Visualization of a cell with fluorescent compounds provides a wide variety of information for the analysis of cell functions. Various activities and structures of a cell can be targeted for staining with fluorescent compounds (Fig. 19). The most commonly stained cell components are cell membranes, proteins, and nucleotides. Small neutral molecules and positively charged molecules can pass through viable cell membranes and remain inside of cells, depending on their reactivity or hydrophilicity. Negatively charged molecules cannot pass through viable cell membranes. Positively charged molecules are usually cell membrane permeable and accumulate in the mitochondria. Ester is a suitable functional group for staining viable cells because it can pass through viable cell membranes, where it is hydrolyzed by cellular esterases into a negatively charged molecule under physiological conditions. Several fluorescein analogs with ester groups in their structure are available for viable cell staining. Succinimidyl ester compounds can also be used to improve the retention of the fluorescent derivative within the cell. These compounds are neutral molecules that pass through cell membranes and covalently conjugate with cell proteins. Covalently-conjugated molecules can stay in the cell for several weeks. Nucleotide staining with fluorescent intercalators is mostly applied to dead cell detection.

Cell Cytosol Staining
Fluorogenic esterase substrates that can be passively loaded into viable cells, such as Calcein-AM, BCECF-AM, Carboxyfluorescein succinimidyl ester (CFSE), and Fluorescein diacetate (FDA), are converted by intracellular esterases into fluorescein analogs with green fluorescence. Calcein and BCECF are converted into electrically neutral molecules by the addition of acetyl or acetoxymethyl groups to their phenolic OH or carboxylic groups, which allows them to freely permeate into the cell. Once converted into fluorescent products by esterase, these compounds are retained by cells because of their negative charges. These esterase substrates, therefore, can serve as cell viability assay probes.

Fluorescent esterase substrates may also be used in cell viability assays in place of tetrazolium analogs such as MTT or WST. The mechanism of the determination of cell viability is different: though both assays determine cell metabolism, esterase substrates detect esterase activity, and tetrazolium salts detect dehydrogenase activity of viable cells. CFSE is also an ester compound that passes through viable cell membranes. Since it has an amine-reactive succinimidyl group, fluorescein derived from CFSE can covalently bind to proteins or other amino groups in the cell or on the cell membrane. This covalently-attached fluorescein is stable enough to trace the cell over several weeks.

Mitochondria Staining
Mitochondria exist in most eukaryotic cells and play a very important role in oxidative metabolism by generating ATP as an energy source. The average number of mitochondria per cell is from 100 to 2,000. Though the typical size is about 0.5-2mm,
# Cell Staining Reagents

Applications: Fluorescence Microscopy, Flowcytometry, Electrophoresis (Nucleic Acid Screening)

## Characteristics of Dye

<table>
<thead>
<tr>
<th>Target</th>
<th>Dye</th>
<th>Excitation</th>
<th>Emission</th>
<th>Excitation filter</th>
<th>Color</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Living cells</td>
<td>BCECF-AM</td>
<td>490 nm</td>
<td>526 nm</td>
<td>B excitation</td>
<td>yellowish green</td>
<td>Fluorescence is produced by hydrolysis inside the cell.</td>
</tr>
<tr>
<td></td>
<td>Calcein-AM</td>
<td>490 nm</td>
<td>515 nm</td>
<td>B excitation</td>
<td>yellowish green</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CFSE</td>
<td>496 nm</td>
<td>516 nm</td>
<td>B excitation</td>
<td>yellowish green</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CytoRed</td>
<td>535 nm</td>
<td>590 nm</td>
<td>G excitation</td>
<td>red</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FDA</td>
<td>488 nm</td>
<td>530 nm</td>
<td>B excitation</td>
<td>yellowish green</td>
<td></td>
</tr>
<tr>
<td>Dead cells</td>
<td>DAPI</td>
<td>360 nm</td>
<td>460 nm</td>
<td>W excitation</td>
<td>blue</td>
<td>Fluorescence is produced by interacting with the nucleus of dead cells.</td>
</tr>
<tr>
<td></td>
<td>PI</td>
<td>530 nm</td>
<td>620 nm</td>
<td>G excitation</td>
<td>red</td>
<td></td>
</tr>
<tr>
<td>Nucleous</td>
<td>AO (dsDNA)</td>
<td>500 nm</td>
<td>526 nm</td>
<td>B excitation</td>
<td>red</td>
<td>Fluorescence is produced by combining with single stranded and double stranded DNA.</td>
</tr>
<tr>
<td></td>
<td>(ssDNA &amp; RNA)</td>
<td>420-460 nm</td>
<td>630-650 nm</td>
<td>B excitation</td>
<td>yellow</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hoechst33258</td>
<td>350 nm</td>
<td>461 nm</td>
<td>W excitation</td>
<td>blue</td>
<td>Fluorescence is produced by combining with the nucleus of living and dead cells.</td>
</tr>
<tr>
<td></td>
<td>Hoechst33342</td>
<td>352 nm</td>
<td>461 nm</td>
<td>W excitation</td>
<td>blue</td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>MitoRed</td>
<td>560 nm</td>
<td>580 nm</td>
<td>G excitation</td>
<td>red</td>
<td>Fluorescence is produced by accumulation in the mitochondria.</td>
</tr>
<tr>
<td></td>
<td>Rh123</td>
<td>507 nm</td>
<td>529 nm</td>
<td>B excitation</td>
<td>yellowish green</td>
<td></td>
</tr>
</tbody>
</table>

## Product Information

**-Cellstain- Series**

<table>
<thead>
<tr>
<th>Product name</th>
<th>Product code</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCECF-AM</td>
<td>B262-10</td>
<td>1 mg</td>
</tr>
<tr>
<td>Calcein-AM</td>
<td>C326-10</td>
<td>1 mg</td>
</tr>
<tr>
<td>Calcein-AM solution</td>
<td>C396-10</td>
<td>1 ml</td>
</tr>
<tr>
<td>CFSE</td>
<td>C375-10</td>
<td>1 mg</td>
</tr>
<tr>
<td>CytoRed solution</td>
<td>C410-10</td>
<td>1 ml</td>
</tr>
<tr>
<td>FDA</td>
<td>F209-10</td>
<td>1 mg</td>
</tr>
<tr>
<td>DAPI</td>
<td>D212-10</td>
<td>1 mg</td>
</tr>
<tr>
<td>DAPI solution</td>
<td>D523-10</td>
<td>1 ml</td>
</tr>
<tr>
<td>PI</td>
<td>P346-10</td>
<td>1 mg</td>
</tr>
<tr>
<td>PI solution</td>
<td>P378-10</td>
<td>1 ml</td>
</tr>
<tr>
<td>AO</td>
<td>A386-10</td>
<td>1 mg</td>
</tr>
<tr>
<td>AO solution</td>
<td>A430-10</td>
<td>1 ml</td>
</tr>
<tr>
<td>Hoechst33258 solution</td>
<td>H341-10</td>
<td>1 ml</td>
</tr>
<tr>
<td>Hoechst33342 solution</td>
<td>H342-10</td>
<td>1 ml</td>
</tr>
<tr>
<td>MitoRed</td>
<td>R237-10</td>
<td>50 µg x 8 vials</td>
</tr>
<tr>
<td>Rh123</td>
<td>R233-10</td>
<td>1 mg</td>
</tr>
</tbody>
</table>
Cell Staining Reagents

Required Materials

Devices, Tools
- CO₂ incubator
- Clean bench
- Fluorescence microscope
- Cytometer or cell counter
- Centrifuge
- Slide glass, cover glass, or chamber slide

Reagents

Living Cell Staining Dyes
- Cellstain- Calcein-AM (product code: C326)
- Cellstain- CFSE (product code: C375)
- Cellstain- FDA (product code: F209)
- Cellstain- BCECF-AM (product code: B262)

Dead Cell Staining Dyes
- Cellstain- DAPI (product code: D212)
- Cellstain- PI (product code: P346)
- Cellstain- CytoRed solution (product code: C410)

Nucleus Staining Dyes
- Cellstain- AO (product code: A386)
- Cellstain- Hoechst 33258 (product code: H341)

Mitochondria Staining Dyes
- Cellstain- MitoRed (product code: R237)
- Cellstain- Rh123 (product code: R233)

Other Reagents
- DMSO, Sterilized Water, PBS(-)

Preparation of Staining Solution

The following is a general protocol for preparing assay solutions. In order to obtain the best results, optimization of staining conditions, such as changing the reagent concentration and staining time will be required.

Some reagents are stable in the solution. However, some reagents are not stable. Please follow the storage conditions for each reagent. Generally, the reagents offered in the solution form are fairly stable. If no microbalance is available to weigh small amounts of the reagent, add an appropriate amount of solvent described in the chart, aliquot, and store it in a freezer.

Dyes for Living Cell Staining

If the reagent is in a solid form, use DMSO to prepare a solution with a certain concentration. Since CFSE has a succinimidyl group, the stability of the prepared DMSO solution is poor. After the preparation of the DMSO solution, aliquot in an appropriate volume and store at -20 °C. The DMSO solution can be used for several months. The working solutions prepared by PBS (-) are not stable enough to store. Discard the remaining working solution after each use.

Staining solutions are not stable for a storage. Discard the remaining staining solution after each use.
Cell Staining Reagents

Dyes for Dead Cell Staining

If the reagent is in a solid form, use sterilized water to prepare a solution and store according to the indicated condition. Prepare DAPI solution with PBS. Working solutions prepared using PBS(-) are not stable enough to store. Discard the remaining working solution after use.

<table>
<thead>
<tr>
<th>Product name</th>
<th>Characteristic</th>
<th>Storage</th>
<th>Mol. Weight</th>
<th>Units</th>
<th>Stock Solution (H2O)</th>
<th>Staining Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Cellstain-DAPI yellow solid</td>
<td>avoid light, freeze</td>
<td>350.25</td>
<td>1 mg</td>
<td>1 mg/ml</td>
<td>1-10 µg/ml (Storage solution diluted by PBS (-))</td>
<td></td>
</tr>
<tr>
<td>-Cellstain-DAPI soln.</td>
<td>light yellow liquid</td>
<td>avoid light, refrigerate</td>
<td>350.25</td>
<td>1 ml</td>
<td>1 mg/ml*</td>
<td></td>
</tr>
<tr>
<td>-Cellstain-PI red-brown solid</td>
<td>avoid light, refrigerate</td>
<td>668.39</td>
<td>1 mg</td>
<td>1 mg/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-Cellstain-PI soln.</td>
<td>red liquid</td>
<td>avoid light, freeze</td>
<td>668.39</td>
<td>1 ml</td>
<td>1 mg/ml</td>
<td></td>
</tr>
</tbody>
</table>

* Use buffer to prepare a solution

Since the dyes directly stain the nucleus, these dyes are considered mutagens. Gloves, safety goggles, and masks are necessary when handling. If it comes in contact with your skin, immediately wash with a copious amount of water.

When disposing remaining dye solution and solution containing staining dyes, follow handling guidelines and regulations, and entrust disposal to an industrial waste disposal company. If the amount of the dye solution is fairly small and disposal rules and regulations of your institute allow, use a paper towel to adsorb and mix it with plastic tubes used for the preparation of the staining dye solution to incinerate.

Dyes for Nucleus Staining

If the reagent is in a solid form, use sterilized water to prepare a solution and store according to the indicated condition. Working solutions prepared using PBS(-) are not stable enough to store. Discard the remaining working solution after use.

<table>
<thead>
<tr>
<th>Product name</th>
<th>Characteristic</th>
<th>Storage</th>
<th>Mol. Weight</th>
<th>Units</th>
<th>Stock Solution (H2O)</th>
<th>Staining Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Cellstain-AO</td>
<td>red-brown</td>
<td>avoid light, refrigerate</td>
<td>301.81</td>
<td>1 mg</td>
<td>1 mg/ml</td>
<td>1-10 µg/ml (Storage solution diluted by PBS (-))</td>
</tr>
<tr>
<td>-Cellstain-AO soln.</td>
<td>orange-yellow liquid</td>
<td>avoid light, freeze</td>
<td>301.81</td>
<td>1 ml</td>
<td>1 mg/ml</td>
<td></td>
</tr>
<tr>
<td>-Cellstain-Hoechst 33258 soln.</td>
<td>yellow liquid</td>
<td>avoid light, refrigerate</td>
<td>533.88</td>
<td>1 ml</td>
<td>1 mg/ml</td>
<td></td>
</tr>
<tr>
<td>-Cellstain-Hoechst 33342 soln.</td>
<td>yellow liquid</td>
<td>avoid light, refrigerate</td>
<td>561.93</td>
<td>1 ml</td>
<td>1 mg/ml</td>
<td></td>
</tr>
</tbody>
</table>

Since the dyes directly stain the nucleus, these dyes are considered mutagens. Gloves, safety goggles, and masks are necessary when handling. If it comes in contact with your skin, immediately wash with a copious amount of water.

When disposing remaining dye solution and solution containing staining dyes, follow handling guidelines and regulations, and entrust disposal to an industrial waste disposal company. If the amount of the dye solution is fairly small and disposal rules and regulations of your institute allow, use a paper towel to adsorb and mix it with plastic tubes used for the preparation of the staining dye solution to incinerate.

Dyes for Mitochondria Staining

Prepare the stock solution using DMSO.

<table>
<thead>
<tr>
<th>Product name</th>
<th>Characteristic</th>
<th>Storage</th>
<th>Mol. Weight</th>
<th>Units</th>
<th>Stock Solution (H2O)</th>
<th>Staining Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Cellstain-MitoRed purple brown solid</td>
<td>avoid light, refrigerate</td>
<td>637.17</td>
<td>50 µg/ml</td>
<td>1 mmol/l*</td>
<td>20 - 200 mmol/l</td>
<td></td>
</tr>
<tr>
<td>-Cellstain-Rh123 brown powder</td>
<td>avoid light, refrigerate</td>
<td>380.82</td>
<td>1 mg</td>
<td>1 mg/ml</td>
<td>20 - 100 mmol/l</td>
<td></td>
</tr>
</tbody>
</table>

* Avoid storing in the solution.

Only mitochondria in the living cells will be stained.
Add $10^4$-$10^5$ cells to each well with cell culture medium and incubate overnight.

Remove cell culture medium and wash cells by PBS(-) buffer.

Remove PBS(-) buffer, add 0.2 ml "Staining Solution" to cells and incubate cells at 37°C for 15-30 min.

Remove "Staining Solution" and wash cells by PBS(-) buffer for 1-2 times.

Observe the fluorescent image under a fluorescence microscope.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Precautions &amp; Tips</th>
</tr>
</thead>
<tbody>
<tr>
<td>Add $10^4$-$10^5$ cells to each well with cell culture medium and incubate overnight.</td>
<td>Use a cytomter or a cell counter to measure cell number. When using a glass bottom plate, the clear image of cells may be obtained. Gently pipette to avoid damaging the cells.</td>
</tr>
<tr>
<td>Remove cell culture medium and wash cells by PBS(-) buffer.</td>
<td>Gently remove the medium and add PBS buffer without damaging the cells. When using a staining dye for staining living cells, the dye will be hydrolized and emit fluorescence if esterase in the media remains. This is one of the factor for a high background, so it is important to wash the cells several times.</td>
</tr>
<tr>
<td>Remove PBS(-) buffer, add 0.2 ml &quot;Staining Solution&quot; to cells and incubate cells at 37°C for 15-30 min.</td>
<td>Refer to the previous section &quot;Preparation of Assay Solution&quot; and prepare the &quot;Staining Solution&quot;. In order to obtain the best fluorescent image, it is necessary to determine the optimal reagent concentration and staining time.</td>
</tr>
<tr>
<td>Remove &quot;Staining Solution&quot; and wash cells by PBS(-) buffer for 1-2 times.</td>
<td></td>
</tr>
</tbody>
</table>

If you use suspension cells, it’s necessary to centrifuge the cell suspension at 500 xg for 3 min before removing the medium, PBS(-) buffer or “Staining Solution”.

---

**Cell Staining Reagents**

**Staining Procedure for a Fluorescence Microscopy (for 24-well plate)**

**Procedure**

1. Add $10^4$-$10^5$ cells to each well with cell culture medium and incubate overnight.
2. Remove cell culture medium and wash cells by PBS(-) buffer.
3. Remove PBS(-) buffer, add 0.2 ml "Staining Solution" to cells and incubate cells at 37°C for 15-30 min.
4. Remove "Staining Solution" and wash cells by PBS(-) buffer for 1-2 times.
5. Observe the fluorescent image under a fluorescence microscope.

**Precautions & Tips**

- Use a cytomter or a cell counter to measure cell number.
- When using a glass bottom plate, the clear image of cells may be obtained.
- Gently pipette to avoid damaging the cells.
- Gently remove the medium and add PBS buffer without damaging the cells.
- When using a staining dye for staining living cells, the dye will be hydrolized and emit fluorescence if esterase in the media remains. This is one of the factor for a high background, so it is important to wash the cells several times.
- Refer to the previous section "Preparation of Assay Solution" and prepare the "Staining Solution". In order to obtain the best fluorescent image, it is necessary to determine the optimal reagent concentration and staining time.

---

If you use suspension cells, it’s necessary to centrifuge the cell suspension at 500 xg for 3 min before removing the medium, PBS(-) buffer or “Staining Solution”.
Cell Staining Reagents

Experimental Example 1
Living HeLa cells were stained with each reagent by following the protocol on page 34.

Living Cell Staining Images
Cellular hydrolysis activity was visualized at strong fluorescence by living cell staining reagents.

- Calcein-AM (x400, B excitation)
- CytoRed (x200, G excitation)
- BCECF-AM (x200, B excitation)
- FDA (x400, B excitation)
- CFSE (x400, B excitation)

Mitochondria Staining Images
MitoRed accumulated on the mitochondria in the living cell.

- MitoRed (x200, G excitation)
- (Visible light)

Nucleus Staining Images
AO and Hochst33258 produced strong fluorescence after binding with nuclear in dead and living cells.

- AO (x200, B excitation)
- Hochst 33258 (x200, U excitation)
**Experimental Example 2**

HeLa cells were fixed with 80% ethyl alcohol and stained by each reagent.

**Dead Cell Staining Images**

The nuclear of all fixed cells was stained with the dead cell staining reagents.

**Experimental Example 3**

**Fluorescent Staining of Fixed Cells**

NIH3T3 cells that were fixated with 3% glutaraldehyde were stained with the nuclic acid staining reagent Hoechst 33258. Then, actin filaments were stained with biotin-labeled phalloidin and anti-biotin antibody labeled with HiLyte Fluor™ 555.*

* HiLyte Fluor™ Dyes (patent pending) manufactured by AnaSpec. Inc.

**Experimental Example 4**

**Flowcytometry Example**

HL60 cells were stained with Calcein-AM, a reagent used to stain the living cells. The cells were then measured using flowcytometry (excitation: 488 nm). The fluorescence of the stained living cells (blue line) increased dramatically compared to the unstained cells (white line).
Living and Dead Cells Staining: -Cellstain- Double Staining Kit

Introduction

-Cellstain - Double Staining Kit combines Calcein-AM (used for fluorescent staining the living cells) and Propidium Iodide (used for a fluorescent staining of the dead cells) for simultaneous staining of the living and the dead cells.

Product Information

-Cellstain - Double Staining Kit

<table>
<thead>
<tr>
<th>Product code</th>
<th>Unit</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS01-10</td>
<td>1 set</td>
<td>Solution A(Calcein-AM) x 4 vials, Solution B(PI) x 1 vial</td>
</tr>
</tbody>
</table>

Required Materials

Devices, Tools

- CO₂ incubator
- Clean bench
- Fluorescence microscope
- Hematocytometer or cell counter
- Slide glass, cover glass
- Multi-pipette (8 or 12 channel: 10-100 µl)

Reagents

- Cellstain - Double Staining Kit (item code: CS01)
  Kit contents
  Solution A: Calcein-AM stock solution (1 mmol/l) 4 vials
  Solution B: PI stock solution (1.5 mmol/l) 1 vial

- Store at -20 °C and protect from light.

- Solution A (Calcein-AM) is easily hydrolyzed by moisture. Tightly close the cap after the use.

- Concentration of Reagent in dye solution: Calcein-AM: 2 mmol/l, PI: 4 mmol/l

- PBS(-)

Preparation

Staining Solution

Bring Solution A and Solution B to room temperature.
Add 10 µl of Solution A and 15 µl of Solution B to 5 ml of PBS (-) and mix.

- Prepare the staining solution only prior to each use.

- PI may be mutagenic, so wear gloves, safety goggles, and mask when handling. If it comes in contact with your skin, immediately wash with a copious amount of running water.

- When disposing of remaining dye solution, follow handling guidelines and regulations and entrust disposal to an industrial waste disposal company. If the amount of the dye solution is fairly small and disposal rules and regulations of your institute allow, use a paper towel to adsorb and mix it with plastic tubes used for the preparation of the staining dye solution to incinerate.
Living and Dead Cells Staining: *Cellstain*- Double Staining Kit

Staining Procedure for a Fluorescence Microscopy

The below procedure is used to stain adherent cells. Please be aware that the staining conditions may vary depending on the cell types and the concentration of the reagent.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Precautions &amp; Tips</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recover the cells to be assayed from a culture flask.</td>
<td>Recover using the trypsin to detach cells, and use a cell scraper if necessary.</td>
</tr>
<tr>
<td>Centrifuge the cell suspension (500 xg for 3 min).</td>
<td>When using Dye reagents for staining living cells, each ester group of the dye will be hydrolyzed and fluoresce if esterase remains in the media. This is one factor for a high background, so it is important to wash cells several times. Use a hematocytometer or a cell counter. Gently pipette to avoid damaging the cells.</td>
</tr>
<tr>
<td>Remove the supernatant of the media, and add PBS (-). At this step, adjust the cell volume to $10^5$ - $10^6$ cells/ml.</td>
<td></td>
</tr>
<tr>
<td>Add 200 µl of the cell suspension to a microtube.</td>
<td></td>
</tr>
<tr>
<td>Add 100 µl of Staining solution to the same tube.</td>
<td>In order to get the best fluorescent image, it is necessary to determine the optimal reagent concentration and a staining time.</td>
</tr>
<tr>
<td>Incubate at 37 °C for 15-30 min with protection from light.</td>
<td></td>
</tr>
<tr>
<td>Place 10 µl of the cell and staining solution on a glass slide and cover with a cover glass.</td>
<td></td>
</tr>
</tbody>
</table>
Living and Dead Cells Staining: *Cellstain*- Double Staining Kit

View the fluorescent image on a fluorescence microscope.

It is possible to observe yellowish-green stained living cells using a 490 nm excitation filter. In addition, red stained dead cells can be observed simultaneously.

It is possible to observe the fluorescence of dead cells stained red using a 545 nm excitation filter.

**How to Determine the Optimum Concentration of Dye**

The best concentration for Calcein-AM and PI depends on the cell type, so it is necessary to determine the concentration when staining each cell. The best concentration can be determined using the following protocol.

**Optimum concentration for PI**

Stain the desired cells with 0.1 - 10 µmol/l of PI. Too high concentration of PI will stain not only nuclear but also cytosol, staining concentration should be adjust to appropriate range.

Fix the cells prior to staining using one of the method below if necessary:
- Treat the cells for 10 min with 0.1% saponin or 0.1 -0.5% digitonin.
- Treat the cells for 30 min with 70% ethanol.

**Optimum concentration for Calcein-AM**

Using the fixed cells to stain with 0.1 - 10 µmol/l of Calcein-AM solution. Determine the concentration range that will not stain all of the fixed cells. Next, using the living cells, determine if the concentration is enough to stain the cells. If sufficient staining has not been obtained, increase the concentration of Calcein-AM.

**Experimental Example**

Simultaneous staining using *Cellstain*- Double Staining Kit

Green fluorescence indicates the living cells stained by Calcein-AM using B excitation filter.
Red fluorescence indicates the dead cells stained by PI using G excitation filter.
## Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>The cells are not stained well.</td>
<td>The staining dye was hydrolyzed or decomposed due to the exceedingly long term storage or incorrect storage conditions.</td>
<td>Check the purchase date of the reagent and the storage conditions. If the reagent has been stored for a year from the purchase date, do not use. The staining dye may not work properly.</td>
</tr>
<tr>
<td></td>
<td>The dye in the working solution was hydrolyzed or decomposed because the solution was not freshly prepared.</td>
<td>Some of the reagent is unstable in a buffer solution. In particular, viable staining dye is fairly unstable in the buffer solution. Prepare a working solution only prior to each use.</td>
</tr>
<tr>
<td></td>
<td>The dye or the working solution was decomposed by the exposure to light.</td>
<td>Light may accelerate the oxidation process of the dyes. Keep the reagent under the proper storage conditions. Protect the working solution from light during the experiment.</td>
</tr>
<tr>
<td></td>
<td>The concentration of the dye in the working solution is too low.</td>
<td>Increase the concentration of the dye in the working solution. If there is no change, use Pluronic F-127 or another low toxic detergent to improve the dye uptake by the cell if it is allowed.</td>
</tr>
<tr>
<td></td>
<td>The viable cell expels the dye due to the cell function.</td>
<td>Use the stained cell as quickly as possible for your experiments.</td>
</tr>
<tr>
<td></td>
<td>Not enough reagent was used for the cells.</td>
<td>Probenecid, a transporter inhibitor, may be used to block the leakage of the dye from the cell.</td>
</tr>
<tr>
<td></td>
<td>The dye remaining insoluble with the solvent.</td>
<td>Since a vacuum centrifuge was used to prepare the dye product, the dye is tightly packed on the bottom of the tube. Use a vortex mixer or ultra sonic bath to dissolve the dye with the solvent completely.</td>
</tr>
<tr>
<td></td>
<td>The dye was decomposed or hydrolyzed</td>
<td>Check the purchase date of the reagent &amp; storage conditions. If the reagent has been stored for over a year from the purchase date, do not use. The staining dye may be decomposed or hydrolyzed.</td>
</tr>
<tr>
<td></td>
<td>The wrong solvent was used to dissolve.</td>
<td>Simultaneous Staining of living and dead cells Use the proper solvent to prepare a dye solution.</td>
</tr>
<tr>
<td></td>
<td>High fluorescent background is observed.</td>
<td>Not enough washing and the dye still remained after the washing process. Repeat washing with PBS(-) or an appropriate buffer to remove excess dye from the cells.</td>
</tr>
<tr>
<td></td>
<td>Too much dye was used for the staining.</td>
<td>Reduce the concentration of the dye in the working solution.</td>
</tr>
</tbody>
</table>
Staining reagents for living cells

Q: What should the powder-type reagent be dissolved in?
A: Please dissolve the reagent in DMSO for viable cell staining reagents. Since DMSO easily absorbs moisture, please use fresh DMSO.

Q: Among all the staining reagents used for living cells, which one remains the longest inside cells?
A: CFSE remains relatively the longest inside the cells. It has been reported in a paper that the fluorescent dye was retained within cells for up to 8 weeks. Also, the fluorescence of Calcein-AM and BCECF-AM have been observed in cells for up to three days. Please refer to the following for more details:

Q: Which staining reagents used for living cells have the lowest cytotoxicity?
A: Calcein-AM and BCECF-AM seem to have the lowest cytotoxicity, Please refer to the following for more details:

Q: What are the characteristics of staining dyes used for the living cells?
A: Refer to the list below for characteristics of each product:
  BCECF-AM: This was originally used to measure pH inside the cell, and is also used as a dye to stain living cells.
  Calcein-AM: This has the least effect on cell function.
  CFSE: After entering into a cell, it combines with the amino base of protein in the cell membrane on the cytoplasm side. As a result, it leaks out of the cell comparatively less than other dyes.
  CytoRed: A compound produced by Dojindo, it possess a higher fluorescence intensity than Calcein-AM.
  FDA: The oldest known dye. It leaks out of the cell relatively quickly.

Q: Are there any papers that report on the toxicity of the dyes?
A: Refer to the below paper comparing the toxicity of Calcein-AM, BCECF-AM, CFDA, and CFSE.

Q: Which dye should be used to stain the bacteria?
A: Since bacterial cells have a cell wall, most cell staining dyes cannot penetrate. For example, Calcein-AM and BCECF-AM will pass through the cell membrane of animal cells, but will not pass through the bacteria cell wall. AO can be used to stain bacteria such as malaria parasites. PI, and DAPI can be used to stain dead bacteria cells. There is a report of using FDA to stain living bacteria. Refer to the paper below for more information:
Nucleus staining reagents (dead cells)

Q: What are the differences between the nucleus staining reagents AO, Hoechst 33258, and Hoechst 33342 other than fluorescent wavelength?
A: The differences are listed below:
AO: It is possible to distinguish between single stranded DNA and double stranded DNA using the difference in fluorescence wavelength when intercalating with a double stranded DNA and when combining with the phosphoric acid of a single stranded DNA. AO passes through the membrane of living cells.
Hoechst 33258, Hoechst 33342: Binds specifically with adenine - thymine base pairs of DNA. They pass through the cell membrane, and stain the DNA of living cells. Hoechst 33342 has a higher membrane permeability. A better staining is possible when cells are fixed.

Q: What is the method of disposal after use?
A: PI is a possible carcinogen. When disposing remaining dye solution and solution containing staining dyes, follow handling guidelines and regulations and entrust disposal to an industrial waste disposal company. If the amount of the dye solution is fairly small and disposal rules and regulations of your institute allow, use a paper towel to adsorb and mix it with plastic tubes used for the preparation of the staining dye solution to incinerate.

Nucleus staining reagents (living / dead cells)

Q: What is the difference among dyes used to stain the nucleus?
A: Some notable differences other than wavelength are listed below:
PI: It does not have base specificity. It binds to all DNA and RNA., but the fluorescence intensity is higher when intercalating and can be used widely among variety of cells.
DAPI: This will bind with the minor groove of a double chain, and has a high affinity for adenine - thimine base pairs.

Mitochondria staining reagents

Q: Why do MitoRed and Rh123 stain the mitochondria?
A: Both MitoRed and Rh123 employ the chemical structure Rhodamine. Rhodamine has the characteristic of gathering to mitochondria after entering the cell, so it is used as a mitochondria staining dye. When too much dye is introduced into the cell, other areas are stained also, so it is necessary to determine the best concentration in advance.