Chapter 9

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

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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

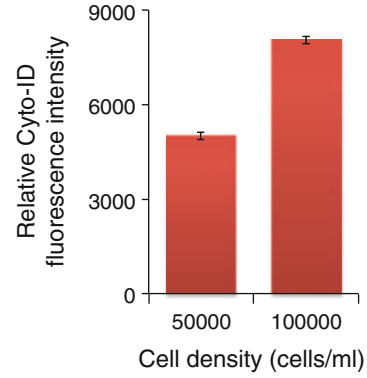

Fig. 1 The relative Cyto-ID levels in K562 cells at different cell densities. K562 cells at the cell densities as indicated were stained with Cyto-ID. The relative Cyto-ID fluorescence intensities were determined using the approach described in Subheading 3.1. Error bars represent standard deviations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

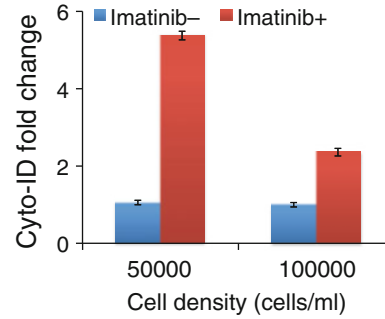

Fig. 2 Imatinib-induced autophagy in K562 cells at different cell densities. K562 cells seeded at the cell densities as indicated were treated with either vehicle (imatinib−) or 2 μM imatinib (imatinib+) for 4 h. The Cyto-ID fold changes were determined using the approach described in Subheading 3.1.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

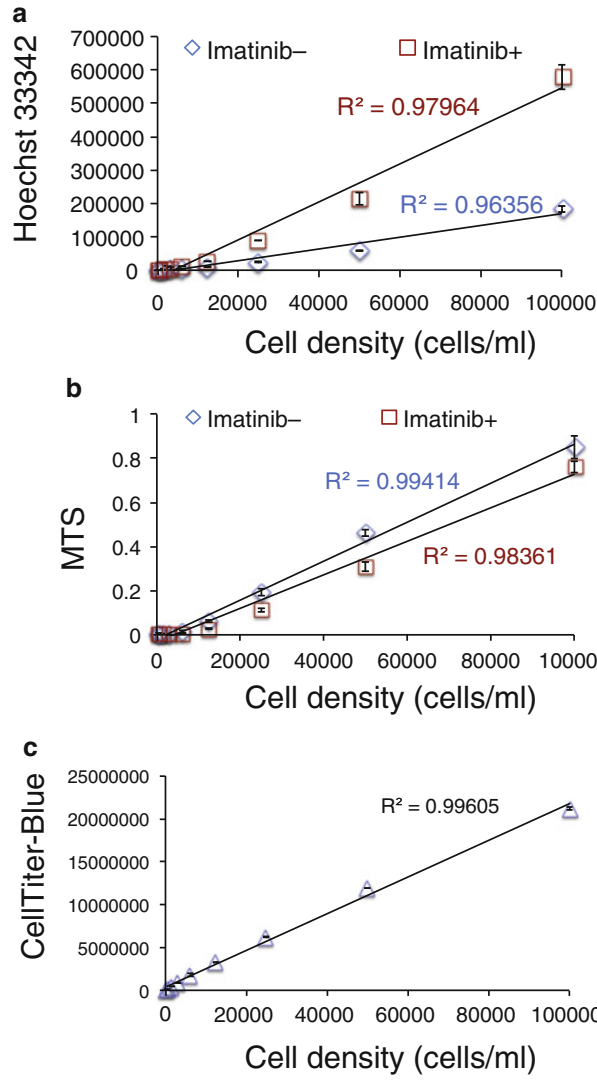

Fig. 3 Cell number determination using Hoechst 33342 staining, MTS viability assay, or CellTiter-Blue viability assay. K562 cells were plated at different cell densities as indicated. Cells were treated with either vehicle (imatinib−) or 2 μM imatinib (imatinib+) for 4 h. The cell number was determined 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.

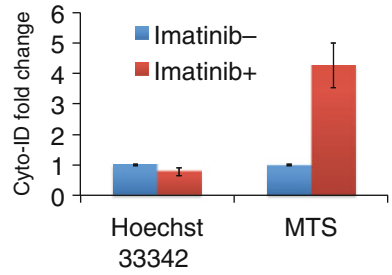

Fig. 4 Determination of imatinib-induced autophagy. K562 cells were plated at a cell density of 5×10^4 cells/ml. Cells were treated with either vehicle (imatinib−) or 2 μM imatinib (imatinib+) for 4 h. The cell number was determined by the Hoechst 33342 staining or MTS assay, respectively. The Cyto-ID 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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