Chapter 6

Quantitation of Chromosome Damage by Imaging Flow Cytometry

Lindsay A. Beaton-Green and Ruth C. Wilkins

Abstract

Biodosimetry is a method for measuring the dose of radiation to individuals using biological markers such as chromosome damage. Following mass casualty events, it is important to provide this information rapidly in order to assist with the medical management of potentially exposed casualties. Currently, the gold standard for biodosimetry is the dicentric chromosome assay, which accurately estimates the dose from the number of dicentric chromosomes in lymphocytes. To increase throughput of analysis following a large-scale mass casualty event, this assay has been adapted for use on the imaging flow cytometer. This chapter describes the methods for the identification and quantification of mono- and multicentric chromosomes using the imaging flow cytometer.

Key words Dicentric chromosome assay, Imaging flow cytometry, Lymphocytes, Chromosome damage, Biodosimetry

1 Introduction

The dicentric chromosome assay (DCA) converts the frequency of dicentric chromosomes scored in mitotic lymphocytes into a dose estimate of ionizing radiation exposure. The DCA is considered to be the gold standard for radiation biodosimetry [1–3], and can be useful in the case of an emergency involving radiological or nuclear material where physical dosimetry is absent. While the assay is sensitive, the time required to prepare slides for microscopy and to score them manually is a limiting factor particularly for high-throughput triage following a large-scale mass casualty event [4, 5].

Recent efforts to increase the sample throughput of the DCA for emergency triage have included the development of the Quickscan slide scoring protocol [6] and improvements in the automated analysis of slides using metaphase finders which both locate the metaphase spreads and identify dicentric chromosomes [7, 8]. Despite these efforts, sample throughput is still limited by

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the time required for the preparation, scanning and scoring of microscope slides.

Previous efforts to develop the DCA method for analysis by flow cytometry have resulted in well-established sample preparation methods in which chromosomes and centromeres were successfully fluorescently labelled [9–12]. However with the sensitivity limitations of conventional flow cytometry, it remains difficult to reliably detect the difference between mono- and dicentric chromosomes or to distinguish dicentric chromosomes from chromosome aggregates or debris [9, 10]. Recently, with the development of new imaging flow cytometry technology, imaging capabilities of microscopy can be combined with the increased throughput of conventional flow cytometers. This has led to the adaptation of microscope-based DCA analysis to an automated cytometry method (flow-DCA) which can now identify and quantify damaged chromosomes [13].

Chapter 6 describes the methods to quantitate chromosome damage in four major sections: sample preparation and culture, chromosome isolation, hybridization and staining, and data acquisition and analysis.

2 Materials

2.1 Lab Equipment

1. Lithium-heparin Vacutainer® tubes (10 mL).
2. Histopaque®-1077 (Sigma-Aldrich Inc.).
3. AccuSpin™ tubes (Sigma-Aldrich Inc.).
4. Centrifugation tubes (15 mL).
5. Ventilated culture flasks (25 cm^2).
6. Safe-Lock tubes (1.5 mL).
7. Filters (0.2 μm).
8. Imagestream^X (EMD-Millipore) with 488 nm laser, 405 nm laser (*see* **Note 1**), 60× magnification and extended depth of field (EDF) option.

2.2 Reagents

1. Complete media: RPMI 1640 with 1 % 2 mM l-Glutamine–Penicillin–Streptomycin solution, 15 % v/v inactivated fetal bovine serum (FBS), 2 % phytohemagglutinin (PHA), and 1 % colcemid (10 μg/mL) (*see* **Note 2**).
2. Isotonic phosphate buffered saline (PBS) (sterile): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 (*see* **Note 3**).
3. Hypotonic solution: 55 mM KCl and 20 mM HEPES (*see* **Note 4**).
4. Chromosome isolation buffer: 10 % v/v each of 20 mM ethylenediaminetetraacetic acid (EDTA) in double-distilled water

(ddH_2O) (*see* **Note 5**), 5 mM ethylene glycol tetraacetic acid (EGTA) in ddH_2O (*see* **Note 6**) and 150 mM Tris–HCl, 800 mM KCl, and 200 mM NaCl in ddH_2O (*see* **Note 7**). 0.1 % v/v each 2-mercaptoethanol and Triton X-100. 0.05 % each of 0.4 M spermine in ddH_2O (*see* **Note 8**) and 1.0 M spermidine in ddH_2O (*see* **Notes 8** and **9**).

5. Hoechst 33258 stock solution: 100 μg/mL in ddH_2O (*see* **Note 10**).
6. PNA probe (F3006, PNA Bio, Thousand Oaks, CA): 5 μM stock in ddH_2O (*see* **Note 11**).
7. $MgCl_2$ buffer (20×): 82 mM Na_2HPO_4, 9 mM citric acid and 20 mM $MgCl_2$ in ddH_2O (*see* **Note 12**).
8. Blocking reagent stock solution (Perkin Elmer, 10×): 2.5 % w/v in ddH_2O (*see* **Note 13**).
9. Hybridization Buffer: 70 % formamide, 10 % (v/v) blocking reagent stock solution (0.25 % w/v. final concentration), 5 % (v/v) $MgCl_2$ buffer, 0.2–0.5 μg/mL fluorescently labeled PNA probe in ddH_2O (*see* **Note 14**).
10. Wash solution (for hybridization): 70 % formamide, 0.1 % (w/v) bovine serum albumin (BSA), and 5 % (v/v) $MgCl_2$ buffer in ddH_2O.

3 Methods

- Unless otherwise noted, all steps are conducted at room temperature (RT) which is assumed to range from 18 to 25 °C.
- Steps are conducted under normal lighting conditions unless otherwise noted.

3.1 Sample Preparation and Culture

1. Draw blood into lithium-heparin Vacutainers®.
2. Irradiate and/or challenge samples as required.
3. To separate lymphocytes bring Histopaque®-1077 to RT and pipet 3.0 mL Histopaque®-1077 into each upper chamber of the AccuSpin™ tubes.
4. Centrifuge at 800×*g* for 30 s to place the Histopaque®-1077 below the frit.
5. Pipet 6.0 mL anticoagulated whole blood into each upper chamber of the AccuSpin™ tubes.
6. Centrifuge at 800×*g* for 15 min at RT (*see* **Note 15**).
7. Carefully aspirate the plasma layer with a Pasteur pipette and discard.
8. Carefully transfer the mononuclear band with a Pasteur pipet to a 15 mL centrifuge tube (*see* **Note 16**).

9. Add 10 mL of isotonic PBS to the mononuclear cells and mix gently.
10. Centrifuge at 250 × *g* at RT for 10 min.
11. Aspirate the PBS and resuspend the pellet (*see* **Note 17**).
12. Repeat **steps 9–11** twice, each time resuspending pellet in 5 mL isotonic PBS.
13. Resuspend the pellet in complete media.
14. Divide cells in complete media, containing colcemid and PHA at an initial concentration of 1×10^6 cells/mL (*see* **Note 18**).
15. Incubate the cultures for 48 h at 37 °C and 5 % CO_2.

3.2 Chromosome Isolation

1. After incubation, transfer culture to 15 mL centrifuge tubes.
2. Spin down cultures at 350 × *g* for 5 min at RT.
3. Aspirate media from the tube, removing as much media as possible.
4. Disrupt the cell pellet by flicking the tube.
5. Resuspend cell pellet by slowly adding 10 mL hypotonic solution (*see* **Note 19**).
6. Incubate at RT for 20 min (*see* **Note 20**).
7. Spin down cells at 350 × *g* for 5 min at RT.
8. Gently aspirate the hypotonic solution from the tubes (*see* **Note 21**).
9. Disrupt the cell pellet by flicking the tube and resuspend the cell pellet by slowly adding 3 mL ice-cold chromosome isolation buffer.
10. Incubate on ice for 15 min.
11. Vortex vigorously for 75 s at the maximum vortex setting to break apart the cells and free the chromosomes into suspension (*see* **Note 22**).
12. Allow chromosome suspension to settle overnight at 4 °C (*see* **Note 23**).

3.3 Hybridization and Staining of Chromosomes in Suspension

1. Set water bath to 80 °C.
2. After chromosomes have been left to settle overnight, carefully remove excess buffer to concentrate the chromosomes (*see* **Note 24**).
3. Add 100 μL of pre-warmed hybridization buffer to the chromosomes, pipette to mix.
4. Denature the chromosome suspension by immersing in a water bath at 80 °C for 5 min (*see* **Note 25**).
5. Hybridize, in the dark, at RT for 60 min.
6. After hybridization, add 500 μL of pre-warmed wash solution to the chromosome suspensions.

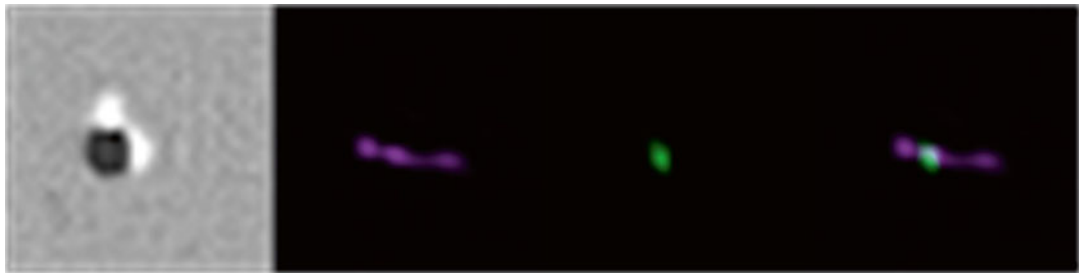

Fig. 1 Example of an object (#1605) in each of the collected channels (channel 5 bright field, channel 1 Hoechst 33258 and channel 2 PNA probe) as well as a composite view of channels 1 and 2 overlayed

7. Incubate for 5 min at 37 °C.
8. Spin down the chromosomes at 1675 × *g* for 10 min at 4 °C.
9. Carefully remove the supernatant, leaving approximately 150 μL (*see* **Note 26**).
10. Add 2 μg/mL of Hoechst 33258 to each sample and let stand for 1 h at 4 °C (*see* **Note 27**).

3.4 Data Acquisition and Analysis

1. Transfer 75 μL of well-mixed sample to a 1.5 mL Safe-Lock tube, and load onto the Imagestream^X.
2. Set the Imagestream^X to 60× magnification and extended depth of field.
3. Set the laser power and cell classifiers to optimize collection of chromosomes while discarding intact cells and unstained debris (*see* **Note 28**).
4. Acquire samples (*see* **Note 29**).
5. Open the Imagestream analysis software package IDEAS.
6. Follow **steps 7–12** to identify the chromosome population; iterate as required to fine-tune the gating strategy.
7. Create a composite view by overlaying channel 1 (Hoechst 33258) and channel 2 (PNA probe) to allow for visual inspection of objects as in Fig. 1.
8. Create a plot of *Intensity* of channel 1 (Hoechst 33258) versus *Intensity* of channel 2 *(PNA)*, where the *Intensity* feature is the sum of the background subtracted pixel values within the masked area of the image. In this way you can select for two-colour positive populations (*see* Fig. 2). Note that cells can also be identified and rejected (identified as "cells" in Fig. 2).
9. Visually investigate identified populations to ensure that gates are including objects of interest without including too many unwanted objects (*see* Fig. 3).
10. Based on the two-color positive population, create a new scatterplot of the *Contrast* of channel 5 (bright field) versus the *Contrast* of channel 1 (Hoechst 33258), where *Contrast*

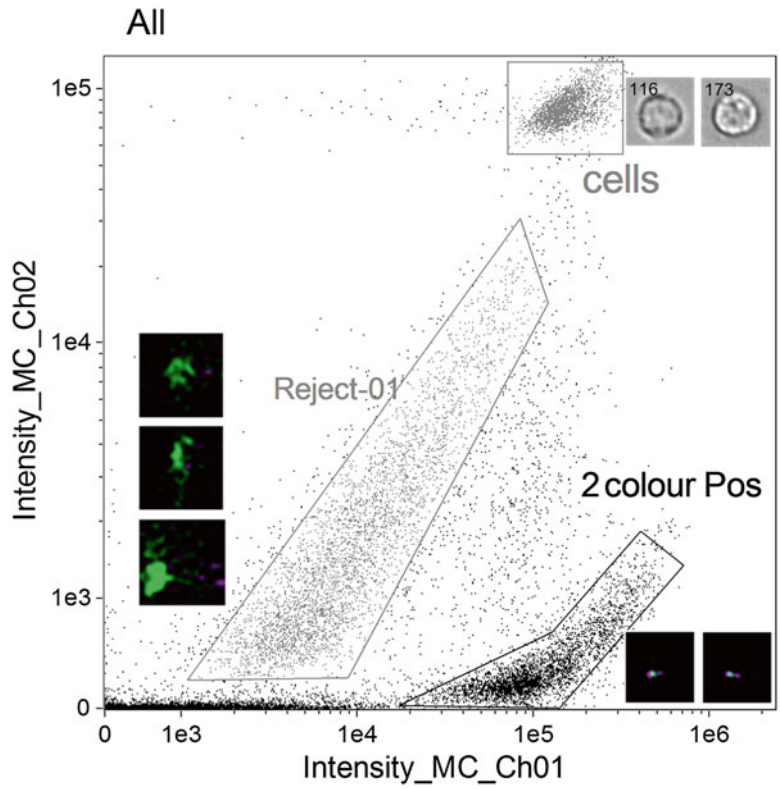

Fig. 2 Scatterplot of *Intensity* of channel 1 (Hoechst 33258) versus *Intensity* of channel 2 (PNA probe) to identify two-colour positive population "2colourPos". Noted on the plot is a population of "cells" which appear to be intact. Also shown is a population identified as "Reject-01" with some example objects to the *left*

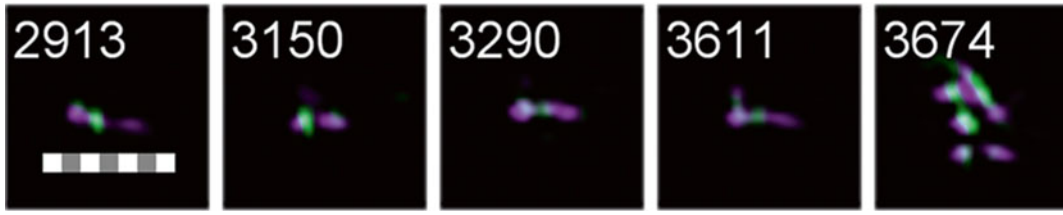

Fig. 3 Selection of objects from the "2colourPos" population with a scale bar of 7 μm for reference. Note that some of the objects appear to be aggregates of multiple chromosomes (object #3674)

measures the sharpness quality of an image, to select a sub-selection of objects with good contrast (*see* Fig. 4, **Note 30**).

11. Again, visually inspect the populations to verify the stringency of the gating strategy. Figure 4 shows a selection of objects from each gate (Reject-02 and goodContrast) and illustrates the difference in image quality.

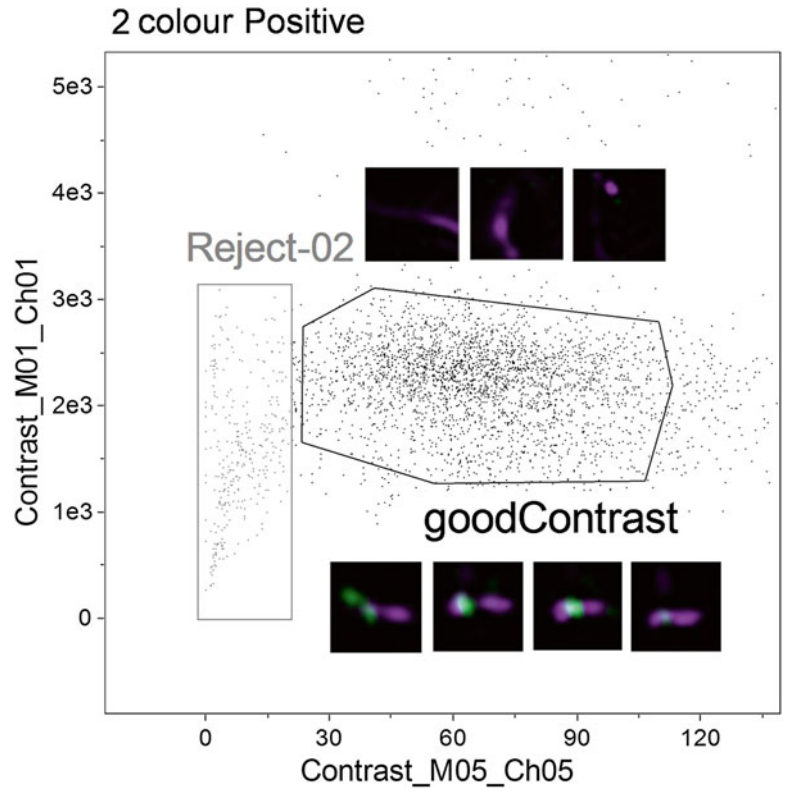

Fig. 4 Based on the two-colour positive chromosome population identified in Fig. 2, plot *Contrast* of channel 5 (bright field) versus the *Contrast* of channel 1 (Hoechst 33258) to identify objects with good contrast. Examples of objects in each of the "good Contrast" and "Reject-02" region are shown alongside the gates

12. From the goodContrast population, create a scatterplot of the *Area* of channel 2 (PNA probe), where *Area* is equal to the number of microns squared in a mask, versus the *Intensity* of channel 2 to remove objects that have too much PNA probe (*see* Fig. 5, **Note 31**).
13. From the centArea population created in **step 12**, create a scatterplot of the *Area* of channel 5 (bright field) versus the *Aspect Ratio Intensity*, defined to be the ratio between the intensity weighted minor axis of the image divided by the intensity weighted major axis of the image, of channel 6 (side scatter), to remove objects that appear to be doublets (Reject-04), leaving a population of apparently single chromosomes (Singles) (*see* Fig. 6).
14. To quantify the chromosome damage, follow **steps 15–17**; iterate as required to fine tune the masking strategy.
15. Create a spot mask based on the centromere signal as shown in Fig. 7 (*see* **Note 32**).

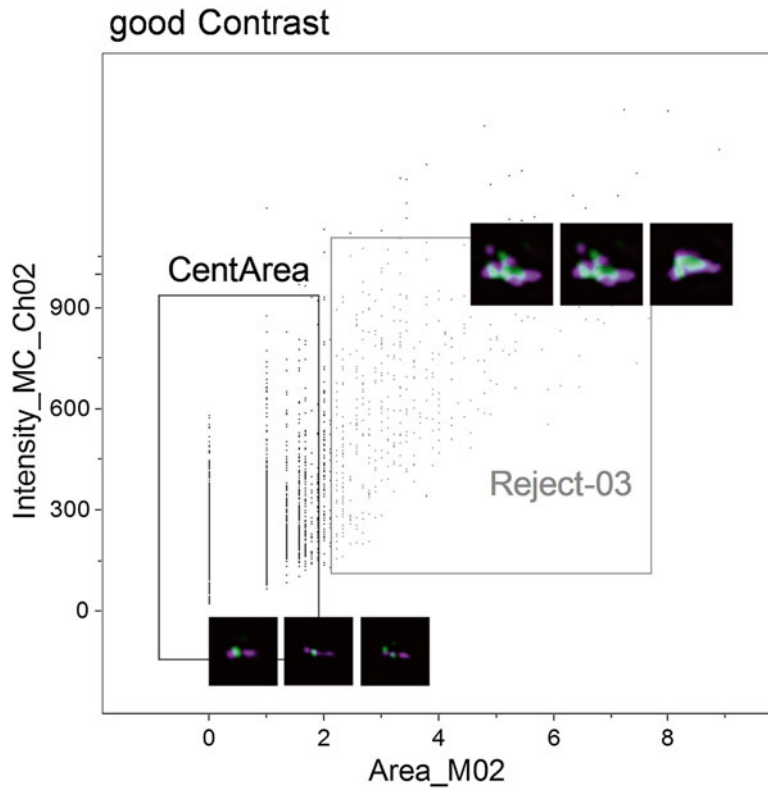

Fig. 5 Scatterplot of the *Area* of channel 2 (PNA probe) versus the *Intensity* of channel 2

16. Plot a histogram of spot counts based on the mask created in **step 15** (*see* Fig. 8).
17. Create gates on the histogram bins for chromosomes with each number of centromeres (spots). Check each of these gates to see if the included objects are good representations of chromosomes with the specified number of centromeres (*see* **Note 33**).

4 Notes

1. If a 405 nm laser is not available, the method can be adapted for use with a different laser and an alternative DNA stain, i.e., propidium iodide on the 658 nm laser.
2. Make fresh on the first day of the experiment. For a 70 mL final volume, start with 56.8 mL RPMI 1640 media and add 0.7 mL l-Glutamine-Penicillin-Streptomycin, 10.5 mL heat-inactivated FBS, 1.26 mL PHA, and 0.7 mL colcemid. Let warm in an incubator until needed.

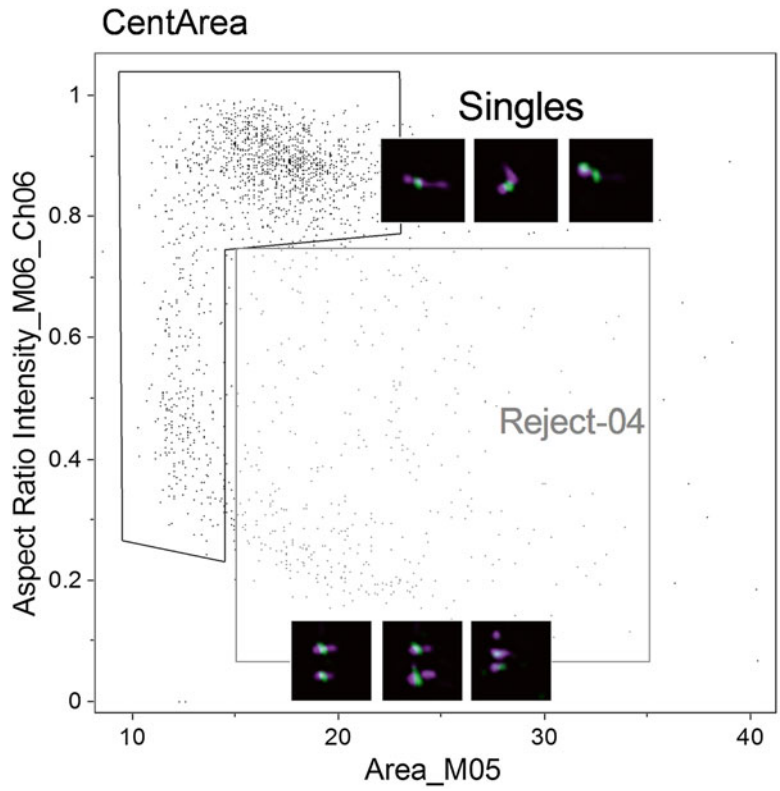

Fig. 6 Scatter plot of the *Area* of channel 5 (bright field) versus the *Aspect Ratio Intensity* of channel 6 (side scatter). Two populations are shown, one of objects considered to be single chromosomes "singles," and another of objects considered to have multiple chromosomes or aggregates "Reject-04." Examples of objects in each population are shown adjacent to the respective gates

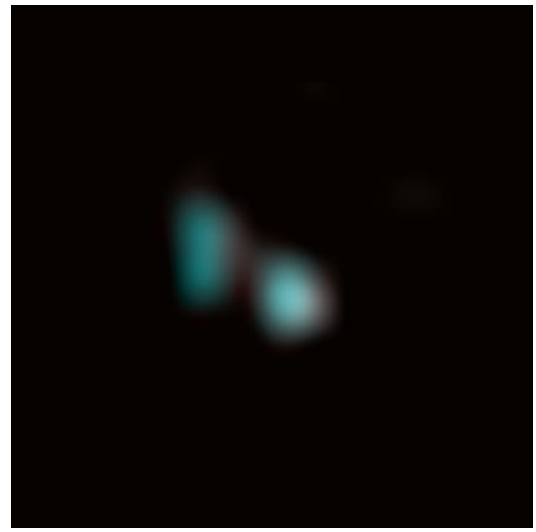

Fig. 7 Masking of PNA probe signal (*see* **Note 32**)

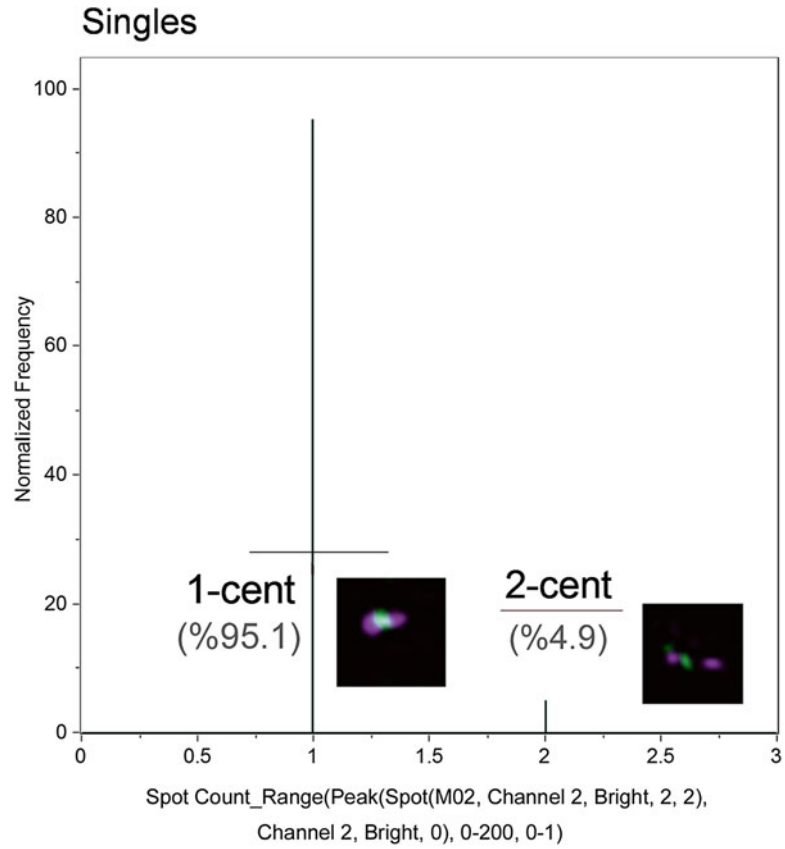

Fig. 8 Histogram of *Spot counts*. These gates represent monocentric (1-cent) and dicentric (2-centric) chromosomes

3. pH 7.4 in ddH_2O and filter sterile. Can be stored at RT for up to a year.
4. pH 7.4 in ddH_2O and filter (0.2 μm) and store at RT for up to several months.
5. Store at RT for up to several months.
6. Dissolve by adding 18 M NaOH dropwise. Store at RT for up to several months.
7. Store at RT for up to several months.
8. Filter (0.2 μm), aliquot, and store at −20 °C for up to several years.
9. Make fresh for each experiment. In a beaker, start with approximately 60 % of final volume ddH_2O; add 10 % each of 20 mM EDTA in ddH_2O, 5 mM EGTA in ddH_2O and 150 mM Tris–HCl, 800 mM KCl, and 200 mM NaCl in ddH_2O. Adjust the pH to 7.2 using 0.5 M NaOH. Add 0.1 % v/v each of 2-mercaptoethanol and Triton X-100. Cover and stir for 15 min.

Transfer to a volumetric flask and top up with ddH_2O. Filter sterile (0.2 μm). Add 0.05 % each of chromosome isolation buffer components 0.4 M spermine in ddH_2O and 1.0 M spermidine in ddH_2O. Keep on ice until required.

10. Aliquot and store at −20 °C. Protect from light.
11. Dissolve 5 nM in 1 mL ddH_2O to make 5 μM stock, aliquot and store frozen. Heat to 70 °C for 10 min before each use. Protect from light.
12. Aliquot and store at −20 °C for up to a year. If the buffer precipitates, warm at 37 °C to resuspend. Working solution can be kept at 4 °C for up to a month.
13. Dissolve by adding 18 M NaOH dropwise. Filter (0.2 μM), aliquot and store at −20 °C for up to a year. Working solution can be kept at 4 °C for up to a month.
14. For each condition, prepare 100 μl of hybridization mixture.
15. Centrifuge brakes can be used but if lymphocyte recovery is low, remove the brakes.
16. If lymphocyte recovery is low, harvest the plasma layer and repeat **steps 6–8** in Subheading 3.1.
17. To ensure a proper wash, aspirate as close to the pellet as possible.
18. A total volume of 10 mL of culture media is used in each vented 25 cm^2 culture flask. Note that including PHA stimulates the lymphocytes to enter metaphase, while the colcemid should prevent the cells from entering second metaphase. This is important as only the cells in first metaphase should be included. The addition of colcemid for 48 h results in smaller, tighter chromosomes, which works well for detection on the Imagestream^X. Time of incubation with colcemid can be varied (3–48 h), requiring only a few hours for some cell cultures.
19. The hypotonic solution will cause the cells to swell and become more fragile; therefore it is important to handle the tubes gently.
20. The incubation time for the hypotonic treatment varies for different cell types. Approximately 20 min works well, or a range between 17 and 22 min is also acceptable. Some testing might be required if using different cell types. *See* ref. [11] for details of other cell types.
21. Handle the tubes gently while aspirating as the cell pellet is more loose after hypotonic treatment.
22. Vortex time varies per cell type and vortex force. Test new cell types to determine vortex settings. Use caution: to prevent

chromosomes from uncoiling and clumping, do not vortex longer than required.

23. If need be, the chromosome suspensions can be stained on the same day (after 3–4 h at 4 °C) but for best results, let the chromosome suspensions settle and rest. Chromosome suspensions can be stored at 4 °C for up to a few weeks.
24. Typically approximately 0.5 mL of chromosomes are left in suspension; this step could result in chromosome loss if they have not settled well, and if the tubes are not handled carefully. Larger volumes can be pipetted with the use of a 1 mL pipette however for smaller volumes, a 200 μL pipette should be used. Unlike the method of Brind'Amour [11], RNase pretreatment was not used.
25. Denaturing requires very specific temperatures and timing—it is important to be as accurate as possible here.
26. Chromosomes do not pellet well in formamide and this step can lead to loss of chromosomes when removing the supernatant. As recommended by Brind'Amour [11], use a 1000 μL pipette to remove most of the volume, and a 200 μL pipette for finer control. Washing was performed only once, but **steps 6–9** in Subheading 3.3 can be repeated if problems are encountered with staining.
27. Stained chromosomes can be left at 4 °C for up to a week. A 405 nm laser is required to excite the Hoechst 33258; alternatively, propidium iodide can be used to stain the DNA and excited with a 658 nm laser.
28. The following acquisition settings were used in our laboratory: bright-field LED, a 488 nm laser at 80 mW and an unfiltered 405 nm laser at 2.5 mW. If, as is the case with most Imagestream^X, the 405 laser is filtered, the power will need to be increased. Images were collected from channel 1 (430–505 nm, DNA signal, Hoechst 33258), channel 2 (505–560 nm, centromere signal, PNA probe), channel 5 (660–745 nm, bright-field signal, LED) and channel 6 (side scatter). Note that bright field can be switched to a different unused channel if channel 5 is required for detection of another fluorophore. Cell classifiers were set to limit acquisition to images with a minimum of integrated intensity (less background) in channel 1 of 25 (arbitrary units [au]) and in channel 2 of 10 au, as well as a maximum area in channel 5 of 35 μm^2. Note that these settings will vary by machine, staining intensity and cell type, and will need to be optimized for different experiments.
29. Number of objects to acquire will vary on sample preparation and on how stringently the cell classifiers are set. It is recommended that smaller data files are collected at first to fine tune

which classifiers work best. Once the acquisition settings are optimized, then more objects of interest (and less debris) can be collected for good statistics without resulting in overly large data files.

30. Note that to optimize the potential of the IDEAS software, it is important to make use of the "Find the Best Feature" approach available to the user. Using the population tagger, the user can define populations of objects of interest (i.e., good chromosomes) as well as populations that the user would like to gate out (i.e., too much background, objects too large). Using the statistics functions, the user can compare the two populations and use the RD-mean statistic to find the features that best differentiate the two populations. As the user builds and applies more masks and features, there are more options to better separate the desired and undesired populations. These features, that best differentiate the populations, can be plotted on a scatterplot to create subsequent gated regions. Iterating through this approach leads to a better final population of desired objects, in this case, a chromosome population. Note that it is also very useful to show the tagged populations on the scatterplots to help create different regions.
31. If the area of the centromere signal is too large, it is difficult to distinguish if this is multiple centromeres.
32. This mask can be fine-tuned: the final mask used for this experiment was *Range*(*Peak*(*Spot*(M02, Channel 2, Bright, 2, 2), Channel 2, Bright, 0), 0–200, 0–1). The *Range* mask provides the ability to mask components in an image within a set area (i.e., 0–200) and set aspect ratio (i.e., 0–1). The *Peak* mask identifies intensity areas from an image with local maxima (i.e., bright peak) or local minima. Finally, the *Spot* mask (on the Bright option) obtains bright regions from the image by eroding the image leaving only bright areas. The spot to cell background ratio and radius thresholds are determined by the user (i.e., 2 and 2).
33. If the gates include too many chromosomes with the wrong number of centromeres, the masking may need to be adjusted. Repeat **steps 15–17** in Subheading 3.4 as needed to optimize the masking strategy. It is not unusual to have some false positive objects; to some extent, this can be taken into account in a calibration curve.
34. The sample preparation method described in the chapter uses a polyamine method to release the chromosomes into suspension. The KCl method has also been successful [13] but it was found that the polyamine method results in chromosomes with less aggregation and more stability than with the KCl method. The imaging flow cytometry method (data acquisition

and analysis) described in this chapter can also be used with chromosomes isolated using the KCl sample preparation method.

35. Samples were analyzed uncompensated after testing whether compensation improved the results. As little spectral overlap was observed, compensation was deemed unnecessary. This should be tested based on the conditions used and proper single-color controls to generate a compensation matrix should be collected.

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