

Protocol

Ethidium monoazide (EMA) for exclusion of dead cells by FACS

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Materials

EMA, Molecular Probes E-1374

Stock solution is prepared as 5 mg/ml in DMSO and stored as single-use aliquots of 20µl in amber eppendorf vials in a dessicator at -20deg. Note that EMA is extremely light-sensitive, so exposure to light must be minimized when making the stock and when using the reagent (up until the step in which the cells are intentionally exposed to light).

Mechanism

Like propidium iodide (PI), EMA diffuses into dead cells and intercalates into DNA. Upon exposure to light, EMA then covalently binds to the DNA. EMA excitation peak is 462nm (blue laser excitation) with an emission peak at 625nm (channel = TRPE).

Purpose and use

EMA is used to identify dead cells by FACS under conditions when other dead cell markers such as PI cannot be used. These conditions include intracellular staining where the permeabilization of the cells would allow leakage of PI into all the cells. When cells are stained with EMA and exposed to light prior to permeabilization, the EMA is covalently linked to the DNA in the dead cells and cannot subsequently leak out. EMA is also used when cells are surface stained only, but must be fixed and cannot be run on the FACS quickly. Fixed cells lose their membrane integrity over time, so they will eventually take up PI. PI can be used with fixed, non-permeabilized, cells; however, the cells must be run within about 12-18 hours of staining.

Need to compensation: Like PI, EMA is detected in the Cy5PE channel. (EMA can also be detected in the TRPE channel.) Unlike PI, this channel must be devoted to EMA since EMA is not very bright. A separate compensation sample should be prepared for EMA. Although the stained cells will be gated out and should therefore not affect the other colors used, any background EMA staining of the live cells will carry-over into other colors and will need to be compensated. For our EMA-only stained compensation sample, we currently use a small aliquot of cells and stain these cells with an excess of EMA (10 to 20x the recommended titer). Since EMA at this titer has high background, the background staining is bright enough to use as the positive stained sample for the compensation control. Note that there will be no negative cells in this sample. Use unstained cells (in another tube) as your negative cells. Alternatively, after washing the EMA control, you can add back unstained cells so that you have a negative population in the same tube. As an alternate compensation sample, live cells can be mixed with heat-killed cells (see below). This ensures adequate representation of dead cells in the compensation sample. Note that EMA is a different fluorescent dye compared with

Cy5PE, PerCP or TRPE, and therefore compensation samples for these dyes cannot be substituted for the EMA compensation sample.

Titration

The amount of EMA to be used to label dead cells prior to analysis by FACS should be determined by a titration. We prepare of mixture of live and dead cells by killing an aliquot of PBMC in a hot water bath (56deg) for 30-45 minutes and then mixing these dead cells with live cells. Based on our titration, we use EMA at 0.5 µg/ml. We take 10 µl of the 5 mg/ml stock and add this to 990 µl of staining media or PBS (50ug/ml). We then use 1.0 µl of this dilution per final staining volume of 100 µl (0.5ug/ml, final dilution, 1:10,000). This titer is not determined simply on the brightness of the stained dead cells, but also on the background staining of the live cells. At the end of this document is an example of a titration (see the procedure below for staining details).

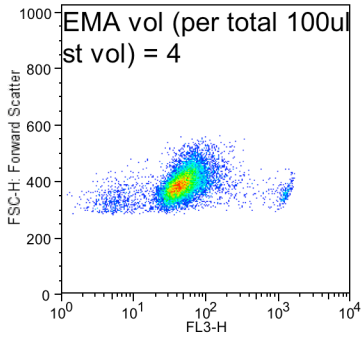
Procedure

We include EMA as one of the reagents when the cells are surface stained. Cells are incubated **under aluminum foil** with the EMA and the surface stains for **10 min** on ice or at room temp. Cells are then transferred to a location under a **bright fluorescent light** source where they are exposed for **10 minutes**. This is a total of 20 minutes staining time. Note that these times can be extended, but should not be shortened. We have found that 10 to 15 minutes of light exposure is optimal – shorter times result in lower levels of staining.

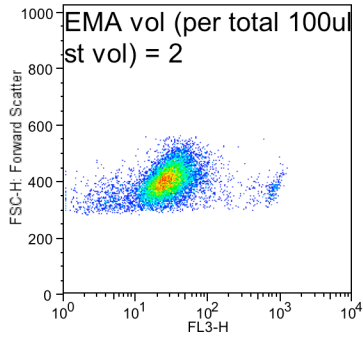
To be safe, intense light exposure is best. For example, an X-ray viewing box can be inverted and placed over the ice bucket containing the cells, or a fluorescent swing-arm desk lamp can be used. Or the cells can be placed closer to the light source in a laminar flow hood by placing them on a stack of boxes or ice buckets in the hood. Light exposure can take place on ice or at room temp. We also expose all the compensation samples to this light to ensure that these controls and the actual multicolor samples have identical light exposure.

Cells are then washed 2x (total of 3 spins when using plates). Cells can then be fixed or permeabilized depending on the planned experiment. Note that light exposure should be minimized during the steps prior to light exposure. We turn off overhead lights and are careful to cover the cells with foil. We suspect that light exposure during this time may lead to higher background levels of EMA staining. . Once the cells have been exposed to light, the special precautions to avoid ambient light exposure are not needed. Of course, cells are stained with other fluorescent markers, so standard precautions to prevent excessive light exposure as per FACS staining protocols should continue to be followed.

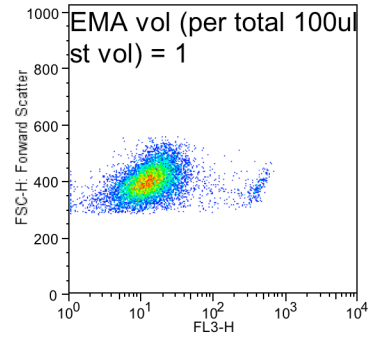
Previous protocol: Note that this procedure differs from our previous protocol where we would wash the cells before exposure to light. However, this older protocol resulted in lower levels of EMA staining (the reagent may have leaked out of the dead cells) and it did not result in lower background staining. Therefore, we feel the current protocol is better.



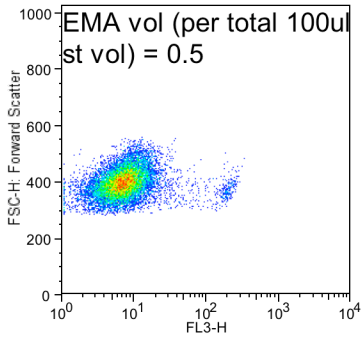
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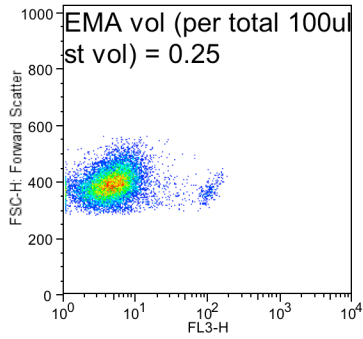
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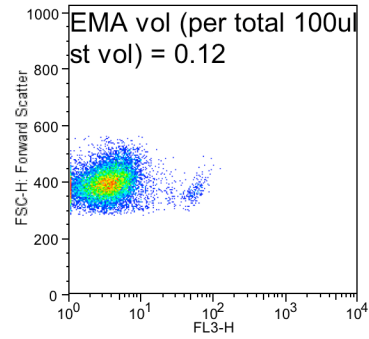
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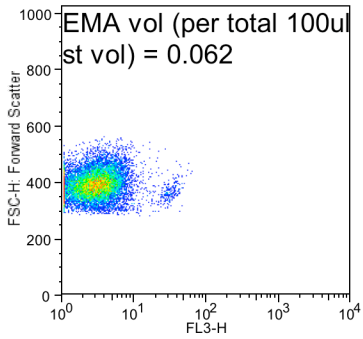
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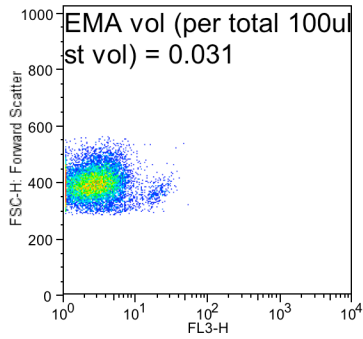
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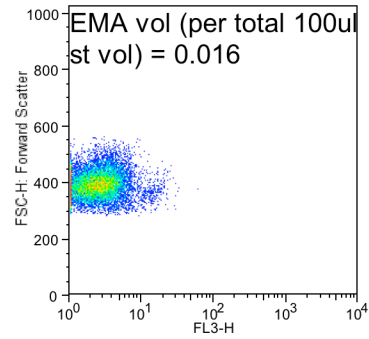
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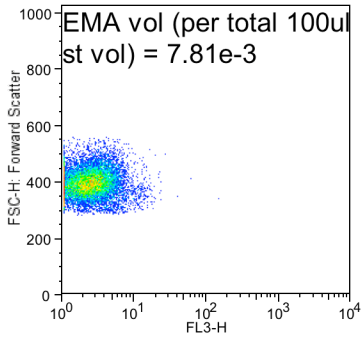
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