Chapter 1

Detection of Apoptotic Versus Autophagic Cell Death by Flow Cytometry

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Abstract

Different modes of regulated cell death (RCD) can be initiated by distinct molecular machineries and their morphological manifestations can be difficult to discriminate. Moreover, cells responding to stress often activate an adaptive response centered around autophagy, and whether such a response is cytoprotective or cytotoxic cannot be predicted based on morphological parameters only. Molecular definitions are therefore important to understand various RCD subroutines from a mechanistic perspective. In vitro, various forms of RCD including apoptosis and autophagic cell death can be easily discriminated from each other with assays that involve chemical or pharmacological interventions targeting key components of either pathway. Here, we detail a straightforward method to discriminate apoptosis from autophagic cell death by flow cytometry, based on the broad-spectrum caspase inhibitor Z-VAD-fmk and the genetic inhibition of ATG5.

Key words Autophagy, Immunogenic cell death, Mitochondrial outer membrane permeabilization, Necrosis, Mitochondrial permeability transition, Necroptosis

1 Introduction

Cell death can be accidental, meaning that its course cannot be altered, or regulated, which means that it can be inhibited or at least retarded by specific pharmacological or genetic interventions [1–3]. Thus, at odds with its accidental counterpart, regulated cell death (RCD) is precipitated by the activation of a genetically encoded molecular machinery, which generally occurs once adaptive responses to stress fail [4–6]. As a notable exception to this tendency, programmed cell death (PCD) constitutes a peculiar case of RCD that is activated in a completely physiological manner, in the context of post-embryonic development or adult tissue homeostasis [1, 7].

During the past three decades, several systems for the classification of RCD have been proposed, based on morphological, biochemical, or functional features [8–14]. It soon became clear

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that defining RCD instances based on their morphology is rather inappropriate, because it suffers from a considerable degree of operator-dependency, and it does not convey any mechanistic information [15, 16]. Nowadays, a molecular classification of RCD, based on objectively quantifiable biochemical parameters, is favored [17]. Thus, apoptosis is currently defined as caspase-3-dependent variant of RCD, while autophagic cell death is defined as a form of RCD that mechanistically impinges on the molecular machinery for autophagy [17, 18]. However, detecting caspase-3 activation or any biochemical manifestations of autophagy in the course of RCD is not sufficient for defining it as apoptotic or autophagic. Indeed, caspase-3 activation occurs in several apoptosis-unrelated settings [19]. Along similar lines, the adaptive response of eukaryotic cells to stress often (if not always) involves an autophagic component, which generally supports (rather than compromises) the reestablishment of homeostasis and cell survival [20–22].

These observations imply that functional assays are required to properly identify apoptotic and autophagic instances of RCD, as well as other forms or RCD including necroptosis [23, 24]. While implementing such functional assays in vivo may be complicated, they can be carried out in vitro in a relatively straightforward manner, by appropriately combining (1) the detection of reliable indicators of cell death, and (2) the use of pharmacological or genetic interventions that inhibit caspase-3 or essential components of the autophagic machinery.

It is widely accepted that plasma membrane permeabilization (PMP) constitutes the most reliable (if not the only) marker of dead cells, at least in vitro [1]. Indeed, while several other biochemical processes can accompany (and be mechanistically involved in) RCD, most (if not all) of them: (1) are not universally associated with it; and (2) are not always irreversible. For instance, caspase-3 is activated not only during the terminal phases of apoptosis, but also in a reversible manner in the course of erythroid differentiation (to which it provides a critical contribution) [25]. Additional events that generally accompany RCD, such as mitochondrial outer membrane permeabilization (MOMP) and phosphatidylserine (PS) exposure, can be monitored to obtain kinetic insights into the process, but are inappropriate as sole biomarkers of cell death [5, 26]. In vitro, PMP, MOMP, and PS exposure can be conveniently measured by flow cytometry, after co-staining living cells with the exclusion dye propidium iodide (PI) and either the mitochondrial transmembrane potential ($\Delta\psi_m$)-sensitive fluorophore 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$), or a fluorescent variant of the PS-binding protein annexin A5 (ANXA5, best known as AnnV) [27].

Several pharmacological agents have been developed to inhibit components of the apoptotic or autophagic machinery, and are now commercially available. For instance, the non-cleavable

caspase substrate *N*-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethylketone (Z-VAD-fmk) is commonly employed as an inhibitor of apoptosis [28, 29], while the phosphoinositide-3-kinase (PI3K) inhibitor 3-methyladenine (3-MA) can be used to block autophagic responses, which often rely on the PI3K-dependent synthesis of phosphatidylinositol-3-phosphate [30, 31]. Still, many of these compounds have specificity issues, warranting the use of targeted genetic tools, including RNA interference and the gene knockout technology. General recommendations for the implementation of appropriate assays involving RNA interference or knockout cells go beyond the scope of this chapter and can be found in the literature [32–34].

Here, we provide a detailed description of a simple, cytofluorometric assay for the discrimination of apoptotic and autophagic cell death in vitro, based on the simultaneous detection of PMP and MOMP exposure in human cancer cells responding to a lethal stimulus in normal conditions, in the presence of Z-VAD-fmk, or upon the small interfering RNA (siRNA)-mediated downregulation of ATG5. With the appropriate variations, this protocol is suitable for the identification of apoptosis and autophagic cell death in most, if not all, cultured mammalian cells.

2 Materials

2.1 Disposables and Equipment

1. 6- and 12-well plates for cell culture.
2. 75 cm^2 flasks for cell culture.
3. 5 mL, 12×75 mm FACS tubes.
4. 1.5 mL microcentrifuge tubes.
5. 15 and 50 mL conical centrifuge tubes.
6. Cytofluorometer: FACScan or FACSVantage (BD, San Jose, USA) or equivalent, equipped with an argon ion laser emitting at 488 nm and controlled by the operational/analytical software CellQuest™ Pro (BD) or equivalent (*see* **Note 1**).

2.2 Cell Maintenance

1. Complete growth medium for human osteosarcoma U2OS cells: Dulbecco modified Eagle's medium (DMEM) containing 3.0 g/L D-glucose, 1.5 mM L-glutamine, supplemented with 100 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer and 10 % fetal bovine serum (FBS) (*see* **Note 2**).
2. Phosphate buffered saline (PBS, 1×): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 in deionized water (dH_2O), adjust pH to 7.4 with 2 N NaOH.
3. Trypsin–EDTA: 0.25 % trypsin–0.38 g/L (1 mM) EDTA×4 Na^+ in Hank's balanced salt solution (*see* **Note 3**).

2.3 RNA Interference

1. siUNR (sense 5′-GCCGGUAUGCCGGUUAAGUdTdT-3′), 100 μM stock solution in dH_2O, stored at −20 °C (*see* **Notes 4** and **5**).
2. siATG5 (sense 5′-UUUCUUCUUAGGCCAAAGGdTdT-3′), 100 μM, stock solution in dH_2O, stored at −20 °C (*see* **Notes 4** and **6**).
3. Transfection reagent: HiPerFect® or equivalent (*see* **Note 7**).
4. Transfection medium: Opti-MEM® with Glutamax™ and phenol red (*see* **Note 8**).

2.4 Pharmacological Treatments and DiOC6(3)/PI Co-staining

1. *N*-benzyloxycarbonyl-Val-Ala-Asp (O-Me) fluoromethylketone (Z-VAD-fmk): 20 mM stock solution in dimethylsulfoxide (DMSO), stored at −20 °C (*see* **Note 9**).
2. Staurosporine (STS): 2 mM stock solution in DMSO, stored at −20 °C (*see* **Note 10**).
3. 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$): 40 μM stock solution in 100 % ethanol,, stored at −20 °C under protection from light (*see* **Notes 11** and **12**).
4. Propidium iodide (PI): 1 mg/mL stock solution in dH_2O, stored at 4 °C under protection from light (*see* **Notes 13** and **14**).

3 Methods

3.1 Cell Maintenance

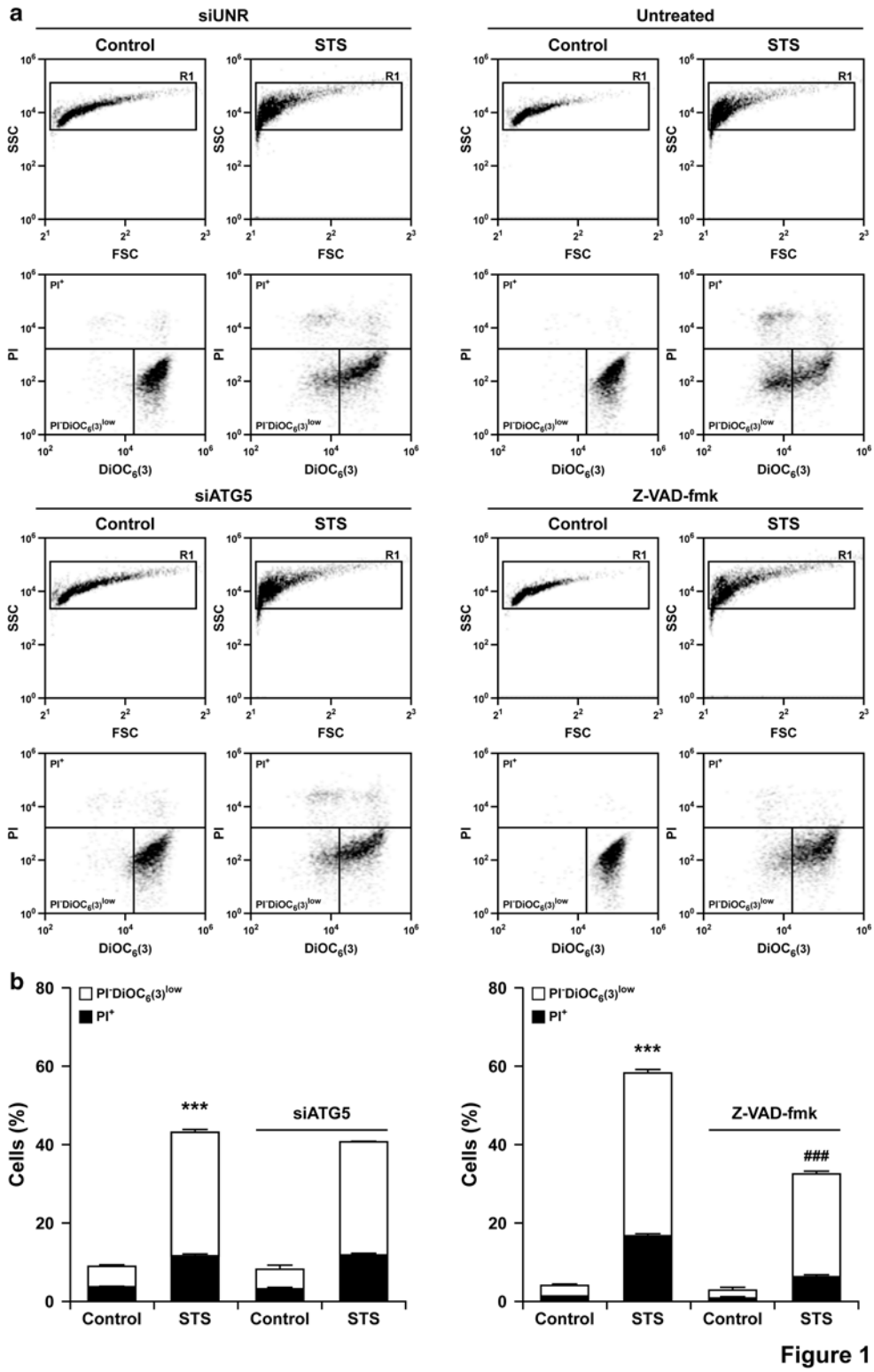
1. U2OS cells are routinely maintained in complete growth medium within 75 cm^2 flasks, in standard culture conditions (37 °C, 5 % CO_2) (*see* **Note 15**).
2. When the culture reach 70–80 % confluence (*see* **Note 16**), discard the supernatant by aspiration, wash gently adherent cells with pre-warmed PBS (*see* **Note 17**), and incubate them with ~3 mL 0.25 % (w/v) Trypsin–EDTA solution for 1–3 min at 37 °C (*see* **Notes 19**).
3. As soon as cells are detached (*see* **Note 20**), add complete growth medium to the cell suspension (*see* **Note 21**).
4. Maintenance cell cultures can be generated by transferring aliquots of the cell suspension to new 75 cm^2 flasks, and propagated as described in **steps 1–3** in Subheading 3.1 (*see* **Notes 15–23**).
5. For RNA interference, seed 2.0×10^5 U2OS cells in 6-well plates, in 2 mL growth medium per well (*see* **Note 24**), and proceed to Subheading 3.2.
6. For pharmacological treatments, seed 1.5×10^5 U2OS cells in 12-well plates, in 1 mL growth medium per well (*see* **Note 25**), and proceed to Subheading 3.3.

3.2 RNA Interference

1. When cells reach a confluence of 40–60 % (*see* **Note 26**), dilute 50 pmol siRNA (final concentration in wells = 25 nM) in 62.5 μL Opti-MEM® (solution A), and 7.5 μL HiPerFect® in 55 μL Opti-MEM® (solution B), and allow both solutions to stand at RT for 5–10 min (*see* **Note 27**).
2. Mix solution A and B gently, and incubate at RT additional for 15–20 min, to allow for the formation of HiPerFect®:siRNA transfection complexes (transfection solution) (*see* **Notes 28** and **29**).
3. Replace growth medium with 1.875 mL complete growth medium.
4. Add 125 μL of the transfection solution to each well (*see* **Note 30**), and incubate plates under standard culture conditions (37 °C, 5 % CO_2).
5. 4–24 h later (*see* **Notes 31** and **32**), detach transfected cells (500 μL trypsin–EDTA per well), seed them in 12-well plates (0.8×10^5 cells in 1 mL growth medium per well) (*see* **Note 25**), and proceed to Subheading 3.3.

3.3 Pharmacological Treatments and DiOC6(3)/PI Co-staining

1. 24 h after seeding non-transfected U2OS cells, as described in **step 6** in Subheading 3.1 (*see* **Note 33**), gently remove supernatant and substitute with 1 mL complete culture medium alone (or containing an equivalent amount of solvent, negative control condition), or supplemented with 1 μM STS (or the cell death inducer of choice), 50 μM Z-VAD-fmk (additional control condition), or 1 μM STS + 50 μM Z-VAD-fmk.
2. Alternatively, 24 h after seeding transfected U2OS cells, as described in **step 3** in Subheading 3.2 (*see* **Note 33**), gently remove supernatant and substitute with 1 mL complete culture medium alone (or containing an equivalent amount of solvent, negative control condition), or supplemented with 1 μM STS (or the cell death inducer of choice).
3. When the stimulation period is over, collect culture supernatants in 5 mL FACS tubes (*see* **Notes 34** and **35**) and detach adherent cells with ~0.5 mL trypsin–EDTA, following a wash with ~0.5 mL pre-warmed PBS (*see* also **steps 2** and **3** in Subheading 3.1, and **Notes 17–19**).
4. Following complete detachment (*see* **Note 20**), add 1 mL complete growth medium to each well (*see* **Note 21**), and transfer cells to the FACS tube containing the corresponding supernatant.
5. Spin down cell suspensions at 300 × *g*, RT, for 5 min.
6. Discard supernatants, resuspend cells in 200–400 μL of staining solution (40 nM $DiOC_6(3)$ in complete growth medium) (*see* **Notes 36–38**), and incubate them for 20–30 min in the dark at 37 °C (5 % CO_2) (*see* **Note 39**).


a
siUNR
Untreated
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Z-VAD-fmk
Control
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siATG5
Z-VAD-fmk
Control
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Figure 1

7. Add PI to each sample at a final concentration of 1 μg/mL, and incubate samples for additional 2–5 min under protection from light (*see* **Notes 40** and **41**).
8. Analyze samples on a conventional flow cytometer allowing for the simultaneous assessment of light scattering parameters (forward and side scatter, FSC and SSC) and fluorescence in two separate channels (e.g., green and red) (*see* **Notes 42–45**).
9. *Bona fide* apoptotic cell death is retarded in the presence of Z-VAD-fmk, but normally accelerated upon the pharmacological or genetic inhibition of core components of the machinery for autophagy (*see* Fig. 1). Conversely, bona fide autophagic cell death is insensitive to Z-VAD-fmk, but can be retarded by the pharmacological or genetic inhibition of autophagy (not shown).

4 Notes

1. The manufacturer recommends to periodically check flow rate, laser alignment and fluorescence stability, to ensure technical reliability from the instrument. Moreover, it is recommended to align/calibrate the cytometer with standard beads for flow cytometry, as per manufacturer's recommendations, prior to each experimental session.
2. Recommended for U2OS cells by the American Type Culture Collection (ATCC, Manassas, VA, USA).
3. Under optimal storage conditions (−20 °C, protected from light), trypsin–EDTA is stable for at least 18 months. Repeated freeze-thawing should be avoided by storing the reagent in aliquots of 2–10 mL. Once thawed, the trypsin–EDTA is stable at 4 °C for approximately 2 weeks.
4. According to the manufacturer, lyophilized siRNA are stable for at least 3 years if stored appropriately (−20 °C, protected from light). Under appropriate storage conditions reconstituted siRNA stock solution are stable for at least 6 months. We recommend storing the reagent in small aliquots (5–20 μL), to avoid repeated freeze-thawing.

Fig. 1 Apoptotic cell death induced by staurosporine. Human osteosarcoma U2OS cells were transfected with a control siRNA (siUNR) or with an ATG5-targeting siRNA (siATG5) for 48 h, as detailed in Subheading 3.2, and then left untreated or treated with 1 μM staurosporine (STS) for additional 24 h. Alternatively, non-transfected U2OS cells were maintained in control conditions or treated with 1 μM STS, alone or in combination with Fig50 μM Z-VAD-fmk, as detailed in Subheading 3.3. Twenty-four later cells were processed for the cytofluorometric quantification of plasma membrane permeabilization and mitochondrial transmembrane potential dissipation as detailed in Subheading 3.3. In panel (**a**), representative *dot plots* are reported. In panel (**b**), quantitative data are reported (means ± SD, $n = 2$ parallel samples, ***$p < 0.001$, as compared to untreated or siUNR-transfected cells; ###$p < 0.001$, as compared to cells treated with STS only; two-sided, unpaired Student's *t* test)

5. siUNR is a custom siRNA unrelated to the human and murine genome [35]. Other commercial or noncommercial siRNAs can be employed to generate appropriate negative control conditions for the siRNA-mediated downregulation of ATG5.
6. Core components of the autophagic machinery other than ATG5, including ATG7 and beclin 1 (BECN1), can be targeted instead, or in parallel. Ideally, an instance of RCD should be tagged as autophagic if (1) it can be delayed by the genetic inhibition of at least two distinct components of the core machinery for autophagy; or (2) it can be delayed by the knockdown/knockout of one component of the core autophagic machinery as well as by chemical inhibitors of autophagy [31].
7. Several liposomal transfection reagents commercially available can substitute for HiPerFect®, including Oligofectamine™ (Gibco®-Life Technologies) and DharmaFECT™ (GE Dharmacon, Lafayette, CO, USA). Each of these reagents attains maximal efficacy with a specific transfection protocol.
8. For most cell types, Opti-MEM® can be replaced by FBS-, antibiotic-, HEPES-, and sodium pyruvate-free growth medium.
9. Under appropriate storage conditions (−20 °C, sealed and protected from light), undissolved Z-VAD-fmk is stable for at least 2 years. If stored at −20 °C and under protection from light, stock solutions are stable for at least 1 year. It is recommended to avoid repeated freeze-thawing by storing the reconstituted product in small aliquots (10–50 μL).
10. According to the manufacturer, STS stock solutions are stable for at least 6 months, if stored at −20 °C and protected from light.
11. If stored at −20 °C and protected from light, $DiOC_6(3)$ stock solution is stable for at least 12 months. Unnecessary exposure to light should be avoided to prevent photobleaching.
12. $DiOC_6(3)$ exhibits excitation/emission peaks at 482/504 nm, respectively.
13. Undissolved PI is stable for at least 12 months under standard storage conditions (at room temperature, and protected from light). PI stock solution is stable for at least 6 months, if stored at 4 °C and protected from light.
14. PI exhibits excitation/emission peaks at: (1) 482/504 nm, respectively, in aqueous solution; and (2) 535/617 nm, respectively, when bound to DNA.
15. The choice of the support for maintenance cultures (i.e., 25 cm^2, 75 cm^2, or 175 cm^2 flasks) depends on the amount of cells needed for experimental determination and other factors (e.g., limited space within incubators). As an indication, a 75 cm^2 flask of U2OS cells at 60–70 % confluence contains approximately $4–5 \times 10^6$ cells.

16. Both under-confluence and over-confluence in maintenance cultures should be avoided, as the former may be associated with a considerable genetic drift in the cell population, and the latter may impose a metabolic burden that affects cell viability.
17. This step ensures the removal of residual traces of FBS, which inactivates trypsin. Washing should not be protracted or harsh to avoid a sizeable loss of cells, especially for cell types that per se are relatively prone to detachment like human colorectal carcinoma HCT 116 cells.
18. TrypLE™ Express can substitute for trypsin–EDTA. As compared to trypsin–EDTA, TrypLE™ Express exhibits improved stability at 4 °C and RT, and does not require inactivation.
19. Optimal detachment time may vary from <1 min to several minutes, depending on cell type and culture conditions. As an indication most cancer cell lines are properly detached in 1–3 min. Over-trypsinization should be avoided, as it can result in cellular damage and/or phenotypic alterations.
20. Detachment can be verified by visual inspection.
21. Addition of complete growth medium at this step ensures the FBS-dependent inactivation of residual trypsin activity.
22. The ATCC recommends to subculture U2OS cells at a ratio of 1:3–1:6. We observed that U2OS cells can be safely subcultured at a ratio 1:8 without noticeable shifts in phenotype and behavioral traits.
23. As a general recommendation, immortalized cells should be kept in the exponential growth phase, and be maintained in culture for a limited, predetermined number of passages. This calls for a relatively large stock of cryopreserved cells.
24. The amount of cells required to generate cultures that are suitable for transfection 24 h later vary quite considerably with cell type and culture conditions.
25. The amount of cells required to generate cultures that are suitable for pharmacological treatment 24 h later vary quite considerably with cell type and culture conditions.
26. In our experience, transfection efficacy drops remarkably when confluence >50 %.
27. These conditions are appropriate for transfecting cells in 1 well of a 6-well plate. They can be readily scaled up to transfect several wells with the same transfection solution.
28. The transfection solution may appear cloudy.
29. Transfection is carried out entirely at RT under a common safety cabinet. However, it is a good practice to maintain siRNA stock solutions and HiPerFect® in ice bath (and to return them to storage conditions immediately after use).

30. Transfection complexes have a very high affinity for the plasma membrane. They should therefore be added to wells dropwise and evenly (covering the whole surface of the growth medium), in order to avoid intra-well variations in transfection efficiency and potential toxicity.
31. Transfection is generally rapid (<4 h), but cells can be allowed to reach 70–80 % confluence in 6-well plates prior to sub-culturing.
32. Ideally, cells should be ready for treatment (*see* also **Note 25**) in 12-well plates as soon as the siRNA-mediated downregulation of ATG5 achieves maximal efficacy. ATG5 and most other proteins are optimally downregulated by siRNAs 48 h after transfection. However, checking transfection efficacy and kinetics by quantitative real-time PCR or immunoblotting in preliminary experiments is strongly recommended.
33. Particularly sensitive cells may require prolonged adaptation times to resume sufficient proliferation.
34. Confluence and general status should be verified on light microscopy before collection.
35. Some forms or RCD are accompanied by the detachment of cells from the substrate. It is therefore important to collect supernatants (unless the experiment is designed as to include only viable cells) to avoid a considerable underestimation of cell death.
36. Fluorochrome-containing solutions should be thoroughly mixed before use to eliminate precipitates. Moreover, they should be shielded from light to minimize photobleaching.
37. At low concentrations (20–40 nM), $DiOC_6(3)$ rapidly accumulates within energized mitochondria in a virtually non-saturable fashion. To label all samples from the same experiment in a homogenous manner, we recommend to: (1) carefully remove supernatants (by aspiration and inversion of FACS tubes on paper); (2) employ a unique staining solution for all samples; (3) repeatedly and thoroughly mix the staining solution throughout the experiment; (4) employ an equal volume of staining solution for all samples; and (5) take particular care at avoiding cell clumps during labeling.
38. At high concentrations (>100 nM), $DiOC_6(3)$ exhibit remarkable self-quenching, which renders it inappropriate to measure $\Delta\psi_m$. Moreover, at high doses, $DiOC_6(3)$ may label other intracellular compartments, including the endoplasmic reticulum.
39. Prolonged incubation with $DiOC_6(3)$ (>40 min) may be toxic for some cell types, while an excessively short labeling time (<20 min) may result in non-homogenous or incomplete staining. Preliminary experiments should address the toxicity of $DiOC_6(3)$ in the experimental setting of choice.

40. As an alternative, PI can be added directly to the staining solution, at the final concentration of 0.5–1 μg/mL (*see* **step 6**, Subheading 3.3). Preliminary experiments to assess the toxicity of PI are recommended in this case.
41. FSC and SSC reflect cell size and the so-called "refractive index," respectively. The refractive index depends on various parameters, including cell shape and granularity.
42. We generally employ channel FL1 for the detection of $DiOC_6(3)$ and channel FL3 for the detection of PI.
43. If >30 samples must be analyzed, we suggest to carry out staining and acquisition on <24 samples at a time, to homogenize the exposure of cells to $DiOC_6(3)$ and PI.
44. Two-color analyses are relatively straightforward and can be carried out on the flow cytometer proprietary software. Alternatively, several software packages for the analysis of cytofluorometric data are available online.
45. To provide adequate statistical power, we recommend to acquire and analyze at least 10,000 events exhibiting normal FSC and SSC values per sample.

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