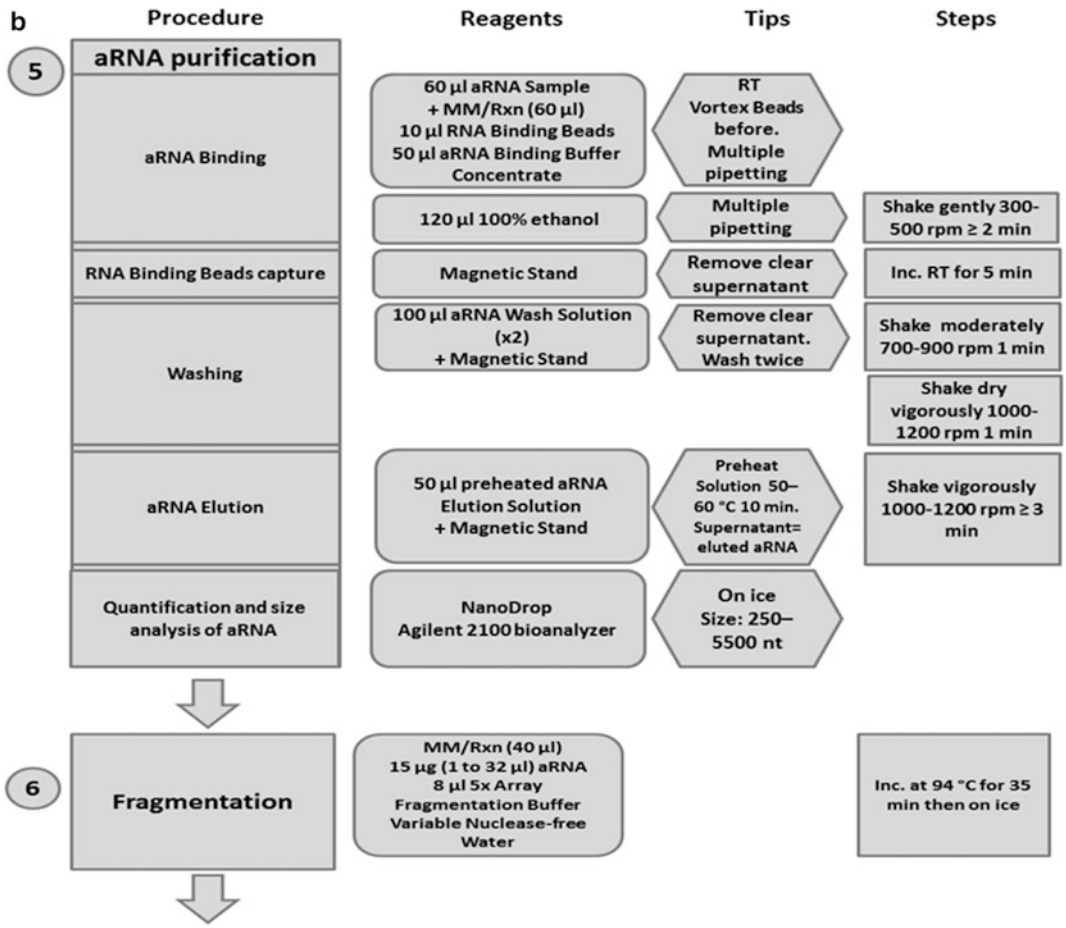

Fig. 3 (continued)

First-Strand Enzyme Mix. Vortex gently and centrifuge briefly. Distribute 5 µl First-Strand Master Mix in PCR tubes and add 5 µl of Total RNA/poly-A Control Mixture (*see* **Note 27**) to a final volume of 10 µl. Vortex gently and microfuge briefly. Incubate for 2 h at 42 °C in a thermal cycler. Afterwards, centrifuge briefly the first-strand cDNA and place the sample on ice.

3. Second-Strand cDNA synthesis (1.5 h): All steps done on ice. Thaw Second-Strand Enzyme Mix and Second-Strand Buffer Mix. Prepare a Second-Strand Master Mix in a nuclease-free tube in this sequence that includes ~5 % overage to cover pipetting error (per one reaction): 13 µl Nuclease-free Water, 5 µl Second-Strand Buffer Mix, and 2 µl Second-Strand Enzyme Mix. Vortex gently and centrifuge briefly. Distribute 20 µl Second-Strand Master Mix to each 10 µl cDNA sample. Vortex gently/Flick 3–4 times and microfuge briefly. Incubate for 1 h at 16 °C followed by 10 min at 65 °C in a thermal cycler

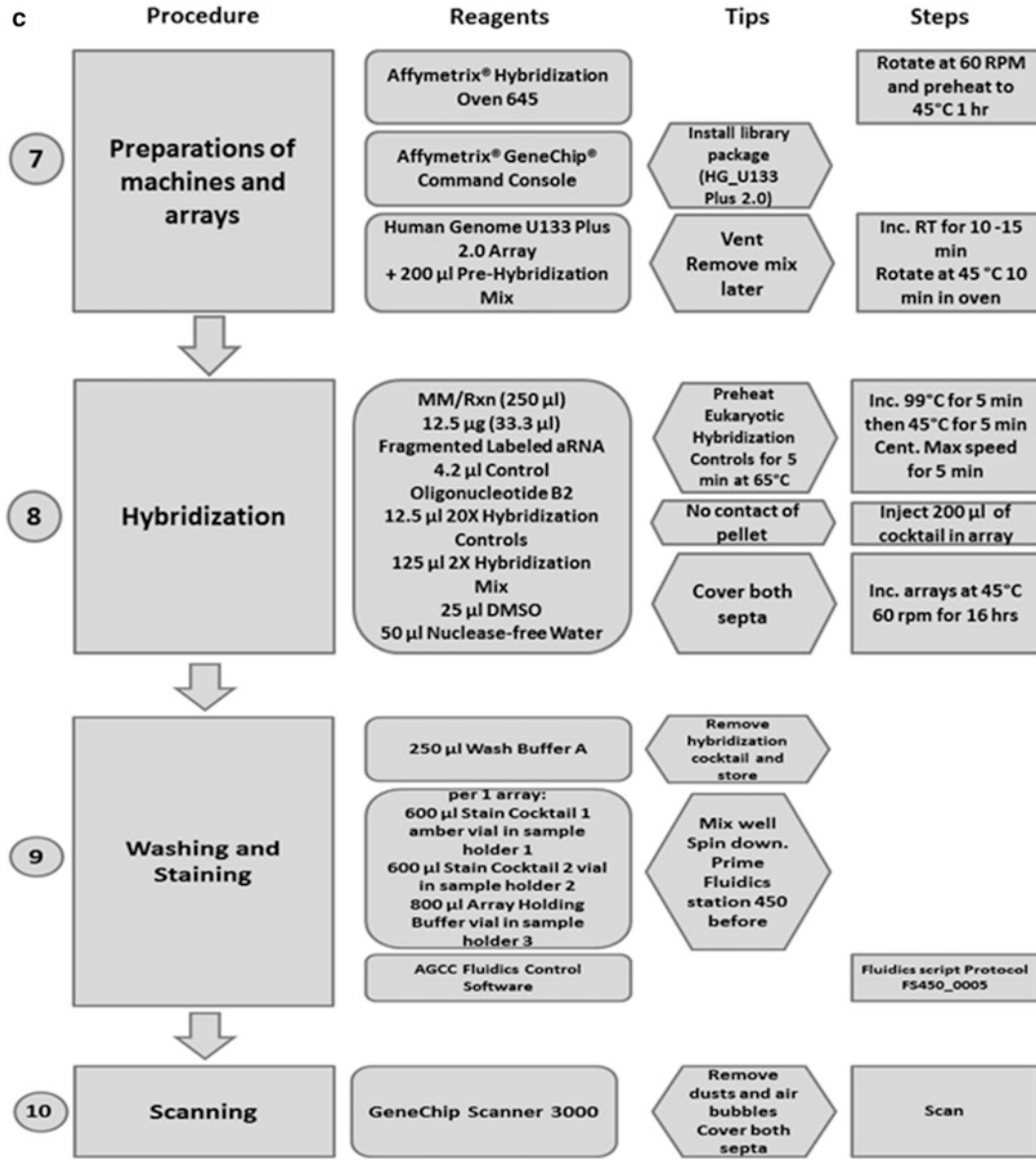

Fig. 3 (continued)

(*see* **Note 28**). Afterwards, centrifuge briefly and put on ice to continue with next step or freeze overnight at −20 °C if desired.

4. In Vitro Transcription (IVT)/Labeling of amplified RNA "aRNA" with biotin (4 or 16 h): At room temperature, prepare an IVT Master Mix in a nuclease-free tube in this sequence that includes ~5 % overage to cover pipetting error (per one reaction of total volume 30 µl): 4 µl IVT Biotin Label, 20 µl IVT Labeling Buffer, and 6 µl IVT Enzyme Mix. Vortex gently and

centrifuge briefly. Distribute 30 μl IVT Master Mix to each 30 μl double stranded cDNA sample. Vortex gently and microfuge briefly. Incubate for 4 or 16 h at 40 °C in a thermal cycler depending on quantity of RNA input (*see* **Note 29**). Place the aRNA on ice briefly to proceed with the next step or freeze immediately at −20 °C for overnight storage.

5. aRNA purification (0.75 h): to remove enzymes, salts, and unincorporated nucleotides. aRNA Binding Mix: Vortex the RNA Binding Beads before use. At room temperature, prepare aRNA Binding Mix in a nuclease-free tube (per one reaction): 10 μl RNA Binding Beads and 50 μl aRNA Binding Buffer Concentrate. aRNA binding: Add 60 μl aRNA Binding Mix to each sample and place each sample in a well of a U-Bottom Plate. Mix by pipetting up and down several times. Add 120 μl 100 % ethanol to each sample. Repeat the mixing step. Shake gently for ≥2 min at 300–500 rpm for the aRNA binding to the RNA Binding Beads. RNA Binding Beads capture: Place the plate on a magnetic stand for approximately 5 min to capture the magnetic beads that will form pellets against the magnets in the magnetic stand leaving a transparent mixture (*see* **Note 30**). Carefully remove the supernatant without disrupting the magnetic beads and remove the plate from the magnetic stand. Washing: Add 100 μl aRNA Wash Solution to each sample, and shake at moderate speed 700–900 rpm for 1 min. Place the plate on a magnetic stand to recapture the RNA Binding Beads as before. Carefully remove the supernatant without disrupting the magnetic beads and remove the plate from the magnetic stand. Repeat the washing step with 100 μl of aRNA Wash Solution and then recapture step. Shake the plate dry vigorously at 1000–1200 rpm for 1 min to evaporate remaining ethanol from the beads. aRNA Elution: Preheat an aliquoted amount of aRNA Elution Solution in 1.5 ml RNase-Free Tube to 50–60 °C for at least 10 min before Elution step. Add 50 μl preheated aRNA Elution Solution to each sample to elute the purified aRNA. Shake vigorously the plate for 3 min or more at 1000–1200 rpm until RNA Binding Beads are fully dispersed. Place the plate on a magnetic stand, and capture the RNA Binding Beads. Transfer the supernatant, which contains the eluted aRNA, to a nuclease-free PCR tube. Storage: Store eluted aRNA at ≤ −20 °C for up to 1 year (avoid multiple freeze-thawing cycles to maintain aRNA integrity) or keep on ice to continue with quantitation and fragmentation. Quantification and size analysis of aRNA: Use NanoDrop Spectrophotometer that only consumes 2 μl of the sample. It is expected to have higher yield from cell lines compared to tissues and more yield after 16 h incubation for a low amount of RNA input. Use Agilent 2100 bioanalyzer with the Agilent RNA 6000 Nano Kit

to check size distribution of aRNA that should be from 250 to 5500 nucleotides mostly between 600 and 1200 nucleotides.

6. Fragmentation (1 h): prepares labeled aRNA for hybridization to GeneChip® 3′ expression arrays. aRNA fragmentation mixture for 49 format array: 15 μg (1–32 μl) aRNA, 8 μl 5× Array Fragmentation Buffer, Nuclease-free Water Variable (up to 40 μl final volume). Incubate the fragmentation reaction at 94 °C for 35 min. After the incubation, put the mixture on ice for the next step or store at −20 °C (or −70 °C for longer-term storage).
7. Preparations of machines and arrays (same as in miRNA microarray) but place the oven at 45 °C. Download and install the Array library file package (HG_U133 Plus 2.0) into AGCC and use Human Genome U133 Plus 2.0 Array chips (*see* **Note 15**). Fill one of the septa of the 49 format array with 200 μl of Pre-Hybridization Mix to wet it and rotate the array at 45 °C for 10 min in the oven.
8. Hybridization (16 h): Thaw reagents Control Oligonucleotide B2 (3 nM), 20× Hybridization Controls, 2× Hybridization Mix, DMSO (*see* **Note 3**), and Nuclease-free Water. Heat the 20× Eukaryotic Hybridization Controls for 5 min at 65 °C. Prepare Hybridization cocktail per one 49 format array (total volume: 250 μl): 12.5 μg (33.3 μl) Fragmented and Labeled aRNA, 4.2 μl Control Oligonucleotide B2 (3 nM), 12.5 μl 20× Hybridization Controls, 125 μl 2× Hybridization Mix, 25 μl DMSO, and 50 μl Nuclease-free Water (*see* **Note 31**). Heat the hybridization cocktail in a heat block to 99 °C for 5 min followed by 45 °C for 5 min. Microfuge the hybridization cocktail at maximum speed for 5 min to collect any insoluble material from the hybridization mixture. Place a clean pipette tip into one of the septa to vent the arrays and extract the Pre-Hybridization Mix with a pipettor through the other septum. Refill the array with 200 μl of hybridization cocktail without contacting insoluble matter at the bottom of the tube. Rotate probe array at 60 rpm in the hybridization oven at 45 °C for 16 h.
9. Washing and Staining (90 min): same step as in miRNA microarray but the chips used are 49 Format Arrays that require filling the array with 250 μl of Wash Buffer A and that require different fluidics script. Wash and stain with Fluidics Station 450 using the fluidics script Protocol FS450_0005 (for 49 format array) (*see* **Notes 20** and **21**): Post Hybridization Wash #1: 10 cycles of 2 mixes/cycle with Wash Buffer A at 25 °C, Post Hybridization Wash #2: 4 cycles of 15 mixes/cycle with Wash Buffer B at 45 °C, first Stain: Stain the probe array for 10 min with Stain Cocktail 1 at 25 °C, Post Stain Wash:

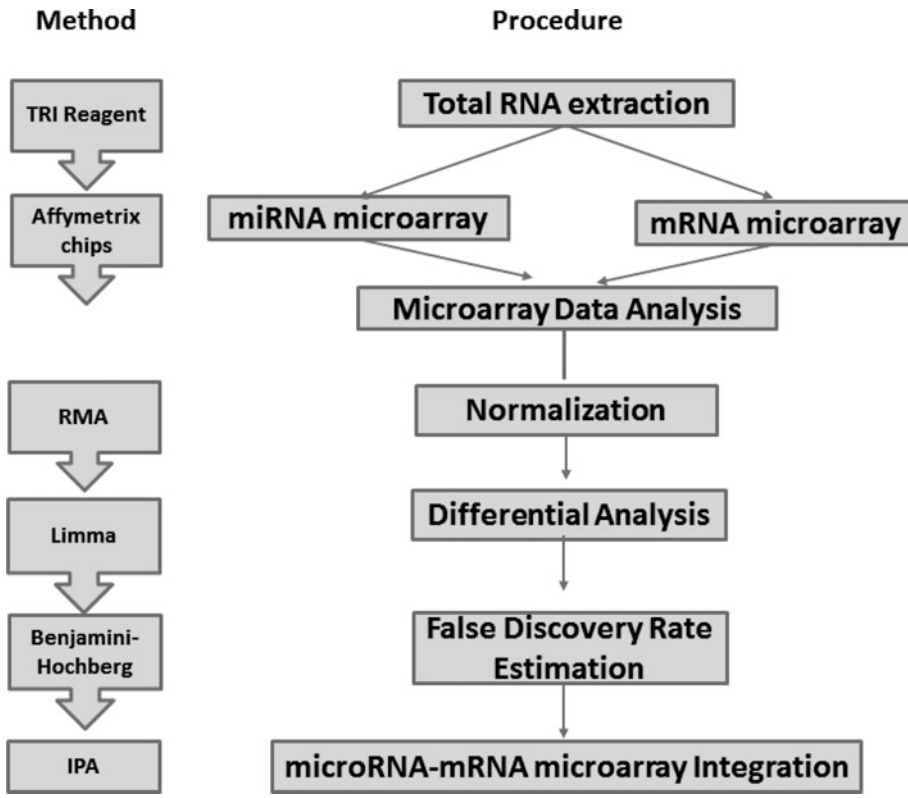

Fig. 4 All Protocol Summary with Integration of mRNA and miRNA microarray data

Wash 10 cycles of 4 mixes/cycle with Wash Buffer A at 30 °C, second Stain: Stain the probe array for 10 min with Stain Cocktail 2 at 25 °C, third Stain: Stain the probe array for 10 min with Stain Cocktail 1 at 25 °C, and Final Wash: 15 cycles of 4 mixes/cycle with Wash Buffer A at 30 °C. Array Holding Buffer: Fill the probe array with Array Holding Buffer.

10. Scanning: same step as in miRNA microarray.

3.4 miRNA-mRNA Integration (Fig. 4)

3.4.1 miRNA and mRNA Microarray Data Analysis

1. miRNA and mRNA microarray Data Analysis Using Bioconductor (http://www.bioconductor.org) packages within R statistical environment miRNA and mRNA Affymetrix microarray data (.CEL files) were analyzed in a similar way.
2. miRNAs from Homo sapiens were extracted from the array. Normalize Data with the Robust Multiarray Average algorithm (RMA).
3. Detect differentially expressed miRNA and mRNA between different CML stem cells/CML cells (e.g., untreated versus treated) samples using Limma by fitting a linear model to normalized expression data for each probe.

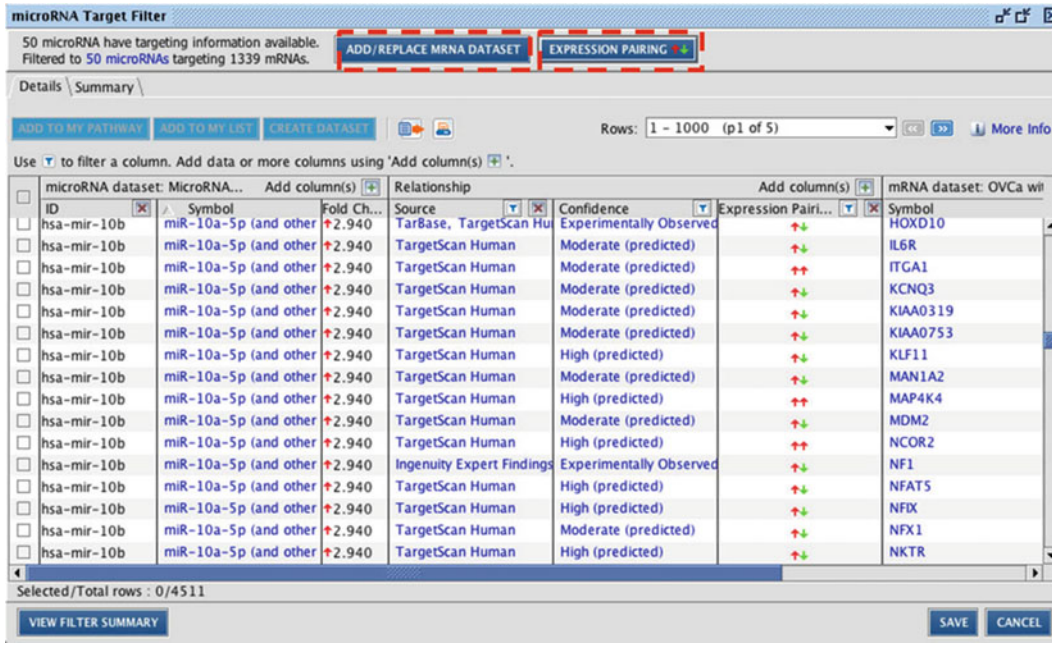

Fig. 5 miRNA-mRNA integration using Ingenuity Pathway Analysis with two buttons highlighted with red dashed line: "Add/Replace Dataset" and "Expression Pairing"

4. Estimate False Discovery Rates (FDRs) using the Benjamini-Hochberg method.

3.4.2 Integration Using Ingenuity Pathway Analysis (IPA) (Fig. 5)

1. Data Format: Using Microsoft Excel, make separate Excel sheets for mRNA and microRNA microarray with data placed in the first sheet only. Limit the data to the significant miRNA and mRNA with p-value <0.05. Use only one header row. Make the first column with Affymetrix ID, second column log ratio/log fold change and third column as adjusted p-value and a last column with miRBase annotation for miRNA (IPA might not identify all of the miRNA Affymetrix ID). Save as type: text (tab delimited).
2. Upload Dataset: Select Upload Dataset from the "New" button present at the upper left of IPA main page. Upload the tab delimited files for miRNA microarray. Keep the select file format option as flexible format. Select contains column header if a header is present in the tab delimited file. Select Identifier Type as Affymetrix or miRBase (mature) depending on which column that you will use ID (first or fourth). No array platforms are present for miRNA so select "Not Specified/applicable." Assign the ID column as "ID" and assign the other columns as "Observation1" (automatically the other column will be named log ratio and p-value) or as "Ignore" if you want to hide the column. Do the same steps for the mRNA microarray but Select Identifier Type as Affymetrix and array

platforms as Human Genome U133 Plus 2.0 Array. Save the Datasets in a folder.

3. Integration of two Datasets: Open Ingenuity Pathway Analysis software. Select New microRNA Target Filter from the "New" button present at the upper left of IPA main page and select miRNA microarray existing dataset. Filter the in silico tool or source used to predict the targets (Ingenuity Expert Finding, Ingenuity ExpertAssist Findings, TargetScan, TarBase, and miRecords) and its confidence. Filter the predicted mRNA pathway, location, molecule type, species, tissue/cell line, disease, and Entrez gene name. Select Add/Replace mRNA dataset and choose the uploaded mRNA microarray dataset (keep the option: limit targets shown to mRNA in this dataset). Select Expression Pairing to integrate miRNA and mRNA data according to the log Ratio.

4 Notes

1. All FlashTag™ Biotin HSR RNA Labeling Kit Reagents are stored at −20 °C with no more than three freeze-thaw cycle. 10× Reaction Buffer, 25 mM $MnCl_2$, 5× FlashTag Biotin HSR Ligation Mix, HSR Stop Solution, ELOSA Spotting Oligos, Nuclease-Free Water, and 27.5 % Formamide are thawed at room temperature, vortexed, and briefly microfuge. ATP Mix, RNA Spike Control Oligos, and ELOSA Positive Control are thawed on ice, microfuged, and kept on ice at all times. PAP Enzyme and T4 DNA Ligase are removed from freezer just before use, flicked, briefly microfuged, and kept on ice at all times without any vortexing.
2. GeneChip® miRNA 3.0 Array stored at 2–8 °C. Refer to the expiration date on the package label. Do not use probe arrays or reagents after the expiration date.
3. GeneChip® Hybridization, Wash, and Stain Kit should be kept at 4 °C. DMSO solidifies 4 °C so completely thaw before use. After the first use, it is recommended to store DMSO at room temperature.
4. All GeneChip® 3′ IVT Express Kit reagents are stored at −20 °C except for aRNA Binding Buffer Concentrate, aRNA Wash Solution Concentrate, aRNA Elution Solution, Nuclease-free Water (10 ml), 5× Array Fragmentation Buffer stored at room temperature. Place also RNA Binding Beads at 2–8 °C.
5. The mixture separates into an organic lower red phenol-chloroform phase, a protein-rich interphase, and a colorless upper aqueous phase that contains RNA.

6. When precipitating RNA from small sample (<106 cells), 5–10 μg of RNase-free glycogen can be added as a carrier to the aqueous phase. It does not inhibit cDNA synthesis or PCR or microarray at concentrations ≤4 mg/ml.
7. RNA pellet might be invisible or might appear as a tiny white pellet at the bottom of the tube depending on the amount of RNA present. In order to know the position of the pellet, try to orient the tubes in the centrifuge the same way so you can estimate where the pellet is in all of the tubes.
8. Make sure there are no residual drops of ethanol on the tube; otherwise the pellet will not dissolve and the A260/280 ratio will be messed up. Do not overdry because the pellet can lose its solubility. Do not dry by vacuum centrifuge.
9. If the A260/230 ratio is lower than expected, it may indicate the presence of contaminants which absorb at 230 nm such as ethanol and TRI Reagent®. Glycogen used for precipitation could also decrease A260/230 ratio. A high A260/230 ratio could be caused by a dirty pedestal upon blank measurement. As for A260/280 ratio, it may be lower than expected if residual TRI Reagent® or other reagent associated with the extraction protocol is present or RNA is of very low concentration (less than 10 ng/ul). A high A260/280 are not indicative of an issue.
10. It is recommended for miRNA 100 Format Arrays (miRNA 3.0 Arrays) to use 130–1000 ng total RNA in 8 μl instead of enriched low molecular weight RNA to prevent sample loss. All steps mentioned are for using total RNA and not enriched low molecular weight RNA. Ethanol precipitation to concentrate samples is not recommended because this procedure might not succeed in precipitating the low molecular weight RNA.
11. Do not make a master mix at FlashTag™ Biotin HSR Ligation step, as auto-ligation can occur.
12. Store 2 μl of biotin-labeled sample on ice for up to 6 h or store at −20 °C for up to 2 weeks to run the ELOSA QC Assay at a convenient time. Affymetrix recommends performing this assay before the use of any FlashTag Biotin HSR labeling reaction on microarrays. This 2 μl biotin-labeled sample will help in troubleshooting possible target preparation issues.
13. Do not touch the bottom of the ELOSA wells with the pipette tip in all the steps of ELOSA QC assay. The ELOSA wells can be stored at 2–8 °C for up to 2 weeks if covered tightly with an adhesive plate sealer to prevent evaporation but, for best results, incubate overnight.

14. Blot dry is performed by expelling the liquid into a sink, and repeatedly tap the inverted plate on a stack of paper towels without inserting any wipes into the ELOSA wells.
15. Equilibrating the arrays to room temperature is important so that the rubber septa would not be prone to cracking that can lead to leaks. Each array has two septa. Before filling the array, the array chamber must be vented by inserting a clean, unused pipette tip into one of the septa; then insert the pipette tip of a micropipettor into the remaining.
16. 20× Eukaryotic Hybridization Controls are premixed biotin-labeled bioB, bioC, bioD (*E. coli* genes) and cre (B. subtilis gene), in staggered concentrations used as spiked controls from GeneChip® Eukaryotic Hybridization Control Kit.
17. All the steps performed in the hybridization step are for 100 Format Array miRNA 3.0 and later designs. It is recommended to use the newest chips with updated probes in reference to most recent miRBase registry.
18. Before washing, arrays can be stored in the Array Holding Buffer at 4 °C for up to 3 h. Allow the arrays to equilibrate to room temperature before washing and staining.
19. Stain Cocktail 1 is light sensitive so store in an amber vial.
20. Fluidics Station 450/250 is used to wash and stain the probe arrays and is operated using Affymetrix GeneChip Command Console. Before using this station, it should be primed to make sure it is filled with the appropriate buffers and ready for running fluidics station protocols. Fill the intake buffer reservoirs on the right side of the instrument with the appropriate Wash A and Wash B solutions. Empty the waste bottle and fill the water reservoir with deionized water. Place three empty 1.5 ml microfuge tubes into the stain holder positions 1, 2, and 3. Place the washblock lever into the engaged/closed position. Push the needle lever into the down position. Run the Prime_450 maintenance protocol from the AGCC Fluidics Control Software.
21. Use fluidics script to identify the compatible scripts after specifying Design Type, Probe Array Type, Labeling Kit, Hybridization, Wash and Stain Procedure, and the Fluidics Station Model. Fluidics Script to Use can be downloaded from: www.affymetrix.com/support/technical/fluidics_scripts.affx.
22. AGCC Fluidics Control Software will scan the barcode with an external barcode reader after selecting the suitable fluidic Protocol. Make sure the wash solutions and water reservoirs contain enough liquid for the run to complete and that the waste bottle has enough room to collect waste for the run.

23. Excessive washing of array will result in a loss of signal intensity. If arrays are not scanned directly, keep it at 4 °C in the dark until ready for scanning for a maximum of 24 h.
24. The Fluidics Station should be shut down if not used within the next 12 h to reduce the risk of salt buildup in the instrument. Before running Shutdown_450 protocol, Wash A and Wash B lines should be placed into deionized water and empty 1.5 ml microfuge tubes should be placed in the stain holders on the front of the instrument. Turn off the Fluidics Station using the toggle switch on the lower left side of the machine after the LCD display indicates the station is no longer primed.
25. Poly-A RNA controls: synthesized polyadenylated transcripts (lys, phe, thr, and dap) for Bacillus subtilis genes premixed at staggered concentrations spiked in RNA samples to check the later hybridization process. The first dilution of the poly-A RNA controls can be stored up to 6 weeks at −20 °C and frozen-thawed up to eight times.
26. Recommended RNA input is 100 ng in 3 μl but the sample quantity could range between 50 and 500 ng. RNA sample should be free of contaminants such as cellular debris or chemicals associated with RNA extraction. RNA integrity could be checked using Agilent 2100 Bioanalyzer with an RNA LabChip® Kit. RNA samples should have high RNA integrity number; otherwise try to increase input amount.
27. Use as a Control RNA sample isolated from HeLa cells (1 mg/ml) that is provided with the kit. Dilute 2 μl of the Control RNA into 18 μl of Nuclease-free Water. Use 1 μl of the diluted Control RNA (100 ng) starting at Reverse Transcription to Synthesize First-Strand cDNA. At IVT/Labeling aRNA step, use a 16 h incubation. Continue with the procedure for making biotin-modified aRNA through aRNA Purification. It is expected that the positive control reaction should produce $\geq$ 50 μg of aRNA.
28. Precool the thermal cycler block to 16 °C because subjecting the reaction to temperatures >16 ° C will compromise aRNA yield. Cover reactions with the heated lid of the thermal cycler even if its temperature cannot be adjusted to match the block temperature. Set the lid temperature to match the block temperature or ~100 °C. If you see condensation, check to make sure that the heated lid feature of the thermal cycler is working properly.
29. IVT incubation period depends on quantity of RNA input: 16 h if 50–250 ng and 4 h if 100–500 ng.
30. The exact capture time of RNA Binding Beads depends on the magnetic stand used and the amount of aRNA in the sample. To increase aRNA recovery, mix well and make sure that the

mixture is clear without any beads before continuing to the next step.

31. All of the steps for Hybridization Step and Washing/Staining Step are for the 49 Format Array Human Genome U133 Plus 2.0.

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Chapter 19

Single-Cell Cytokine Profiling to Investigate Cellular Functional Diversity in Hematopoietic Malignancies

Jonathan J. Chen, Minsuk Kwak, and Rong Fan

Abstract

Single-cell analysis of cytokine production is increasingly recognized as an important method to understand the inflammatory microenvironment and hematopoietic disease state. Certain cytokines are critical to the regulation of lineage specification, and the aberrant production of these cytokines can contribute to lineage reprogramming. Here, we describe of a platform combining subnanoliter microchambers and a high-density antibody barcode array for the study of single-cell cytokine secretions in hematopoietic cancer cell populations.

Key words Single-cell cytokine profiling, Immunophenotyping, Functional heterogeneity, High-throughput multiplexed analysis, Cytokine secretions, Pathogenesis

1 Introduction

Although there have been many studies detailing the intricacies of blood production, there is much to be discovered about the lineage specification of hematopoietic progenitor cells and how abnormal cytokine function can lead to disease states. Chronic myeloid leukemia (CML) is a disease that stems from the aberrant proliferation in hematopoietic production and is highly associated with a heterogeneous malignant niche favoring disease development [1]. It is widely accepted that tumor development is the evolutionary process of somatic mutation and selection, which can result in substantial intratumor genetic heterogeneity [2]. In addition, phenotypic and functional heterogeneity within a primary tumor has been reported in a variety of human cancers including CML [3].

There has been an increasing need to be able to study the cytokine production from single cells. The overproduction of proinflammatory cytokines is associated with many blood disorders [4]. Certain cytokines serve as the critical regulators of lineage

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specification, suggesting the hematopoietic lineage output can be reprogrammed by aberrant cytokine production [4]. Moreover, the cytokine-driven inflammatory state contributes to the symptoms and disease progression characteristic of hematological malignancies [5]. Detecting and molecular profiling of cytokine-secreting cells in CML will provide novel insights into the molecular basis of CML and likely find a place in the clinic for early diagnosis and therapeutic stratification. However, due to heterogeneous cellular composition of bone marrow compartments and functional heterogeneity of cytokine-secreting cells, new assays for measuring multiple cytokines at the single-cell levels are needed to perform more informative molecular profiling. Recently, single-cell cytokine profiling of hematopoietic cells from myeloproliferative neoplasms (MPN) showed the significant up-regulation of a spectrum of proinflammatory cytokines and the increased heterogeneity in cytokine secretion [6]. Other comparable methods for single-cell cytokine analysis can be used to investigate the roles of cytokine productions in the pathogenesis of CML.

Fluorescent-activated cell sorting (FACS) is the current go-to for single-cell analysis, and while FACS is typically used for detecting and sorting cell phenotypes via surface markers, it has been expanded to detect intracellular proteins [7]. However, intracellular cytokine staining does not give true secretion analysis, and the fixing requirement prevents any further analysis to be completed. ELISpot/FLUOROSpot are the current mainstay for true single-cell secretion analysis; however, they are not very highly multiplexed. Recent bead-based immunoassays can give highly multiplexed analysis of secreted proteins; however, this is limited to population measurements [8].

New immunophenotyping technologies have emerged as new tools that are able to provide highly multiplexed analysis of secreted proteins at single-cell resolutions [9–11]. CyTOF is capable of measuring a diverse variety of proteins at the single-cell level; however, this is mostly limited to intracellular and surface proteins [12]. We achieve single-cell cytokine profiling via a high-throughput single-cell secretomic analysis platform for the simultaneous detection of 14–42 unique proteins [6, 11, 13]. Through the integration of a subnanoliter microchamber and high-density antibody barcode array, our single-cell platform can analyze the secretion of cell lines and primary samples to reveal the functional heterogeneity among the single-cell secretomic signatures within a population.

2 Materials

2.1 Components of Silicon Master Mold (Barcode Flow Patterning and Cell Capture)

1. 4 in. p-type CZ silicon wafer (Silicon Quest Int'l).
2. Patterned Photomask (*see* **Note 1**).
3. MF-319 Microposit developer (Shipley).
4. S1813 Microposit positive photoresist (Shipley).
5. SU-8 2010 developer (MicroChem).
6. SU-8 2010 negative photoresist (MicroChem).
7. Chlorotrimethylsilane (TMCS) (Sigma-Aldrich).

2.2 Components of PDMS Chip (Barcode Flow Patterning and Cell Capture)

1. Silicon Master Mold (Barcode Flow Patterning and Cell Capture).
2. Polydimethylsiloxane (PDMS) Component A (Potting compound) and B (Crosslinking Agent) (*see* **Note 2**).
3. Aluminum Foil.
4. 70 % Ethanol.
5. 100 % Isopropanol.
6. PDMS Hole Puncher (Schmidt).
7. Magic Tape 810 (Scotch).

2.3 Components of Barcode Chip Fabrication

1. PDMS Flow Patterning Chip.
2. SuperChip Microarray Poly-L-Lysine Glass Slide (Thermo Scientific).
3. 23G Metal Pins.
4. Microbore Tubing 0.02 in. (Tygon).
5. 23G Luer Stub Adapter (Becton Dickinson).
6. Nitrogen Gas.
7. Purified Capture Antibodies, ELISA compatible (*see* **Note 3**).
8. Phosphate Buffered Solution (PBS).
9. Blocking Solution: 3 % BSA in PBS (*see* **Note 4**).
10. DI Water.

2.4 Components of Single-Cell Assay

1. PDMS Cell Capture Chip.
2. Barcode Chip.
3. Media (*see* **Note 5**).
4. Cell Suspension.
5. Plexiglass Clamps.
6. 18-8 Stainless Steel Socket Head Cap Screw 4-40 Thread, 5/8″ Length.
7. Ball-Point L-Key 3/32″ Hex.

8. Blocking Solution: 3 % BSA in PBS.
9. Biotinylated Antibody, ELISA compatible (*see* **Note 6**).
10. Streptavidin–Fluorophore.
11. Microscope with automatic image stitching.
12. Genepix Array Scanner (Molecular Devices).

3 Methods

All procedures are performed at room temperature unless specified.

3.1 Silicon Master Mold Fabrication

3.1.1 The Entire Procedure Should Be Performed in the Cleanroom (Dust-Free) Environment

1. Heat up hot plates to 120 °C.
2. Place a new silicon wafer on the hot plate and bake for 5 min.
3. Carefully put the wafer on the center of photoresist spinner's wafer holder and fix its position by applying the vacuum on the wafer holder.
4. Remove any dust or particle by blowing with nitrogen air.

3.1.2 Fabrication of Flow Patterning Master Mold

5. Photoresist Coating: Apply appropriate quantity of photoresist SPR 220 i-line 1.5 (*see* **Note 7**) (from Shipley) on the wafer (4 in.), and spin-coated at 900 rpm for 5 s and at 3000 rpm for 1 min (*see* **Note 8**).
6. Soft Bake: The coated wafer is baked on a hotplate at 100 °C for 3 min.
7. Cool Down: The temperature of the hot plate is gradually cooled down to 55 °C (decrease by 5 °C/min) (*see* **Note 9**).
8. Exposure: The wafer is then exposed to 150 mJ/cm^2 UV light using EVG 620 contact/proximity mask aligner. Note: Measure the lamp intensity of the mask aligner. Divide 150 mJ/cm^2 to get the required exposure time.
9. A postexposure bake: The wafer is baked on a hotplate at 120 °C for 3+ minutes and the wafer is cooled down to room temperature in a similar fashion.
10. Developing: The wafer is developed with Microposit MF-319 developer for ~1 min.
11. Following, Dry Reactive-Ion Etching (DRIE) is used for wafer pattern etching.
12. After DRIE, treat with TMCS for 20 min via vapor deposition in a vacuum chamber.

3.1.3 Fabrication of Single-Cell Capture Microchamber Mold

13. Photoresist Coating: Apply appropriate quantity of SU-8 negative photoresist (from MicroChem) on the wafer. Spin-coat with the following program: 500 rpm for 20 s and 2000 rpm for 60 s (*see* **Note 8**).

14. Soft Bake: Place the coated wafer on the hot plate initially set at 65 °C. Increase the temperature by 5 °C every minute. Once it reaches 95 °C, bake for 15 min.
15. Cool Down: Slowly cool down the temperature of the hot plate to 55 °C (decrease by 5 °C/min).
16. Exposure: The wafer is exposed using EVG 620 contact/proximity mast aligner. Set the aligner parameters as following: Soft Contact/Exposure time = 3 s/Interval = 20s/Separation = 10 cycles.
17. A Postexposure Bake: Same as soft bake and cool-down (**step 13** and **14**). Developing: The wafer is developed with SU-8 developer for 7–8 min with consistent, gentle shaking in a glass dish. Clean the wafer with isopropanol (IPA) and air-dry to remove any excess liquid on the wafer surface.

3.2 Barcode Flow Patterning Chip Fabrication

1. Using filtered compressed air (*see* **Note 10**), remove dust off the surface of the Flow Patterning Silicon Master Mold and ensure there are no particles on the surface (*see* **Note 11**).
2. Create a vessel for the silicon master mold with aluminum foil and place the master mold into the vessel (*see* **Note 12**).
3. Mix PDMS Potting agent and PDMS curing agent at 10:1 ratio (*see* **Note 13**). Pour PDMS mix (50 g) into the vessel over the silicon master mold. Vacuum the mold and PDMS for 1 h to degas (*see* **Note 14**).
4. Transfer PDMS and mold to the oven and bake for 2 h at 80 °C (*see* **Note 15**). After baking, remove the mold and cured PDMS and let cool at room temperature.
5. Carefully remove the cured PDMS (*see* **Note 16**) from silicon wafer and keep it clean and dust free.
6. Using a razor blade, cut the patterned area out to appropriate size for a poly-L-lysine glass slide. Create the inlet and outlet holes via a PDMS hole puncher (*see* **Note 17**).
7. Clean the chip by sonicating the chip in 70 % ethanol for 20 min twice, followed by sonication in 100 % isopropanol for 20 min. Blow dry the surface of the chip with filtered air and place in oven at 80 °C for 2 h to ensure complete removal of liquids (*see* **Note 18**).
8. Using Scotch magic tape (*see* **Note 19**), cover the patterned area. Firmly push tape onto the PDMS and remove tape to remove and particulates (*see* **Note 20**). Repeat this step until dust and particulate free.
9. Using filtered compressed air, remove dust and particulates from poly-L-lysine-coated glass slide and place onto flow patterning chip (*see* **Note 21**).

10. Using a microscope, check for crosstalk, dust, air bubbles, or blockages (*see* **Note 22**).
11. Thermal bond the glass slide to the PDMS chip by baking in oven for 2 h at 80 °C and cool to room temperature before proceeding (*see* **Note 23**). The Bonded PDMS and glass slide will be referred to as the Flowpattern Assembly in the following steps.

3.3 Cell Capture Chip Fabrication

1. Follow **steps 1–5** from Subheading 3.2 using the Cell Capture Chip Silicon Master Mold.
2. Using a razor blade, cut the patterned area out to appropriate size to cover the barcode area of the flow patterned chip previously marked. Store in a clean dust-free container until use in assay.

3.4 Flow Patterning of Barcode Chip

1. Insert hollow pins into tubing with Luer Stub adapters at the other end, and use a pipette to draw up 1–2 μL of primary antibody into the pin (*see* **Note 24**).
2. Insert the pin into the inlet hole of the Flowpattern Assembly and note the channel it was placed in (*see* **Note 25**).
3. Connect the other end to pressure regulated nitrogen gas.
4. Repeat **step 2–3** with the appropriate number of antibodies for cytokine analysis.
5. Slowly open the nitrogen flow to 2 psi using a pressure regulator (*see* **Note 26**).
6. Keep the nitrogen running overnight or until channels are flowed through.
7. Turn off air flow and remove pins (*see* **Note 27**).
8. Submerge Flowpattern Assembly in 3 % BSA and remove flow patterning PDMS chip and set aside (*see* **Note 28**).
9. Remove the barcode chip and block the surface of the barcode chip with 3 % BSA for 1 h.
10. Wash with PBS 2× followed by DI water 2× (*see* **Note 29**).
11. Using filtered compressed air, blow the surface of the barcode chip dry.
12. Store at 4 °C until use (*see* **Note 30**).

3.5 Single-Cell Assay

Schematic of workflow and representative image of the single-cell platform is shown in Figs. 1 and 2, respectively.

1. Sonicate Cell Capture Chip in 3 % BSA for 20 min (*see* **Note 31**).
2. Place cell capture chip onto bottom plate of clamp (*see* **Note 32**).
3. Fill the chambers with Media by pipetting 1 mL of Media on to the chip (*see* **Note 33**).

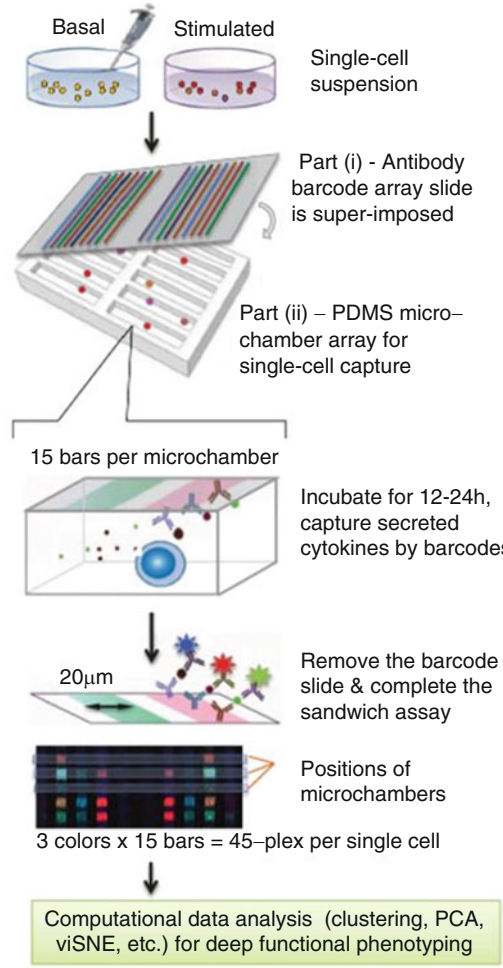

Fig. 1 Schematic of high-throughput profiling of single cells in basal and stimulated conditions for secreted effector proteins

4. Tilt off the Media so that there is only a thin layer on top of the chip.
5. Pipette 200 μL of 0.3 million cells/mL cell suspension onto cell capture area evenly and let cells settle for 10 min (*see* **Note 34**).
6. Place barcode chip lengthwise onto cell capture chip with the antibody barcode facing the cell chambers (*see* **Note 35**).
7. Place top clamp on and screw all sides down evenly.
8. Image chip with microscope and incubate at 37 °C for 12–24 h.
9. After incubation, submerge assembly into 3 % BSA and disassemble the clamps.
10. Remove the barcode chip from the cell capture chamber (*see* **Note 36**) and rinse with 3 % BSA.

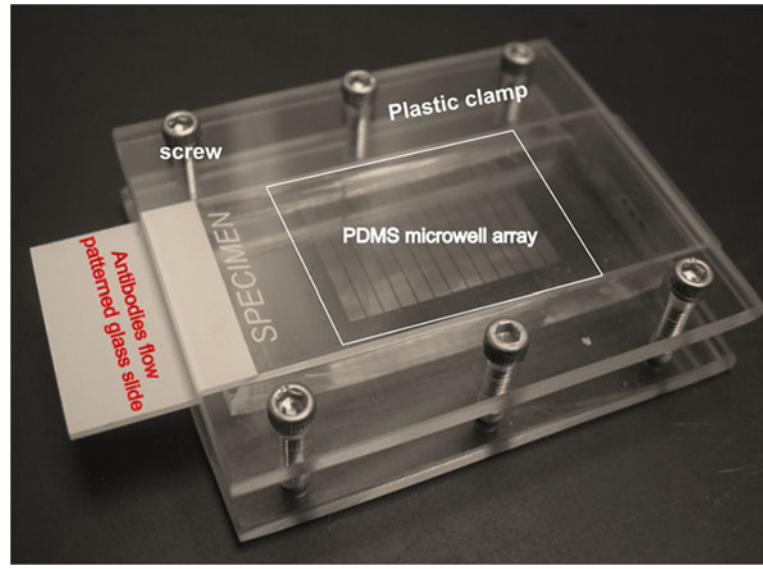

Fig. 2 Representative image of Single-Cell Analysis Platform assembly

11. Place the Barcode chip on a flat level surface and add 200 μL secondary antibody (1:200 dilution in 3 % BSA). Incubate with the secondary antibody for 45 min.
12. Wash the chip with 3 % BSA, and add 200 μL of Streptavidin–-fluorophore (1:100 dilution in 3 % BSA). Incubate with Streptavidin–fluorophore for 30 min.
13. Wash the chip with 3 % BSA and block for 30 min with 3 % BSA.
14. Wash with PBS 2× followed by DI Water 2× and blow dry with filtered air.
15. Scan dry chip using a Genepix array scanner (*see* **Note 37**).
16. The Data from the Genepix array scan combined with the positions of microchambers and cell counts (Fig. 3) are used for further analysis (Fig. 4).

4 Notes

1. Pattern can be obtained via Rong Fan Lab.
2. PDMS RTV 615 (Momentive) or PDMS Sylgard 184 (Dow Corning) can be used.
3. Make sure the antibodies are monoclonal to prevent nonspecific signals.
4. It is best to prepare the blocking solution fresh.
5. Use the same media in which the cells are cultured.

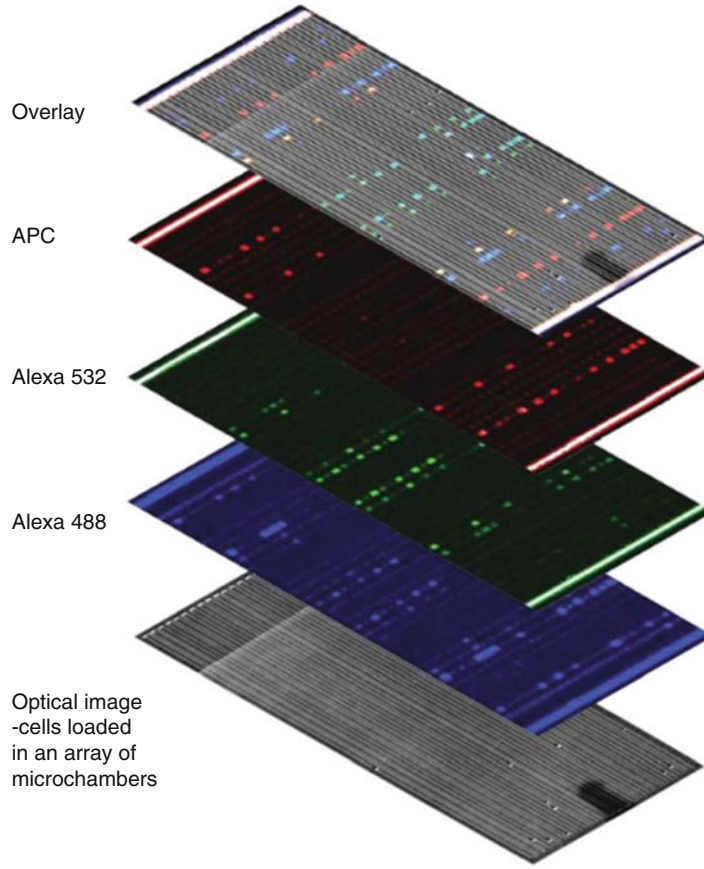

Fig. 3 Representative optical images of microchambers loaded with U937-derived macrophage cells and the corresponding scanned fluorescence images showing protein detection with three colors

6. Make sure these are a different clone to those of the capture antibodies to prevent nonspecific signal.
7. The appropriate quantity is enough to cover ~3/4 of the wafer. When making the flow patterning master mold, use the positive photoresist. When making the Cell Capture mold, use negative photoresist.
8. Make sure there are no bubbles in the photoresist when pouring. If there are bubbles present, the resulting pattern will have defects. Varying the spin speed will change the photoresist thickness for a given size of wafer (i.e., 4″ in our protocol). Check the photoresist manufacturer's technical specifications for spin speed curves (see the link—http://www.microchem.com/PDFs_Dow/SPR%20220%20DATA%20SHEET%20R%26H.pdf).
9. Gradual cool down is required to avoid cracking of the photoresist.

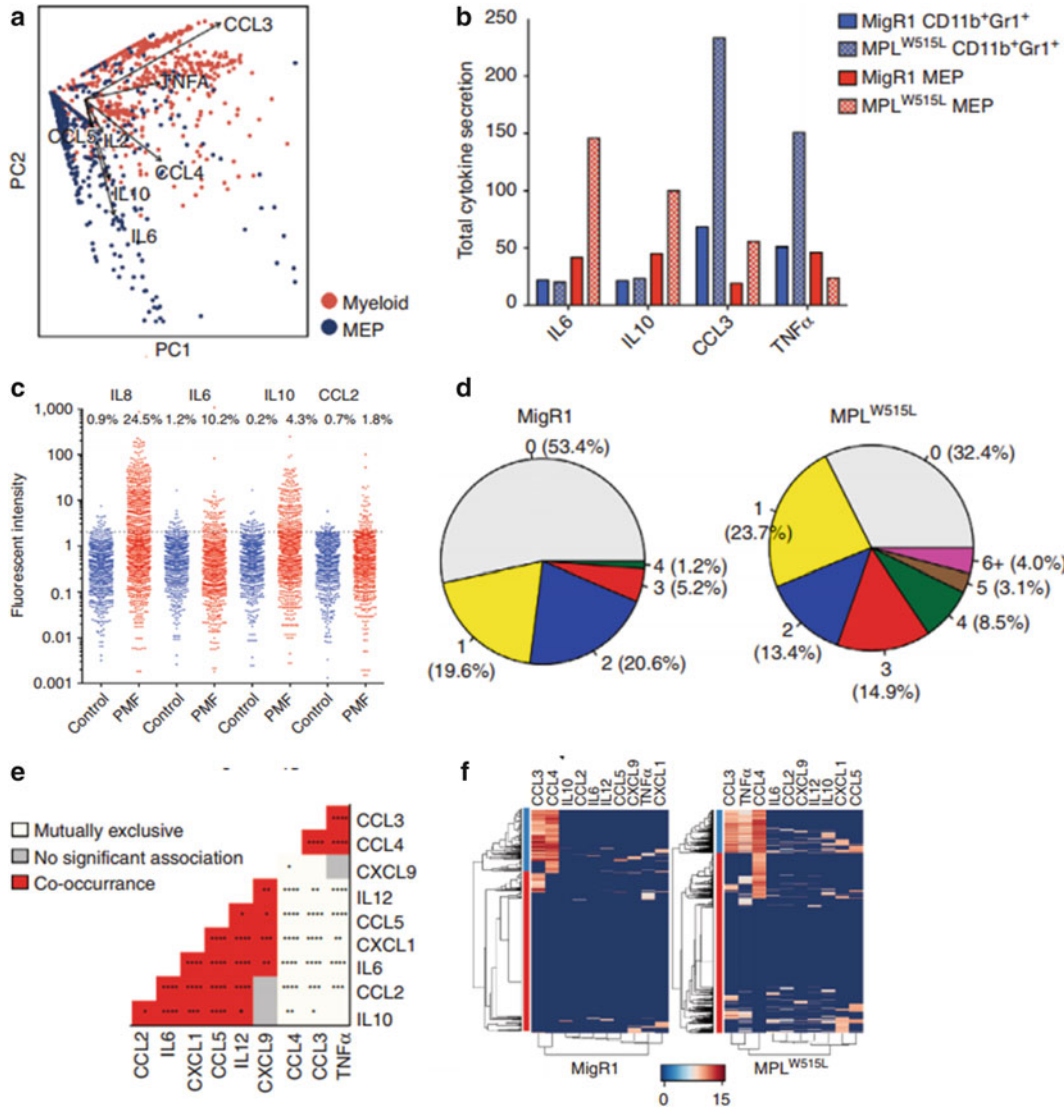

Fig. 4 (**a**–**e**) Analysis performed using data from Multiplexed Single-Cell Secretion Profiling. (**a**) Principle Component Analysis (**b**) Secretion Levels (**c**) Normalized fluorescent intensities for different cytokines (**d**) Polyfunctionality (**e**) Mutual exclusivity analysis (**f**) Hierarchical clustering of single cells

10. The air can be filtered using a one-way filter on the outlet to ensure it is free of dust and residue.
11. Dust crossing the channels can cause them to be connected after the soft lithography process. This renders the channel unusable.
12. Make the vessel as close to the wafer size as possible with a height of ~1 cm.

13. Stir the mixture vigorously for 30 s. Make sure the components A and B are thoroughly mixed so that they will crosslink and cure evenly.
14. Ensure that all the air bubbles are removed from the PDMS.
15. When baking, make sure the surface is level to ensure a uniform chip thickness.
16. Take care to not flex the silicon master mold. If flexed, the silicon master mold can break easily.
17. There are 20 inlet and 20 outlet templates in the pattern. These are the areas where the holes should be created.
18. Complete removal of the liquid is necessary for making a complete bond between the PDMS and the glass slide in the following steps.
19. Scotch brand magic tape is the only tape that does not leave residue.
20. Use a tweezer to apply force onto the tape and PDMS. Make sure the tape is sticking to the PDMS.
21. Again, make sure this step is dust free. If possible, perform this step in a clean room/hood.
22. Mark down the channels with defects to not be used during flow patterning.
23. Make sure you have markers for the area that is flow patterned. You will want to know where your barcode is when performing the assay.
24. When drawing up liquid, make sure there are no air bubbles. Air bubbles will slow down the flow patterning speed.
25. Insert pin 2–3 mm deep, if the pin is too deep, there is risk of delamination when the air is turned on.
26. If the pressure is released too fast, there is risk of delaminating the PDMS bond to the glass slide.
27. Take care in removing the pins as to not delaminate the chip.
28. Carefully remove the chip without smearing antibodies on the glass slide.
29. Make two 50 mL tubes of PBS and two tubes of DI water. Dip the slides in the tubes and shake gently for 10 s.
30. Chip can be stored for 2 weeks.
31. Make sure the air in the cell capture chambers are removed completely.
32. Apply a layer of BSA to the cell capture chip while you are preparing the next steps to keep the surface of the chip from drying out.

33. Dab the corner of the chip with a kimwipe to remove excess BSA before adding the Media. Do not touch the chambers with the kimwipe.
34. Examine the chip with a microscope to make sure cells have fallen into the cell chambers.
35. Make sure the barcode chip is even along the cell capture chip and not askew.
36. Carefully remove the glass slide as to not smear the antibodies on the barcode chip.
37. The chip can be stored in a desiccator away from light.

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Erratum to: An Integrative Analysis of microRNA and mRNA Profiling in CML Stem Cells

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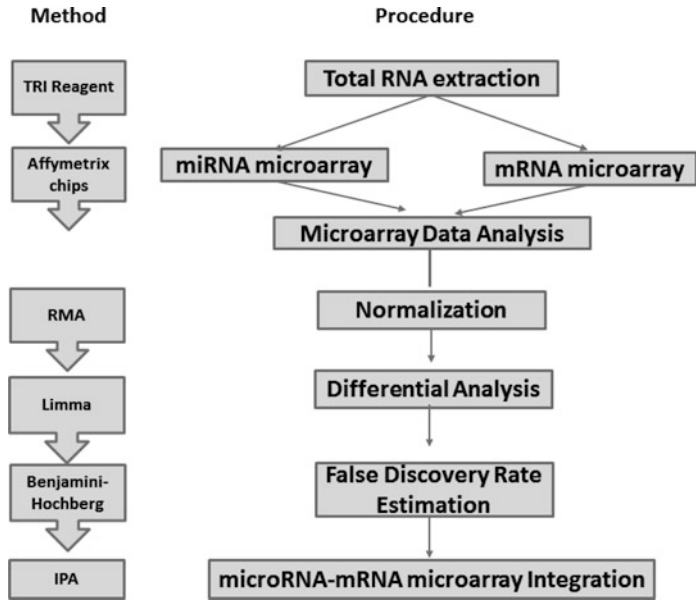
The old figure was inadvertently placed in the final files and so the below updated figure must replace the existing figure in chapter 18.



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