Detection of Extracellular Phosphatase Activity of Heterotrophic Prokaryotes at the Single-Cell Level by Flow Cytometry

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ABSTRACT

Monitoring cell activity using substrates, which turn fluorescent due to biological activity, allows observing the presence and dynamics of sub-populations, and provides a very valuable insight in ecological studies. The phosphatase substrate ELF97 phosphate (ELF-P) is a useful tool to detect and quantify phosphatase activity (PA) of microorganisms at the single-cell level. Most of the studies dealing with PA at the single-cell level focus on autotrophic cells and only few concern heterotrophic prokaryotes (referred as bacteria in the text). While flow cytometry is a promising tool to assess the single-cell analysis, only microscopy tools have been used until now to measure the ELF labeling associated with bacteria expressing PA. Therefore, we have developed a new protocol that enables the detection of ELF alcohol (ELFA), the product of ELF-P hydrolysis, making possible the specific identification of bacteria showing PA using flow cytometry. *Curr. Protoc. Cytom.* 49:11.18.1-11.18.8. © 2009 by John Wiley & Sons, Inc.

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BASIC PROTOCOL

The analysis of microbes at the single-cell level provides a very valuable insight in ecological studies. In particular, monitoring cell activity using substrates, which turn fluorescent due to biological activity, allows observing the presence and dynamics of sub-populations expressing or not this activity. As far as the ecosystem functioning is concerned, among other important interactions, it is fundamental to better understand interactions between microbes and the phosphate (P) cycle, a key element in the ecosystems (Karl, 2000). The detection of extracellular phosphatase activity (PA) at the single-cell level by flow cytometry should bring new insights in that field of research. The phosphatase substrate ELF97 phosphate (ELF-P) is used to detect and quantify cell-attached extracellular PA at the single-cell level and has been successfully applied for assessing the P status of phytoplankton (Gonzalez-Gil et al., 1998; Dyhrman and Palenik, 1999; Nedoma et al., 2003; Nicholson et al., 2006). Following hydrolysis, this water-soluble nonfluorescent compound precipitates at the site of enzyme activity forming ELF-alcohol (ELFA), which is insoluble and highly fluorescent. This induced fluorescence enables PA to be detected at the single-cell level. Recently, the technique has been successfully extended to marine heterotrophic prokaryotes in culture samples, the ELFA-labeling being detected using microscopy (Van Wambeke et al., 2008).

In this unit, we describe a new protocol that enables the detection by flow cytometry of ELFA-labeled heterotrophic prokaryotes (referred as bacteria in the text) in fresh and marine samples. Bacteria from natural samples must be disaggregated and, in oligotrophic waters, concentrated before analysis by flow cytometry. The incubation time necessary to reach a stable percentage of active cells with the maximal fluorescence intensity must

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be determined for each sampling site. For that purpose, a suitable time kinetic curve must be done.

In order to discriminate between bacteria expressing or not expressing PA it is obviously mandatory to detect both. One problem is that most of them are not naturally fluorescent when analyzed by flow cytometry. The solution consists in staining their nucleic acids with a fluorochrome to induce fluorescence in order to discriminate bacteria from other nonliving particles (inorganic particles and/or debris) with similar size and shape. The protocol described in this unit performs the detection and discrimination of signals from both ELFA (the product of ELF-P hydrolysis under PA) and the DAPI counterstain necessary to distinguish between bacteria expressing PA (both ELFA and DAPI-labeled) or not expressing PA (only DAPI labeled).

Materials

Fresh marine or freshwater sample (i.e., non-preserved)
Tween 80 diluted to 100 mg/liter (polyoxyethylenesorbitan monooleate; Sigma)
ELF-P solution (see recipe)
PBS/formalin solution (see recipe)
0.2 μm-filtered sample
Internal standard microsphere mixture: 2 μm-beads (Fluoresbrite, Polysciences) in sheath fluid
Sheath fluid made of 0.2 μm-filtered distilled water
DAPI solution (see recipe)

15-ml Beckman polyallomer centrifuge tubes
Centrifuge (capable of generating acceleration $\geq$20,000 $\times$ *g*) with rotor adapted to 15-ml Beckman polyallomer centrifuge tubes
Sterile pipets
Vortex
Syringe filters, 0.2-μm pore (Sartorius)
A flow cytometer equipped with:
- UV light source (351 nm)
- 440-nm long-pass dichroic mirror to separate DAPI fluorescence from ELFA fluorescence
- 375- to 435-nm band-pass filter for isolating DAPI fluorescence
- 490- to 570-nm band-pass filter for isolating ELFA fluorescence
- 620-nm long-pass filter for the detection of Chlorophyll *a* fluorescence
- Flow cytometry software to acquire and analyze the data

12 $\times$ 75–mm flow cytometry test tubes

Prepare instrument and sample

1. Add a volume of fresh sample in a Beckman polyallomer centrifuge tube.

   Typically, 14 ml in oligotrophic marine waters (in oligotrophic areas, bacterial abundance is 10^5 to 10^6 cell/ml).

2. Add a volume of Tween 80 diluted to 100 mg/liter to obtain a 10 mg/liter final concentration.

3. Centrifuge 30 min at 20,000 $\times$ *g*.

   Centrifugation should be performed at the temperature from where the sample was taken (e.g., for a sample taken at 5 meters, if the temperature at 5 meters was 18°C, then centrifuge the samples at 18°C).

4. Carefully remove the supernatant by aspiration with a sterile pipet.

5. Add 100 µl of the 1/20 ELF-P solution on the pellet. Vortex to resuspend the pellet. Incubate in the dark for a period determined according to time kinetics results.

   Perform the incubation at the temperature from where the sample was taken (e.g., for a sample taken at 5 meters, if the temperature at 5 meters was 18°C, then incubate the samples at 18°C).

   Time kinetics of ELFA labeling must be done prior to each analysis in order to determine the appropriate incubation time (Duhamel et al., 2009).

6. Add 400 µl of PBS/formalin solution. Gently mix by hand.

   This step stops the ELF-P hydrolysis and allows samples to be compared (Duhamel et al., 2009).

7. Add 1.5 ml of a 0.2 µm-filtered sample (prepared by filtering the same sample water through a 0.2-µm syringe filter). Gently mix by hand.

   At this step, ELFA-labeled samples can be stored in liquid nitrogen for up to 4 months before counting (Duhamel et al., 2008).

8. Turn on the flow cytometer prior to analysis, allowing sufficient time (∼1 hr) for the laser output to stabilize. Use 0.2-µm-filtered distilled water as sheath fluid.

9. With amplifiers in linear mode and processors set for pulse integration, align the instrument with 2-µm-diameter fluorescent microspheres (prepared in sheath fluid) to optimize the particle (cells) excitation and the signal collection for forward scatter (FS), DAPI fluorescence, ELFA fluorescence, chlorophyll *a* fluorescence, and 90° light scatter (side scatter or SS).

10. When the alignment is done, set amplifiers for logarithmic processing for the analyses (for nondigital instruments).

11. Add 100 µl DAPI staining solution to an appropriate 12 × 75–mm flow cytometry tube containing 1 ml of the ELFA-labeled sample. Gently mix by hand. Incubate 15 min at room temperature, in the dark.

12. Add 10 µl of a microsphere solution to the samples before analysis. Vortex the sample.

    These microsphere/fluorospheres are used as an internal standard (there is no specific concentration). They are used for quality control and to standardize the cell fluorescence.

Acquire sample data

13. Analyze a sufficient amount of sample in order to collect a sufficient amount of cells for statistical relevance. Store the data in an FCS file.

14. Display the data according to the following cytograms: (1) SS intensity versus pulse width, (2) ELFA fluorescence intensity versus DAPI fluorescence intensity (Fig. 11.18.1B,C) and (3) Chlorophyll *a* fluorescence intensity versus DAPI fluorescence intensity (Fig. 11.18.1D). Set region boundaries for the events of interests on (1) and gate this cluster on (2) and (3).

15. Use compensation to subtract the fluorescence "spillover" between DAPI and ELFA fluorescences.

    The spectral overlap between ELFA and DAPI fluorochromes makes compensation necessary to clearly separate cells labeled or not labeled by ELFA. Compensation is the process by which the fluorescence "spillover" originating from a fluorochrome other than the one specified for a particular photomultiplier tube is subtracted as the percentage of the signal from other photomultiplier tube. Because a digital compensation is performed, the raw data are not affected and compensation can be made a posteriori.

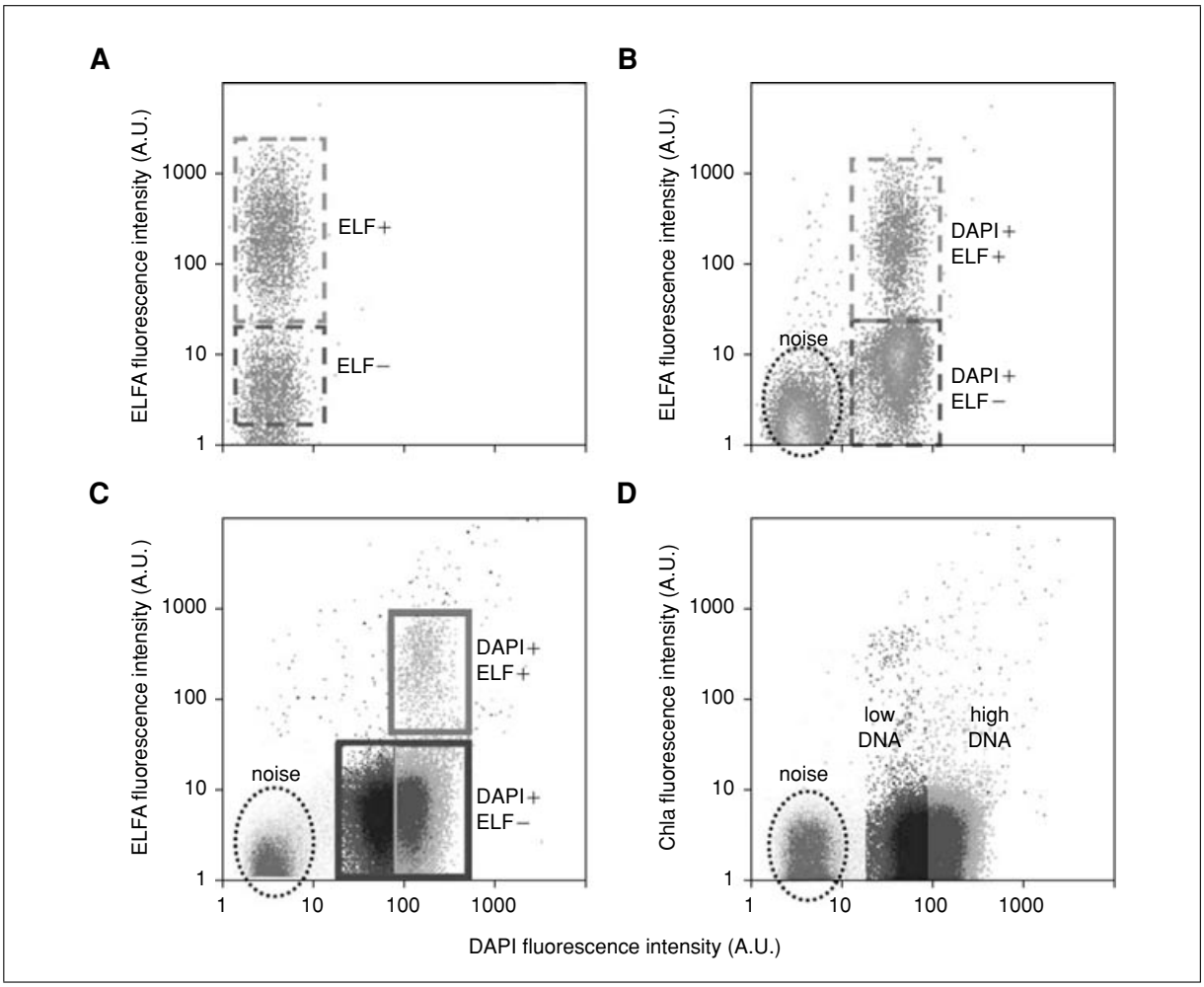

Figure 11.18.1 Examples of cytograms obtained for culture samples (*Alteromonas infernus*) labelled with ELF only (**A**) or ELF+DAPI (**B**) and for marine samples (SOFCOM observation station, Mediterranean Sea) labeled with ELF+DAPI (**C**, **D**). Axes of the cytograms are ELFA or chlorophyll *a* (Chla) (Y-axis) and DAPI (X-axis) fluorescence intensity in arbitrary units (A.U.).

16. Set region boundaries for the ELFA-labeled and non-ELFA-labeled bacterial population(s) and for the cluster of 2-μm microspheres to obtain data for each group on (2). Set region boundaries for the heterotrophic bacterial and phytoplanktonic population(s) on (3). Gate the cluster of *Prochlorococcus*-like cells on (3) to remove these events on (2) (See Marie et al., 2000).

Determine incubation time

17. Repeat steps 1 to 7 on a series of subsamples and incubate them during different times. The scale of incubation times depends on the samples (i.e., from minutes in high-activity samples to hours in low-activity samples). For an unknown sample we suggest preparing ten subsamples and stopping the ELF-P incubation (see step 6) after 0 min, 15 min, 30 min, 45 min, 1 hr, 2 hr, 4 hr, 6 hr, 12 hr, and 18 hr (one subsample for each incubation time).

Analyze sample data

18. Using sample data from steps 13 to 16, determine the abundance of non-ELFA-labeled and of ELFA-labeled bacterial cells (Fig. 11.18.1B,C). Determine the median ELFA fluorescence intensity of the ELFA-labeled bacterial population (E) and of the 2-μm beads (B). Normalize the data (E/B) for comparison among samples and to account for day-to-day variation (quality control).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

DAPI solution

Prepare an aqueous stock solution of 0.5 mg/ml 4′,6-diamidino-2-phenylindole (DAPI; Sigma), and store up to 6 months at −20°C in the dark. For staining solution, dilute the DAPI stock solution to 25 μg/ml in deionized, distilled water, and store up to 1 month at 4°C in the dark. Pass this staining solution through a 0.2-μm sterile syringe filter before use.

ELF-P solution

Prepare a 500 μl dilution 1/20 of component A in component B of the Endogenous Phosphatase Detection Kit (Molecular Probes, cat. no. E6601). Filter this solution through a spin filter of 0.2-μm porosity just before use. Prepare immediately before use.

PBS/formalin solution

Prepare a 10 mM phosphate-buffered saline (PBS; see recipe) solution. Mix this solution with formaldehyde to 5% final (formalin, supplied as 37% formaldehyde). Adjust pH to 7.5 using high-purity HCl and NaOH solutions. Filter through a 0.2-μm sterile syringe-filter just before use. Store up to 6 months at 4°C.

PBS solution, 10 mM

8 g NaCl
0.2 g KCl
1.44 g Na_2HPO_4
0.24 g KH_2PO_4
Adjust volume to 1 liter with distilled water
Adjust pH to 7.5 using high-purity HCl and NaOH solutions and autoclave 15 min at 120°C
Store up to 6 months at 4°C

COMMENTARY

Background Information

Marine microbes play a very important role in biogeochemical cycles. Because of their biodiversity, the diversity of their metabolisms, and their physiological heterogeneity (live/dead, active/inactive cells) it is necessary to study them at the cell level. Flow cytometry has become a technique of choice to reach this goal, providing a fast, reliable, and multiparametric analysis of cells at the single-cell level. Phosphatase activity (PA) is measured to determine the phosphate (P) status of planktonic communities (Elser and Kimmel, 1986; Nicholson et al., 2006; Mackey et al., 2007). Although bulk measurements [with 4-methylumbellyferyl phosphate (MUF-P) nitrophenyl phosphate, 3-O-methylfluoresceine phosphate, 3,6-fluorescein diphosphate, and Attophos] have been widely used to quantify the kinetic parameters of PA (Gonzalez-Gil et al., 1998; Hoppe, 2003; Labry et al., 2005), they do not provide information about the origin of the activity. The substrate ELF97 [2-(5′-chloro-2′-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone]-phosphate (ELF-P) is used to detect and quantify cell-attached extracellular PA (Nedoma et al., 2003; Dignum et al., 2004b). By enzymatic hydrolysis, the water-soluble ELF-P is converted into ELF-alcohol (ELFA) which is insoluble, specific, photostable, and highly fluorescent (Huang et al., 1993) and precipitates at the site of enzyme activity. This enables PA to be detected at the single-cell level (Gonzalez-Gil et al., 1998; Dyhrman and Palenik, 1999). ELFA labeling has been traditionally observed by epifluorescence microscopy providing essentially qualitative results (Gonzalez-Gil et al., 1998; Dyhrman and Palenik, 1999;

Nedoma et al., 2003; Van Wambeke et al., 2008). Analysis of ELFA-labeled cells by flow cytometry was first applied on phytoplankton (Gonzalez-Gil et al., 1998; Dignum et al., 2004a,b) following its initial development for histological applications (Telford et al., 1999). In these studies, the distinction between positive (ELFA-labeled) and negative (unlabeled ELFA) phytoplankton cells was based on the detection of both autofluorescent pigments and ELFA fluorescence (Gonzalez-Gil et al., 1998; Dignum et al., 2004a,b).

Critical Parameters and Troubleshooting

In contrast to phytoplankton, heterotrophic bacteria are not naturally fluorescent. To detect them using flow cytometry and discriminate them from inorganic particles and/or debris, it is necessary to use a counterstaining step of their nucleic acids. In flow cytometry applications, nucleic acids are commonly stained with fluorochromes that are excited in the blue (YOYO-1, YO-PRO-1, PicoGreen, etc.) or in the blue and UV wavelengths (like SYBR series) and emit in the green wavelengths. Since ELFA (max excitation: 360 nm, max emission: 530 nm) also emits in the green wavelengths, counterstaining of ELFA-labeled cells using these fluorochromes is not an option. We thus tested two popular fluorescent dyes emitting over a different wavelength range: 4′,6-diamidino-2-phenylindole (DAPI; max excitation: 358 nm, max emission: 461 nm) and propidium iodide (PI; max excitation: 535 nm, max emission: 617 nm). Cell labeling using DAPI and ELFA, both excitable in UV but emitting at distinct wavelengths, enables the spectral separation of the two dye emissions and thus enables to discriminate the ELFA-positive cells (both DAPI and ELFA-labeled) from the ELFA-negative cells stained only with DAPI. Nevertheless, we observed a spectral overlap between ELFA and DAPI emission wavelength, which makes compensation treatment necessary in order to accurately separate the population of DAPI-only-labeled particles from the population of dual- (ELFA- and DAPI-) labeled particles (Fig 11.18.1B,C). DAPI has the advantage of allowing discrimination of the naturally fluorescent phytoplankton cells. PI, as chlorophyll *a*, emits in the red wavelength making the discrimination of autotrophic from heterotrophic cells impossible. Nevertheless, it should be noted that among phytoplankton, only the prokaryotic autotrophic cells (cyanobacteria) would survive the 20,000 × *g* centrifugation step.

Before making samples for ELF labeling it is important to prepare samples for time kinetics of ELFA labeling in order to determine the appropriate incubation time necessary to reach a stable percentage of active cells with the maximal fluorescence intensity (see step 17). Indeed, this is a condition to make sample comparisons more relevant (Duhamel et al., 2009). Figure 11.18.2 presents results of the evolution of the absolute fluorescence (median fluorescence × cell number in the ELF+ region) for time kinetics made in different samples (culture, marine water, and freshwater). In these typical examples, the time necessary to reach a plateau phase varies widely among samples, being smaller as the cells are under P-limited conditions.

Anticipated Results

Typical results are shown in Figures 11.18.1 and 11.18.2. Figure 11.18.1 displays typical cytograms obtained for culture samples labeled with ELF only (Fig. 11.18.1A) or ELF+DAPI (Fig. 11.18.1B) and for marine samples (Bay of Marseille, SOFCOM observation station, Mediterranean Sea) labeled with ELF+DAPI (Fig. 11.18.1C). It is common to distinguish two different populations of bacteria (Low DNA and High DNA; Fig. 11.18.1C,D) in natural seawater samples that differ both in scatter and in DNA content. The freshwater samples that we have studied until now (Plešné and Prášilské Lakes, Czech Republic) did not present these two populations of bacteria.

Figure 11.18.2 provides typical results from time kinetics studies in culture samples (*Alteromonas infernus*; Fig. 11.18.2A), the Prášilské Lake (in the Czech Republic; Fig. 11.18.2B), and the Mediterranean Sea (Fig. 11.18.2C). The incubation time necessary to reach a stable percentage of active cells with a maximal fluorescence intensity (illustrated by the plateau phase in absolute fluorescence versus time, Fig. 11.18.2) varies widely between samples (i.e., 90 min, 60 min, and 6 hr for culture, lake, and marine samples, respectively).

Time Considerations

Most of the effort is expended on preparation of samples. Sample concentration takes 30 min. According to the time kinetic results, the samples' incubation time varies widely between samples, from minutes to hours. DAPI labeling takes 15 min. Instrument alignment takes ∼20 min, and data acquisition takes 2 min for samples with ∼10^7 cells/ml. Sample

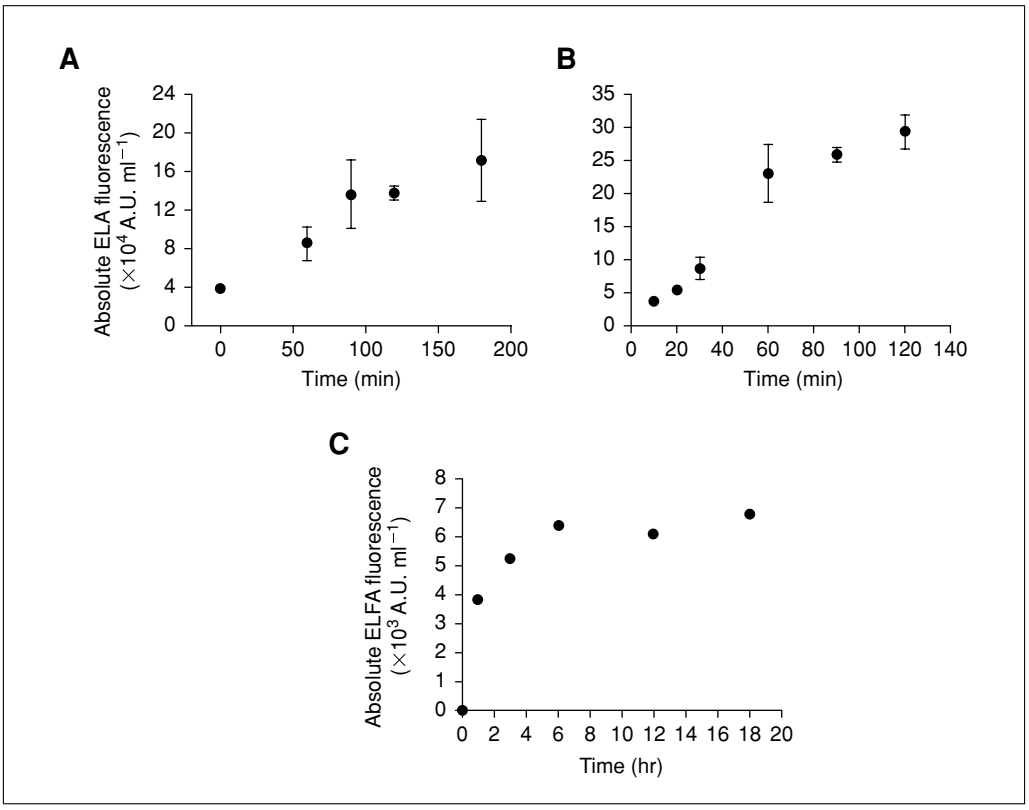

Figure 11.18.2 Time course following the absolute ELFA fluorescence ($\times 10^4$ A.U. ml^{-1}, A, B and $\times 10^3$ A.U. ml^{-1}, C) in culture samples (*Alteromonas infernus*) (**A**), the Prášilské Lake (Czech Republic) (**B**), and the Mediterranean Sea (SOFCOM observation station) (**C**), incubated with ELF-P for different time [notice the different scale of time between culture or lake samples (in minutes) and marine samples (in hours)]. Error bars represent the standard deviation between triplicates.

counterstaining with DAPI at 4 to 6 min intervals can be convenient. Once the region boundaries of the different populations of interests are set up (with gating and compensation treatments, when necessary) data analysis should be <5 min/sample.

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Key References

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