Cellular biology

Imaging protocol handbook
Trusted collection of imaging protocols

invitrogen
Introduction

This guide contains step-by-step instructions for common imaging protocols, including tips for a successful experiment, and lists of needed supplies. Invitrogen™ reagents are recognized all over the world as key components for publication-quality cellular images and analysis. We offer cellular structure and functional probes, labels, and counterstains, as well as tools for achieving stunning cellular images. With the right preparation and a clear plan of action, you can obtain useful and beautiful cell images.

Visit thermofisher.com/cellimaging to learn more.
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Cellular biology

Cell proliferation
Click-iT EdU Imaging Kits
DNA synthesis-based cell proliferation assay

Introduction
In this assay, the modified thymidine analog EdU (5-ethynyl-2'-deoxyuridine), is efficiently incorporated into newly synthesized DNA and fluorescently labeled with a bright, photostable Invitrogen™ Alexa Fluor™ dye in a fast, highly specific, mild click reaction.

Materials

- Invitrogen™ Click-iT™ EdU Imaging Kit (Cat. No. C10338, C10340, C10337, C10339). Refer to the user manual for component detail.
- PBS (Cat. No. 10010-023)
- Fixative (e.g., Invitrogen Image-iT Fixation/Permeabilization Kit (Cat. No. R37602) or 4% formaldehyde in PBS (Cat. No. R37814))
- Permeabilization reagent (e.g., Invitrogen™ Image-iT™ Fixation/Permeabilization Kit (Cat. No. R37602), or Thermo Scientific™ Triton™ X-100 (Cat. No. A16046.AE))
- 3% bovine serum albumin (BSA) in PBS, pH 7.4
- Deionized water (Cat. No. 751-610)
- 18 × 18 mm coverslips
- Optional: 6-well microplate

Protocol

Prepare stock solutions

1. Allow vials to warm to room temperature.

2. Add 2 mL DMSO (component C) or an aqueous solution to component A to make a 10 mM EdU stock solution. Store at −20°C.

3. Make 1X Click-iT EdU reaction buffer by adding 36 mL of deionized water to component D. Store at 2–8°C.

4. Make Alexa Fluor™ azide by adding 70 μL DMSO (component C) to component B, then mixing well. Store at −20°C.

This protocol is continued on page 2
Click-iT EdU Imaging Kits, cont.
DNA synthesis-based cell proliferation assay

5. Make 10X Click-iT EdU buffer additive by adding 2 mL deionized water to component F and mixing. Store at –20°C.

6. Dilute Invitrogen™ Hoechst™ 33342 dye (component G) 1:2,000 in PBS to obtain a 1X solution.

Label cells with EdU

1. Plate cells on coverslips and incubate overnight.

2. Dilute 10 µL of 10 mM EdU stock solution in 5 mL of pre-warmed tissue culture medium to make a 20 µM EdU labeling solution (enough for 10 coverslips).

3. Remove half of the medium from the cells.

4. Replace with an equal volume of EdU labeling solution (final concentration of 10 µM).

5. Incubate cells under appropriate growth conditions and treatments for 2 hours. Slow-growing cells may require a longer incubation time.

6. Proceed immediately to fixation and permeabilization.

Fix and permeabilize cells

1. Transfer each coverslip into one well of a 6-well plate.

2. Add 1 mL of 3.7% formaldehyde in PBS to each well.

3. Incubate for 15 minutes at room temperature.

4. Remove formaldehyde and wash twice with 1 mL of 3% BSA in PBS.

5. Remove wash solution and add 1 mL of 0.5% Triton X-100 in PBS to each well.

6. Incubate for 20 minutes at room temperature.

Figure 1. Eliminate long detection procedures with Click-iT EdU. A rat ileum tissue section was detected using the red-fluorescent Click-iT EdU Alexa Fluor 594 Imaging Kit (Cat. No. C10084). EdU staining is completed in 80 min., while BrdU protocols require harsh permeabilization and overnight anti-BrdU detection. Nuclei were counterstained with blue-fluorescent Hoechst 33342 dye (Cat. No. H1399).
Detect EdU

1. Make 1X Click-iT EdU buffer additive by diluting the 10X solution created above 1:10 in deionized water. Use this solution within 8 hours.

2. Prepare Click-iT reaction mix according to the table below. Add ingredients in the order listed in the table.

<table>
<thead>
<tr>
<th>Reaction component*</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>5</th>
<th>10</th>
<th>25</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuSO₄ (component E)</td>
<td>20</td>
<td>40</td>
<td>80</td>
<td>100</td>
<td>200</td>
<td>500</td>
<td>1 mL</td>
</tr>
<tr>
<td>Alexa Fluor azide</td>
<td>1.2</td>
<td>2.5</td>
<td>5</td>
<td>6</td>
<td>12.5</td>
<td>31</td>
<td>62</td>
</tr>
<tr>
<td>1X Click-iT EdU reaction buffer</td>
<td>430 µL</td>
<td>860 µL</td>
<td>1.8 mL</td>
<td>2.2 mL</td>
<td>4.3 mL</td>
<td>10.7 mL</td>
<td>21.4 mL</td>
</tr>
<tr>
<td>1X Click-iT EdU buffer additive</td>
<td>50 µL</td>
<td>100 µL</td>
<td>200 µL</td>
<td>250 µL</td>
<td>500 µL</td>
<td>1.25 mL</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>Total volume</td>
<td>500 µL</td>
<td>1 mL</td>
<td>2 mL</td>
<td>2.5 mL</td>
<td>5 mL</td>
<td>12.5 mL</td>
<td>25 mL</td>
</tr>
</tbody>
</table>

* Note: Add the reaction components in the order listed in the table.

3. Remove permeabilization buffer from cells and wash twice with 1 mL of 3% BSA in PBS.

4. Remove the wash solution.

5. Add 0.5 mL of Click-iT EdU reaction mix to each well. Rock the plate briefly to ensure even distribution of reaction mix.

6. Incubate the plate for 30 minutes at room temperature, protected from light.

7. Remove the reaction mix and wash each well once with 1 mL of 3% BSA in PBS.

Additional labels—label with antibodies and stain DNA

1. Wash each well with 1 mL of PBS. Remove the wash solution.

2. Add 1 mL of 1X Hoechst 33342 stain solution per well.

3. Incubate for 30 minutes at room temperature, protected from light.

Optional

Perform antibody labeling of the samples following manufacturer recommendations. Search our extensive portfolio of high-quality antibodies at thermofisher.com/antibodies.

This protocol is continued on page 4
Click-iT EdU Imaging Kits, cont.
DNA synthesis-based cell proliferation assay

4. Remove the Hoechst 33342 solution.

5. Wash each well twice with 1 mL of PBS.

6. Remove the wash solution.

Image

1. Cells labeled with Click-iT EdU are compatible with all methods of slide preparation, including wet mount and prepared mounting media.

2. Image cells with appropriate filters listed below:

<table>
<thead>
<tr>
<th>Invitrogen™ dye</th>
<th>Hoechst™ 33342</th>
<th>Alexa Fluor™ 488</th>
<th>Alexa Fluor™ 555</th>
<th>Alexa Fluor™ 594</th>
<th>Alexa Fluor™ 647</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excitation/emission (nm)</td>
<td>350/461</td>
<td>495/519</td>
<td>555/615</td>
<td>590/615</td>
<td>650/670</td>
</tr>
<tr>
<td>Standard filter set</td>
<td>DAPI</td>
<td>FITC</td>
<td>RFP</td>
<td>Texas Red</td>
<td>Cy5</td>
</tr>
</tbody>
</table>
Click-iT Plus EdU Imaging Kits protocol
DNA synthesis-based cell proliferation assay

Introduction
In this assay the modified thymidine analogue EdU (5-ethynyl-2'-deoxyuridine) is efficiently incorporated into newly synthesized DNA and fluorescently labeled with a bright, photostable Alexa Fluor dye in a fast, highly specific, mild click reaction. Because of the mild reaction conditions, the Invitrogen™ Click-iT™ Plus EdU assays can accurately determine cell proliferation while preserving cell morphology, DNA integrity, antigen-binding sites, and the fluorescent signal from GFP.

Materials
- Click-iT Plus EdU Imaging Kit (Cat. No. C10637, C10638, C10639, C10640). Refer to the user manual for component detail.
- PBS (Cat. No. 10010-023)
- Fixative (e.g., Invitrogen™ Image-iT™ Fixation/Permeabilization Kit (Cat. No. R37602), 3.7% Formaldehyde in PBS (Cat. No. R37602))
- Permeabilization reagent (e.g., Invitrogen™ Image-iT™ Fixation/Permeabilization Kit (Cat. No. R37602), Thermo Scientific™ Triton™ X-100 detergent (Cat. No. A16046.AE))
- 3% bovine serum albumin (BSA) in PBS, pH 7.4
- Deionized water (Cat. No. 751-610)
- 18 x 18 mm coverslips
- Optional: 6-well microplate

Protocol

Prepare stock solutions
1. Allow vials to warm to room temperature.
2. Add 2 mL DMSO (component C) or an aqueous solution to component A to make a 10 mM EdU stock solution. Store at –20°C.
3. Make 1X Click-iT EdU reaction buffer by transferring the solution (4 mL) in the component D bottle to 36 mL of deionized water. Store any remaining solution at 2–8°C.

Critical notes
This protocol can be used for:
- Detecting DNA synthesis using an Invitrogen™ EVOS™ imaging system

This protocol should not be used for:
- Flow cytometry; for a flow cytometry protocol, see Click-iT EdU Protocol for Flow Cytometry

Protocol tips
- For in vivo experiments, additional EdU can be purchased separately (Cat. No. A10044, E10187).
- Fixation/permeabilization reagents such as methanol and saponin can be used instead of the included Triton X-100 detergent.

This protocol is continued on page 6
Click-iT Plus EdU Imaging Kits protocol, cont.
DNA synthesis-based cell proliferation assay

4. Make 10X Click-iT EdU buffer additive by adding 2 mL deionized water to component F and mixing until fully dissolved. Store at –20°C.

5. Dilute Hoechst 33342 stain (component G) 1:2,000 in PBS to obtain a 1X solution.

Label cells with EdU

1. Plate cells on coverslips and incubate overnight.

2. Dilute 10 µL of 10 mM EdU stock solution in 5 mL of pre-warmed tissue culture medium to make a 20 µM EdU labeling solution (enough for 10 coverslips).

3. Remove half of the medium from cells.

4. Replace with an equal volume of EdU labeling solution (final concentration of 10 µM).

5. Incubate cells under appropriate growth conditions for two hours.

6. Proceed immediately to fixation and permeabilization.

Fix and permeabilize cells

1. Transfer each coverslip into one well of a 6-well plate.

2. Add 1 mL of 3.7% formaldehyde in PBS to each well.

3. Incubate for 15 minutes at room temperature.

4. Remove formaldehyde and wash twice with 1 mL of 3% BSA in PBS.

5. Remove wash solution and add 1 mL of 0.5% Triton X-100 in PBS to each well.

6. Incubate for 20 minutes at room temperature.

Note
The choice of incubation time depends on the cell growth rate; thus, optimization may be required.

This protocol is continued on page 7
Detect EdU

1. Make 1X Click-iT EdU buffer additive by diluting the 10X solution created above 1:10 in deionized water. Prepare this solution fresh and use the solution on the same day.

2. Prepare Click-iT Plus reaction mix according to the table below.

3. Remove permeabilization buffer from cells and wash twice with 1 mL of 3% BSA in PBS.

4. Remove the wash solution.

5. Add 0.5 mL of Click-iT Plus reaction mix to each well. Rock the plate briefly to ensure even distribution of reaction mix.

6. Incubate the plate for 30 minutes at room temperature, protected from light.

7. Remove the reaction mix and wash each well once with 1 mL of 3% BSA in PBS.

Figure 2. Multicolor imaging with the Click-iT Plus EdU Alexa Fluor 647 compatible with GFP.

This protocol is continued on page 8
Click-iT Plus EdU Imaging Kits protocol, cont.
DNA synthesis-based cell proliferation assay

Stain DNA

1. Wash each well with 1 mL of PBS. Remove the wash solution.
2. Add 1 mL of 1X Hoechst 33342 stain solution per well.
3. Incubate for 30 minutes at room temperature, protected from light.
4. Remove the Hoechst 33342 solution.
5. Wash each well twice with 1 mL of PBS.
6. Remove the wash solution.

Image

1. Cells labeled with Click-iT Plus EdU are compatible with all methods of slide preparation, including wet mount and prepared mounting media.
2. Image cells with appropriate filters listed below.

<table>
<thead>
<tr>
<th>Invitrogen™ dye</th>
<th>Hoechst™ 33342</th>
<th>Alexa Fluor™ 488</th>
<th>Alexa Fluor™ 555</th>
<th>Alexa Fluor™ 594</th>
<th>Alexa Fluor™ 647</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excitation/emission (nm)</td>
<td>350/461</td>
<td>495/519</td>
<td>555/615</td>
<td>590/615</td>
<td>650/670</td>
</tr>
<tr>
<td>Standard filter set</td>
<td>DAPI</td>
<td>FITC</td>
<td>RFP</td>
<td>Texas Red</td>
<td>Cy5</td>
</tr>
</tbody>
</table>

Optional
Perform antibody labeling of the samples at this time, following the recommendations from the manufacturer of the primary and secondary antibody.

Note
Protect samples from light during these incubations.
Introduction
The Invitrogen™ Click-iT™ EdU Colorimetric IHC Detection Kit is a novel alternative to the BrdU assay. EdU (5-ethynyl-2’-deoxyuridine) is a nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis. After the target tissue is fixed and embedded in paraffin, a click reaction covalently attaches the biotin-azide to the alkyne group on the incorporated EdU. Next, streptavidin-peroxidase (horseradish peroxidase) is added to the sample and attaches to the biotin group. Finally, addition of the DAB (peroxidase) substrate results in the colorimetric detection of proliferating cells.

Materials
• Click-iT EdU Colorimetric IHC Detection Kit (Cat. No. C10644)
• 1X Phosphate-buffered saline (PBS) (e.g., Gibco™ DPBS (Cat. No. 14190-144 or 14190-250))
• 3% H₂O₂ in PBS
• 18 × 18 mm coverslips (for standard microscopy)
• Optional: Invitrogen™ EdU (Cat. No. A10044, E10187, E10415)

Protocol
Prepare stock solutions
1. EdU is readily soluble in DMSO, alcohol, water, or aqueous buffers. Depending on your application, prepare an appropriate stock solution of EdU in DMSO or aqueous buffer. To make a 10 mM solution of EdU (component A), add 4 mL DMSO or aqueous solution (e.g., buffer, saline) to component A and mix well. You can store the 10 mM EdU stock solution at ≤–20°C for up to 1 year.

EdU has a characteristic 288 nm absorption peak that can be used to accurately quantitate stock solutions by absorbance using the extinction coefficient of 12,000 cm⁻¹ M⁻¹ in methanol. A 10 mg/mL solution (39.6 mM) when diluted 1:1,000 in methanol gives an absorbance of 0.475 at 288 nm.

2. 1X Click-iT EdU Reaction Buffer (component B) working solution: Transfer all of the solution (4 mL) in the component B vial to 36 mL of deionized water. Rinse the component B vial with some of the diluted Click-iT EdU Reaction Buffer to ensure the transfer of all of the 10X concentrate. To make smaller amounts of 1X Click-iT EdU Reaction Buffer, dilute volumes from the component B bottle 1:10 in deionized water. After use, store any remaining 1X solution at 2–8°C. When stored as directed, this 1X solution is stable for up to 6 months.

This protocol is continued on page 10
Click-iT EdU Colorimetric IHC Detection Kit, cont.

3. 10X Click-iT EdU Reaction Buffer Additive (component F) stock solution: Add 2 mL of deionized water to the component F vial, then mix until fully dissolved. After use, store any remaining stock solution at ≤−20°C. When stored as directed, this stock solution is stable for up to 1 year. If the solution develops a brown color, it has degraded and should be discarded.

4. Biotin azide (component D) stock solution: Add 70 μL of DMSO (component E) to the component D vial and mix well. After use, store any remaining stock solution at ≤−20°C. When stored as directed, this stock solution is stable for up to 1 year.

5. 1X Click-iT EdU Wash Buffer: Dilute the Click-iT EdU Wash Buffer (component H) 1:20 in 1X PBS.

The following protocols describe how to perform the Click-iT EdU Colorimetric IHC Detection assay on FFPE tissue samples.

EdU labeling
10 mg of EdU (component A) will be sufficient for labeling 1–2 mice depending on the size of the mouse and the treatment method (intraperitoneal injection or drinking water).

In initial experiments, we recommend testing a range of EdU concentrations to determine the optimal concentration. The optimal concentration may vary depending upon the duration of the pulse, with lower concentrations recommended for longer incubations. General recommendations for EdU labeling are listed below.

<table>
<thead>
<tr>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flatworm (marine)</td>
<td><em>BioProbes</em> 61</td>
</tr>
<tr>
<td>Rat</td>
<td>Scientific poster, ASCB 2007</td>
</tr>
<tr>
<td>Zebrafish larva</td>
<td><em>BioProbes</em> 57</td>
</tr>
<tr>
<td>Zebra finch</td>
<td>Scientific poster, ASCB 2007</td>
</tr>
</tbody>
</table>

* Visit thermofisher.com/edu for links to PubMed entries, scientific posters, or detailed protocols.

This protocol is continued on page 11
Deparaffinize tissue sections

1. To deparaffinize tissue sections, place the slides in a rack and perform the wash steps listed in the table below in a Coplin staining jar, or use standard deparaffinization rehydration protocols.

<table>
<thead>
<tr>
<th>Tissue deparaffinization procedure</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>5 minutes</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>3 minutes</td>
</tr>
<tr>
<td>95% EtOH</td>
<td>3 minutes</td>
</tr>
<tr>
<td>85% EtOH</td>
<td>3 minutes</td>
</tr>
<tr>
<td>75% EtOH</td>
<td>3 minutes</td>
</tr>
<tr>
<td>50% EtOH</td>
<td>3 minutes</td>
</tr>
<tr>
<td>1X PBS</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>

2. To quench endogenous peroxidase enzymes, immerse the slides in a solution of 3% H₂O₂ in PBS for 10 minutes at room temperature.

3. Rinse three times in 1X PBS for 2 minutes each.

4. Digest the tissue sections with trypsin-EDTA (component G) to aid in antigen retrieval. Optimum digestion time depends on the tissue type. Most other methods of antigen retrieval have been tested and may be used in place of trypsin digestion. A DNA unmasking step is not required. See the table below for general recommendations.

<table>
<thead>
<tr>
<th>Recommended Trypsin-EDTA treatment for various tissues</th>
<th>Species, tissue type</th>
<th>Incubation time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse, embryonic cardiac</td>
<td>0–5 minutes</td>
<td>Room temperature</td>
<td></td>
</tr>
<tr>
<td>Mouse, adult cardiac</td>
<td>20–30 minutes</td>
<td>Room temperature</td>
<td></td>
</tr>
<tr>
<td>Rat Mammary</td>
<td>10 minutes</td>
<td>Room temperature</td>
<td></td>
</tr>
<tr>
<td>Rat Intestine</td>
<td>20–30 minutes</td>
<td>Room temperature</td>
<td></td>
</tr>
<tr>
<td>Rat Uterine</td>
<td>30 minutes</td>
<td>37°C</td>
<td></td>
</tr>
<tr>
<td>Zebra fish, adult Caudal fin</td>
<td>0–5 minutes</td>
<td>Room temperature</td>
<td></td>
</tr>
</tbody>
</table>

Note: Covering the tissue with a coverslip during the incubation steps will allow the tissue to be covered uniformly. Dry the slide edges prior to adding trypsin-EDTA to prevent wicking.
5. Remove the trypsin-EDTA solution by washing the tissue sections 3 times in 1X PBS for 2 minutes each. If using a coverslip, tip the slide to remove the coverslip before proceeding with the wash step.

## EdU detection

1. Prepare a working solution of 1X Click-iT EdU Reaction Buffer Additive by diluting the 10X solution 1:10 in deionized water. Prepare this solution fresh and use it on the same day. Discard any unused 1X solution.

2. Prepare the Click-iT EdU reaction mix according to the table below. It is important to add the reaction components in the order listed in the table; otherwise, the reaction will not proceed optimally.

<table>
<thead>
<tr>
<th>Reaction component*</th>
<th>Number of coverslips</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1X Click-iT Reaction Buffer (from “Prepare stock solutions” step 2)</td>
<td>439 µL</td>
</tr>
<tr>
<td>CuSO₄ (component C)</td>
<td>10 µL</td>
</tr>
<tr>
<td>Biotin azide (from “Prepare stock solutions” step 4)</td>
<td>1.2 µL</td>
</tr>
<tr>
<td>1X Click-iT™ EdU Reaction Buffer Additive (from “Edu detection” step 1)</td>
<td>50 µL</td>
</tr>
<tr>
<td>Total volume (approximate)</td>
<td>500 µL</td>
</tr>
</tbody>
</table>

* Note: Add the reaction components in the order listed in the table.

3. Immediately after preparation, add 0.5 mL of the Click-iT EdU reaction mix (from step 2) to the prepared tissue sections and allow the solution to spread completely over the surface of the tissue.

4. Incubate for 30 minutes at room temperature.

5. **Optional**: Wash the tissue sections with the 1X Click-iT EdU Wash Buffer (from step 5 in “Prepare stock solutions”) for 15 minutes. This optional step reduces the background signal.

---

**Note**

This protocol uses 500 µL of Click-iT EdU reaction mix per tissue section. Lower volumes can be used as long as all others are maintained at the same ratios.

**Critical note**

Use the Click-iT EdU reaction mix within 15 minutes of preparation.

**Note**

We recommend using a cover slip or a humidified chamber to protect against evaporation.

**Note**

For step 5, we recommend using the wash chamber included in the kit.

---

This protocol is continued on page 13
6. Wash the tissue sections 3 times for 2 minutes each with 1X PBS at room temperature.

7. Add 2 drops of the 1X Streptavidin-Peroxidase Conjugate (component I) and incubate at room temperature for 30 minutes in a humidified chamber.

8. Remove unbound Streptavidin-Peroxidase Conjugate by washing the tissue sections 3 times for 2 minutes each with 1X PBS at room temperature. If using a coverslip, tip the slide to remove the coverslip before proceeding with the wash step.

9. Rinse briefly in deionized water and remove residual water without allowing the tissue to dry out.

10. For each slide to be developed, prepare 200 μL of 1X DAB reaction mixture by combining 10 μL of DAB Chromogen (component K) with 190 μL of DAB Substrate Buffer (component J) in a centrifuge tube immediately before use. This results in a 1:20 dilution of the DAB Chromogen in DAB Substrate Buffer.

11. Add 200 μL of the 1X DAB reaction mixture (1:20 dilution of DAB Chromogen; from step 10) to each tissue section and incubate at room temperature for 1–10 minutes depending on desired signal intensity. Discard any unused 1X solution.

12. Wash each tissue section thoroughly with deionized water and counterstain, if desired. Mount the tissue sections in standard aqueous or hard mounting medium before imaging. You can also image the tissue sections prior to mounting, if desired.

Note
Covering the tissue with a coverslip during incubation steps will allow the tissue to be covered uniformly by the reaction components. Dry the slide edges prior to adding the Streptavidin-Peroxidase Conjugate to prevent wicking.

Critical note
Prepare the 1X DAB reaction mixture immediately before use. Discard any unused 1X solution.

Note
Depending on the desired signal strength, you may need to optimize the 1:20 recommended DAB Chromogen dilution. Dilutions of 1:100 to 1:200 for the DAB Chromogen may be needed if the signal develops too rapidly.

Figure 3. Labeling of proliferating cells in mouse cardiac tissue using the Click-iT EdU Colorimetric kit and Movat’s pentachrome stain.
Cellular biology

Cell viability
LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells
Two-color dual-parameter cell viability assay

Introduction
This kit permits quick and easy determination of cell viability using two common microscope filters (FITC and RFP) to discriminate live from dead cells by simultaneously staining with green-fluorescent calcein AM to indicate intracellular esterase activity and red-fluorescent ethidium homodimer-1 to indicate loss of plasma membrane integrity.

Materials
- Cells growing in culture
- Invitrogen™ LIVE/DEAD™ Viability/Cytotoxicity Kit (Cat. No. L3224). Refer to the user manual for component detail.
- Gibco™ Dulbecco’s Phosphate-Buffered Saline (DPBS) (Cat. No. 14040-117)
- Invitrogen™ EVOS™ imaging system with FITC and RFP filters

Protocol
1. Culture cells in an appropriate medium and vessel for microscopy.
2. Thaw vials.
3. Add 5 µL calcein AM (component A) and 20 µL ethidium homodimer-1 (component B) to 10 mL DPBS to create the staining solution.
4. Remove the medium from the cells.
5. Add 100–200 µL of the staining solution directly to the cells.
6. Incubate 30 minutes at 20–25°C.
7. Image the cells.

Critical notes
This protocol can be used for:
- Identifying live and dead cells using an Invitrogen™ EVOS™ imaging system
This protocol should not be used for:
- Flow cytometry

Protocol tips
- Use stock solutions within 1 day.
- Optimal dye concentrations are likely to vary depending on cell type; use the highest dye concentration that gives minimal background.
- The stains in this kit do not survive fixation or permeabilization.

Critical notes
Figure 4. Cell viability staining performed with calcein AM and ethidium homodimer-1 from the LIVE/DEAD Viability/Cytotoxicity Kit.

Spectral information and storage

<table>
<thead>
<tr>
<th>Invitrogen™ dye</th>
<th>Excitation/emission</th>
<th>Standard filter set</th>
<th>EVOS Light Cube</th>
<th>Storage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcein AM</td>
<td>494/517 nm</td>
<td>FITC or GFP</td>
<td>GFP</td>
<td>–20°C</td>
</tr>
<tr>
<td>Ethidium homodimer-1</td>
<td>528/617 nm</td>
<td>RFP</td>
<td>RFP</td>
<td>–20°C</td>
</tr>
</tbody>
</table>
ReadyProbes Cell Viability Imaging Kit, Blue/Red
Two-color nuclear staining assay for cell viability

Introduction
This kit can be used to quickly and easily determine the viability of cells. Invitrogen™ NucBlue™ Live ReadyProbes™ Reagent stains the nuclei of all cells, while propidium iodide stains only the nuclei of dead cells.

Materials
- Cells growing in culture
- Invitrogen™ ReadyProbes™ Cell Viability Imaging Kit, Blue/Red (Cat. No. R37610)
- Invitrogen™ EVOS™ imaging system with DAPI and RFP or TRITC filters

Protocol
1. Culture cells in an appropriate medium and vessel for microscopy.
2. Add 2 drops each of NucBlue Live ReadyProbes Reagent and propidium iodide per mL of medium to label the cells.
3. Incubate for 5–30 minutes.
4. Image the cells.

Critical notes
This protocol can be used for:
- Identifying live and dead cells using an Invitrogen™ EVOS™ imaging system

This protocol should not be used for:
- Flow cytometry

Protocol tip
- In some cases, more or fewer drops of dye may be needed to achieve optimal staining intensity.

Spectral information and storage

<table>
<thead>
<tr>
<th>Invitrogen™ dye</th>
<th>NucBlue Live ReadyProbes Reagent</th>
<th>Propidium iodide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excitation/emission</td>
<td>360/460 nm</td>
<td>528/617 nm</td>
</tr>
<tr>
<td>Standard filter set</td>
<td>DAPI</td>
<td>TRITC or RFP</td>
</tr>
<tr>
<td>EVOS Light Cube</td>
<td>DAPI</td>
<td>TRITC or RFP</td>
</tr>
<tr>
<td>Storage conditions</td>
<td>Room temperature</td>
<td>Room temperature</td>
</tr>
</tbody>
</table>

Figure 5. Jurkat cell viability determination using the ReadyProbes Cell Viability Imaging Kit.
Cellular biology

Cell structure
**Introduction**

MitoTracker probes are cell-permeant mitochondrial stains that contain a mildly thiol-reactive chloromethyl moiety. Following incubation, MitoTracker dyes passively diffuse across the plasma membrane and accumulate in the mitochondria of live cells. These dyes are offered in a range of wavelengths that can all be used for mitochondrial localization in multicolor experiments.

**Materials**

- Invitrogen™ MitoTracker™ dye (Cat. No. M7514, M7510, M7511, M7512, M7513, M22425, or M22426). Refer to the user manual for component detail.
- DMSO
- Suitable buffer or growth medium for live-cell imaging
- Optional: Aldehyde-based fixatives such as formaldehyde for cell fixation
- Optional: Aldehyde-based detergents such as Triton™ X-100

**Protocol**

**Preparing stock solutions**

To prepare a stock solution, dissolve the lyophilized Invitrogen™ MitoTracker™ mitochondrion-selective probe in high-quality, anhydrous dimethylsulfoxide (DMSO) to a final concentration of 1 mM; the molecular weight (MW) is indicated on the product label. The reduced rosamine MitoTracker probes (Cat. No. M7511, M7513) are quite sensitive to oxidation, especially in solution, and must be stored under argon or nitrogen, at ≤–20°C and protected from light. It is preferable to use solutions of the dihydro derivatives immediately after they are prepared. Store all other solutions of the MitoTracker dyes frozen at ≤–20°C and protected from light.

**Cell preparation and staining**

The concentration of probe for optimal staining varies by application. The initial conditions suggested here are guidelines that may be modified based on the particular cell type or on other factors, such as the permeability of the cells or tissues to the probe. In general, the reduced rosamine MitoTracker probes are loaded at 3- to 5-fold higher concentrations than other MitoTracker probes.

This protocol is continued on page 18
Preparing staining solutions. Dilute 1 mM MitoTracker stock solution (see “Preparing stock solutions”) to the final working concentration in an appropriate buffer or growth medium. Because the reduced forms of the MitoTracker probes are susceptible to potential oxidases in serum, we do not recommend using complete media with these dyes.

1. Staining adherent cells. Grow cells on coverslips inside a petri dish filled with the appropriate culture medium. When cells have reached the desired confluency, remove the medium from the dish and add pre-warmed (37°C) staining solution containing MitoTracker probe (prepared in step 1). Incubation for 15–45 minutes under growth conditions appropriate for the particular cell type is generally sufficient but may need to be optimized. After staining is complete, replace the staining solution with fresh pre-warmed medium or buffer and observe the cells using an Invitrogen™ EVOS™ imaging system or fluorescence microplate reader. If the cells are to be fixed and permeabilized, continue to the section on fixation and permeabilization after staining.

2. Staining suspension cells. Centrifuge to obtain a cell pellet and aspirate the supernatant. Resuspend the cells gently in pre-warmed (37°C) staining solution containing the MitoTracker probe (prepared in step 1). Incubation for 15–45 minutes under growth conditions appropriate for the particular cell type is generally sufficient but may need to be optimized. After staining is complete, re-pellet the cells by centrifugation and resuspend cells in fresh pre-warmed medium or buffer. Cells can be analyzed by flow cytometry, microplate-based analysis, or fluorescence microscopy. If immobilized cells on coverslips are needed, use poly-D-lysine to coat the slides or coverslips before mounting. If the cells are to be fixed and permeabilized, continue to section on fixation and permeabilization after staining.

Figure 6. Multicolor staining of bovine pulmonary artery endothelial (BPAE) cells. Cells were stained with Alexa Fluor 488 NPCP (Ms), MitoTracker Red CMXRos, and Alexa Fluor 350 phalloidin.
Optional: Fixation and permeabilization after staining

After staining live cells with a MitoTracker dye, it is often useful to fix and permeabilize the cells for subsequent manipulations. Most of the MitoTracker dyes are well-retained following fixation and permeabilization using the protocol described here. However, MitoTracker Green FM and MitoTracker Red FM are not retained well after fixation.

1. **Wash the cells.** After staining, wash the cells in fresh, pre-warmed buffer or growth medium.

2. **Fix the cells.** Carefully remove the medium/buffer covering the cells, and replace it with freshly prepared, pre-warmed buffer or growth medium containing 2–4% formaldehyde. For MitoTracker Red CMXRos, we have found that fixing with 3.7% formaldehyde in complete growth medium at 37°C for 15 minutes works well for endothelial cells.

3. **Rinse the cells.** After fixation, rinse the cells several times in buffer.

4. **Optional: Permeabilize the cells.** If permeabilization is needed for subsequent steps such as immunocytochemistry, incubate fixed cells in a buffer containing detergent such as Triton X-100. Following permeabilization, rinse the cells in buffer and proceed with immunocytochemistry. We found that incubating the endothelial cells for 10 minutes in PBS containing 0.2% Triton X-100 works well. Alternatively, the cells can be permeabilized by incubating in ice-cold acetone for 5 minutes, and then washed in PBS.
Phalloidin reagents for actin labeling

Introduction
Phalloidin is a bicyclic peptide that belongs to a family of toxins isolated from the deadly *Amanita phalloides* “death cap” mushroom and is commonly used in imaging applications to selectively label F-actin in fixed cells, permeabilized cells, and cell-free experiments. Phalloidin conjugates have similar affinity for both large and small filaments and bind in a stoichiometric ratio of about one phallotoxin per actin subunit in both muscle and non-muscle cells; they reportedly do not bind to monomeric G-actin.

Materials
- Fluorescent or biotin phalloidin (refer to the table on page 21)
- DMSO, Anhydrous (Cat. No. D12345)
- PBS (1X), pH 7.4 or equivalent imaging-grade PBS (Cat. No. 10010049)
- Invitrogen™ Image-iT™ Fixative Solution (4% formaldehyde, methanol-free), or equivalent methanol-free formaldehyde (Cat. No. FB002)
- Biotin-binding conjugate, such as streptavidin or NeutrAvidin™ fluorescent or enzyme conjugate (for use with Biotin-XX Phalloidin only)
- 1-Palmitoyl-sn-glycero-3-phosphocholine
- Optional: Triton™ X-100 Surfact-Amps™ Detergent Solution (or equivalent detergent), or imaging-grade acetone (Cat. No. 85112)
- Optional: Bovine serum albumin (BSA) (Cat. No. 23209)
- Optional: Invitrogen™ Image-iT™ FX Signal Enhancer (Cat. No. I36933)
- Optional: Invitrogen™ BlockAid™ Blocking Solution (Cat. No. B10710)
- Optional: DNA counterstain, one of the following, or equivalent:
  - Invitrogen™ NucBlue™ Fixed Cell ReadyProbes™ Reagent (Cat. No. R37606)
  - Invitrogen™ SYTOX™ Deep Red Nucleic Acid Stain (Cat. No. S11381)
- Optional: Invitrogen™ ProLong™ Glass Antifade Mountant, or equivalent slide mounting solution (Cat. No. P36980)

This protocol is continued on page 21
Under standard experimental conditions for cultured cells, preparing a stock solution in anhydrous DMSO yields superior staining intensity and retention of F-actin structural integrity compared to alcohol-based and aqueous solvents.

Note
One unit/assay of fluorescent phalloidins is equivalent to 0.5 µL of the DMSO stock solution.
Phalloidin reagents for actin labeling, cont.

- **Methanol stock solution:** Dissolve the vial contents in 1.5 mL of methanol to yield a 40X stock solution at a concentration of approximately 6.6 µM, which provides for 200 assays/mL.

Prepare Biotin-XX Phalloidin
- Dissolve the vial contents in 0.5 mL of methanol to yield a final concentration of 100 units/assays per mL, which is equivalent to approximately 20 µM.

Stain formaldehyde-fixed cells
This protocol describes the staining procedure for adherent cells that are grown on glass coverslips.

1. Wash the sample two times with pre-warmed PBS.

2. Fix the sample in 3.7% methanol-free formaldehyde solution in PBS for 15 minutes at room temperature.

3. Wash the sample two or more times with PBS.

4. Permeabilize the sample in 0.1% Triton X-100 in PBS for 15 minutes.

5. Wash the sample two or more times with PBS.

6. Optional: When multiplexing with antibodies, incubate the sample in BlockAid Blocking Solution (Cat. No. B10710) or a similar blocking solution containing 1% BSA for 30–45 minutes at room temperature. Search our extensive portfolio of high-quality antibodies at thermofisher.com/antibodies.

7. Optional: Incubate the sample in Image-iT FX Signal Enhancer (Cat. No. I36933) for 20–30 minutes to enhance the signal. Note: If antibody staining is desired, perform the primary and secondary antibody incubation separately, between step 7 and step 8, according to the manufacturer’s protocol. Labeling samples with phalloidins can be combined with secondary antibody staining. Ensure that the correct dilutions of each reagent are used in the staining solution.

**Note**
- One unit/assay of fluorescent phalloidins is equivalent to 5 µL of the methanolic stock solution.

**Note**
- Cells are stained using a higher concentration of Invitrogen™ Biotin-XX Phalloidin compared to fluorescent phalloidins.
- Cells stained with Biotin-XX Phalloidin require a fluorescent or enzyme-conjugated avidin or streptavidin detection reagent.

**Critical note for step 2**
Avoid methanol-containing fixatives. Methanol can disrupt actin during the fixation process. We recommend using methanol-free formaldehyde, such as Image-iT Fixative Solution (4% formaldehyde, methanol-free) (Cat. No. FB002).

**Note for step 4**
Certain samples can require permeabilization in an acetone solution at −20°C in a glass petri dish.

**Note**
The staining protocol that is described here is compatible with most signal amplification techniques that are used for ICC, IHC, or FISH (such as Invitrogen™ Tyramide SuperBoost™ signal amplification).
8. Prepare the staining solution as indicated in table below.

<table>
<thead>
<tr>
<th>For...</th>
<th>Do this...</th>
</tr>
</thead>
</table>
| Fluorescent phalloidin staining solution | 1. Dilute the stock solution.  
  • For a DMSO stock solution—dilute 0.5 µL of the 40X stock solution in 200 µL of PBS for each coverslip to be stained.  
  • For a methanol stock solution—dilute 5 µL of the 40X methanol stock solution in 200 µL of PBS for each coverslip to be stained.  
  2. Add 1% bovine serum albumin (BSA) to reduce nonspecific background staining. |
| Biotin-XX Phalloidin staining solution  | 1. Dilute 10 µL of the methanol stock solution in 200 µL of PBS for each coverslip to be stained.  
  2. Add 1% bovine serum albumin (BSA) to reduce nonspecific background staining.                                                                                                                                 |

Incubate the sample in the staining solution as indicated:

**For fluorescent phalloidins**—Add the fluorescent phalloidin staining solution to each coverslip, then incubate for 30–60 minutes at room temperature. Place the coverslips in a covered container to prevent evaporation during the incubation.

**For Biotin XX Phalloidin**—Add the Biotin-XX Phalloidin staining solution to each coverslip, then incubate for 15 minutes at room temperature.

9. *Optional:* If needed, add a DNA counterstain, such as NucBlue Fixed Cell ReadyProbes Reagent (Cat. No. R37606) or SYTOX Deep Red Nucleic Acid Stain (Cat. No. S11381) for fixed or dead cells.

10. Wash the sample two or more times with PBS.

11. For long-term storage, mount the sample in a curing or hard-setting aqueous mountant, such as ProLong Glass Antifade Mountant (Cat. No. P36980). Specimens that are prepared in this manner retain actin staining for at least six months when stored in the dark at 2–6°C.

Note

When staining more than one coverslip, adjust the volumes accordingly. For a stronger signal, use 2–3 times more of the staining solution per coverslip.

Note

If you are using Biotin-XX Phalloidin, follow the procedure that is recommended for the specific enzyme to develop enzyme activity. For example, prepare a 10 µg/mL solution of fluorescent or enzyme-conjugated streptavidin in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.3% Triton X-100, and 1% BSA. Prepare enough to add 100 µL to each coverslip. Add 100 µL of the fluorescent or enzyme-conjugated streptavidin solution to each coverslip, then incubate for 30 minutes at room temperature.

Critical note

Do not use mountants that contain organic solvents.

Note

If the samples are not mounted, or are mounted using non-curing mountants, imaging the cells immediately is highly recommended. Phalloidin conjugates lose their signal intensity with increased storage time. The rate of signal loss during storage can differ depending on the type of conjugate, mountant, or storage temperature used. Our experiments indicate that cells mounted in Invitrogen™ SlowFade™ Diamond Antifade Mountant or Invitrogen™ SlowFade™ Glass Antifade Mountant retain actin staining for two weeks or more (when stored at ~20°C with multiple freeze/thaw cycles).

This protocol is continued on page 24
Phalloidin reagents for actin labeling, cont.

**Simultaneously fix, permeabilize, and stain cells**
Phalloidins are only stable for a short amount of time in 4% formaldehyde fixation buffers. This protocol describes a rapid one-step fixation, permeabilization, and labeling procedure.

1. Prepare a 1 mL solution containing 50–100 µg/mL of 1-palmitoyl-sn-glycero-3-phosphocholine and 3.7% methanol-free formaldehyde, then add 25–50 µL of the fluorescent phalloidin methanol stock solution.

2. Add the solution to the sample, then incubate for 20 minutes at 4°C.

3. Rapidly wash the sample three times with PBS.

4. Mount the coverslip, then image the cells.

<table>
<thead>
<tr>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>We recommend using the fluorescent phalloidin methanol stock solution for this procedure. For information on preparing the methanol stock solution, see “Prepare fluorescent phalloidins” on page 21.</td>
</tr>
</tbody>
</table>
Introduction

Invitrogen™ CellLight™ BacMam 2.0 reagents provide an easy way to label specific structures in live cells. Simply add the ready-to-use constructs to cells, incubate overnight, and image the next day. These constructs express fluorescent fusion proteins targeted to specific intracellular structures. The fluorescent protein is introduced using a simple transduction step, using the BacMam 2.0 technology, which does not require molecular biology techniques to carry out—it works like a cell stain.

Materials

- CellLight BacMam 2.0 reagent, refer to selection guide below
- Cell culture medium
- Optional: 4% formaldehyde in PBS
- Optional: 0.2% Triton X-100 in PBS

Protocol

CellLight reagents are provided as solutions of 1 x 10^8 particles/mL. The following protocol was optimized using adherent cells. Cells can also be labeled in suspension prior to plating.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>C10582</td>
<td>C10506</td>
<td>C10583</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>B10383</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endoplasmal reticulum</td>
<td>C10590</td>
<td></td>
<td>C10591</td>
</tr>
<tr>
<td>Early endosomes</td>
<td>C10586</td>
<td></td>
<td>C10587</td>
</tr>
<tr>
<td>Late endosomes</td>
<td>C10588</td>
<td></td>
<td>C10589</td>
</tr>
<tr>
<td>Golgi</td>
<td>C10592</td>
<td></td>
<td>C10593</td>
</tr>
<tr>
<td>Histones histone 2B</td>
<td>C10594</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysosomes</td>
<td>C10596</td>
<td>C10507</td>
<td>C10597</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>C10600</td>
<td>C10508</td>
<td>C10601</td>
</tr>
<tr>
<td>Nucleus</td>
<td>C10602</td>
<td></td>
<td>C10603</td>
</tr>
<tr>
<td>Peroxisomes</td>
<td>C10604</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>C10607</td>
<td></td>
<td>C10608</td>
</tr>
<tr>
<td>Talin</td>
<td>C10611</td>
<td></td>
<td>C10612</td>
</tr>
<tr>
<td>Tubulin</td>
<td>C10613</td>
<td>C10509</td>
<td>C10614</td>
</tr>
<tr>
<td>BacMam 2.0 transduction control</td>
<td>B10383</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unconjugated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Null virus (control)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

General guidelines

- CellLight reagents work with most cell types between 10 and 50 particles per cell (PPC).
- For best results, transduce cells at a confluence of no more than 70%.
- The Invitrogen™ BacMam Enhancer Kit (Cat. No. B10107) is generally not required for CellLight BacMam 2.0 reagents. However, its use has been shown to boost expression in some challenging cell types such as Jurkat.
- For optimal results you may need to alter the PPC, volume, cell density, temperature, or incubation time. Following the PPC, adjusting the volume is the next best parameter to change to optimize protein expression.

This protocol is continued on page 26

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CellLight BacMam 2.0 reagents, cont.

Day 1. Labeling

1. Plate the cells at the desired density and allow them sufficient time to adhere. CellLight BacMam reagents work best when used on low-passage-number cells.

2. Calculate the appropriate volume of CellLight reagent for the number of cells.

   \[
   \text{Volume of CellLight reagent (mL)} = \frac{\text{Number of cells} \times \text{desired PPC}}{1 \times 10^8 \text{ CellLight particles/mL}}
   \]

   Where the number of cells is the estimated total number of cells at the time of labeling, PPC is the number of particles per cell, and \(1 \times 10^8\) is the number of particles per mL of the reagent.

3. Mix the CellLight reagent several times by inversion to ensure a homogeneous solution. **Do not vortex.**

4. Add the volume of CellLight reagent calculated in step 2 directly to the cells in complete cell medium, and mix gently.

5. Return the cells to the culture incubator overnight (≥16 hours).

Day 2. Imaging

1. Image the cells using the appropriate instrument filter sets.

   **Optional:** You may fix your cells with formaldehyde. To fix cells, treat with 4% formaldehyde solution in PBS for 10–30 minutes at room temperature. You can permeabilize the cells following fixation with 0.2% Triton X-100 solution in PBS for 5 minutes at room temperature.

Note

Fluorescence may be lost with methanol fixation. If methanol fixation is necessary for downstream analyses, you may detect CFP and GFP proteins using an anti-GFP antibody; RFP can be detected with an anti-RFP antibody. These antibodies are available separately. Search our extensive portfolio of high-quality antibodies at thermofisher.com/antibodies.

Figure 8. Labeling of HeLa cells with CellLight reagents. HeLa cells were transduced with Invitrogen™ CellLight™ Mitochondria-RFP reagent (Cat. No. C10505) and Invitrogen™ CellLight™ Talin-GFP reagent (Cat. No. C10611) for 24 hours, then labeled with Invitrogen™ NucBlue™ Live ReadyProbes™ Reagent (Cat. No. R37605) for 15 minutes. For photobleach protection, cells were incubated with Invitrogen™ ProLong™ Live Antifade Reagent for 90 minutes before imaging on an Invitrogen™ EVOS™ cell imaging system.
Introduction
Invitrogen™ Tubulin Tracker™ reagents provide fluorescent staining of polymerized tubulin in live cells. They are designed to readily permeate live cells, thus providing more uniform labeling and better selectivity compared to other methods of detection.

Materials
- Tubulin Tracker reagent (Cat. No. T34075, T34076, T34077, T34078, T34079)
- Live specimen, such as cultured or primary cells, 3D cell cultures, spheroids, or organoids
- Invitrogen™ Anhydrous DMSO (Cat. No. D12345)
- Live cell–compatible buffer:
  - Gibco™ HBSS with calcium and magnesium (Cat. No. 24020117)
  - Gibco™ FluoroBrite™ DMEM (Cat. No. A1896701)
- Optional: Invitrogen™ Probenecid, Water Soluble (Cat. No. P36400)
- Optional: Invitrogen™ Pluronic™ F-127 (20% Solution in DMSO) (Cat. No. P3000MP)
- Optional: Invitrogen™ NucBlue™ Live ReadyProbes™ Reagent (Cat. No. R37605)

Protocol
Prepare and stain the cells with Tubulin Tracker Green reagent

1. Vortex the intermediate stock solution well, refer to the “Before you begin” section.

2. Dilute the intermediate stock solution to 1X in live–cell compatible buffer. Lower concentrations can be used in certain cell types. Use only solution freshly made on the same day.

3. Apply a sufficient amount of the final staining solution to cover the cells adhering to the vessel.

This protocol is continued on page 28
4. Incubate for 30 minutes at 37°C and 5% CO₂. Optional: A nuclear staining reagent can be added at a 1X concentration. When staining a 3D cell culture such as spheroid or organoid, the same final staining concentration is recommended, but with a prolonged incubation time to allow complete penetration of the label.

5. Rinse the cells 3 times in a live cell–compatible wash buffer at 37°C. Optional: Add 1X probenecid to the wash buffer to minimize efflux of probe during rinse and imaging steps. Do not leave cells in probenecid for more than 2 hours, as it can interfere with some cellular functions.

6. Image and analyze cells in buffer. Samples should be viewed within a few hours after staining, as staining intensity will diminish with time.

Prepare and stain cells with Tubulin Tracker Deep Red reagent

1. Dilute the stock solution to 1X in live cell–compatible buffer. Lower concentrations can be used in certain cell types. Use only solution freshly made on the same day.

2. Apply a sufficient amount of the final staining solution to cover the cells adhering to the vessel.

3. Incubate for 30 minutes at 37°C and 5% CO₂. Optional: A nuclear staining reagent can be added at 1X concentration. When staining a 3D cell culture such as spheroid or organoid, the same final staining concentration is recommended, but with a prolonged incubation time to allow complete penetration of the label.

4. Rinse the cells 3 times in a live cell–compatible wash buffer at 37°C. Optional: Add 1X probenecid to the wash buffer to minimize efflux of probe during rinse and imaging steps. Do not leave cells in probenecid for more than 2 hours as it can interfere with some cellular functions.

5. Image and analyze cells in buffer. Samples should be viewed within a few hours after staining, as staining intensity will diminish with time.

Figure 9. Cell treated with Tublin Tracker Deep Red reagent.

Figure 10. Live-cell labeling using Invitrogen™ CellMask™ Deep Red plasma membrane stain. Live bovine pulmonary arterial endothelial cells were labeled with CellMask Deep Red plasma membrane stain (Cat. No. C10046), tubulin-selective Tubulin Tracker Green dye (Oregon Green 488 Taxol, bis-acetate (Cat. No. T34075)), mitochondrion-selective MitoTracker Red CMXRos dye (Cat. No. M7512), and blue-fluorescent Hoechst 33342 nuclear stain (Cat. No. H24192). Labeled cells were then imaged using standard fluorescence microscopy, and the image was deconvolved using Huygens software (Scientific Volume Imaging).
LysoTracker and LysoSensor probes

Introduction
Invitrogen™ LysoTracker™ dyes are cell-permeant lysosome markers that target and track acidic organelles in live cells. LysoTracker markers are comprised of a fluorophore bound to weakly basic amines. Though the mechanism of LysoTracker retention is relatively unknown, it is likely due to protonation with retention in the organelles’ membrane. These lysosomal markers accumulate in organelles that have a low pH and are only partially protonated at neutral pH.

Materials
• Invitrogen™ LysoTracker™ or LysoSensor™ dye (Cat. No. L7525, L12491, L7526, L7528, L12492, L7533, L7535, L7545, L22460)
• Growth medium or buffer

Protocol
LysoTracker and LysoSensor dyes

1. Dilute the 1 mM probe stock solution to the final working concentration in the growth medium or buffer of choice. For the LysoTracker probes, we recommend working concentrations of 50–75 nM and for the LysoSensor probes at least 1 µM. To reduce potential artifacts from overloading, the concentration of dye should be kept as low as possible.

2. For adherent cells, grow cells on coverslips inside a petri dish filled with the appropriate culture medium. When cells have reached the desired confluence, remove the medium from the dish and add the pre-warmed (37°C) probe-containing medium. Incubate the cells for 30 minutes to 2 hours under growth conditions appropriate for the cell type. Then replace the loading solution with fresh medium and observe the cells using an Invitrogen™ EVOS™ imaging system fitted with the correct filter set. If the cells do not appear to be sufficiently stained, we recommend either increasing the labeling concentration or increasing the time allowed for the dye to accumulate in the lysosomes.

Critical note
Before opening, allow the vial to warm to room temperature, and then briefly centrifuge the vial in a microcentrifuge to collect the DMSO solution at the bottom of the vial.

Note
The concentration of probe for optimal staining will vary depending on the application. Staining conditions may need to be modified depending upon the cell type and the permeability of the cells or tissues to the probe.

Note
If the cells are incubated in dye-free medium after staining, we often observe a decrease in fluorescent signal and cell blebbing.

Note
Kinetic studies on the internalization of the Invitrogen™ LysoTracker™ Green DND-26 and Invitrogen™ LysoSensor™ Yellow/Blue DND-160 (PDMPO) probes indicate that the rates of uptake of these dyes into living cells can occur within seconds. Unfortunately, these lysosomal probes can exhibit an “alkalizing effect” on the lysosomes, such that longer incubation with these probes can induce an increase in lysosomal pH. We suggest that these probes are useful pH indicators only when they are incubated with cells for 1–5 minutes at 37°C.

This protocol is continued on page 30

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3. For suspension cells, centrifuge to obtain a cell pellet and aspirate the supernatant. Resuspend the cells gently in pre-warmed (37°C) probe-containing medium. Incubate the cells for 30 minutes to 2 hours under growth conditions appropriate for the cell type (see note above regarding internalization rate of these probes). Re-pellet the cells by centrifugation and resuspend in fresh pre-warmed medium. Observe the cells using an EVOS imaging system fitted with the correct filter set. If the cells do not appear to be sufficiently stained, we recommend either increasing the labeling concentration or increasing the time allowed for the dye to accumulate in the lysosomes.

LysoSensor Yellow/Blue dextran

1. To prepare a stock solution, reconstitute the lyophilized Invitrogen™ LysoSensor™ Yellow/Blue Dextran to 50 mg/mL in phosphate-buffered saline, pH 7.4. Store the stock solution at or below –20°C, protected from light.

2. Dilute the stock solution to a final working concentration in the growth medium or buffer of choice. We recommend a working concentration of 1–5 mg/mL.

3. For adherent cells, grow cells on coverslips inside a petri dish filled with the appropriate culture medium. When cells have reached the desired confluence, remove the medium from the dish and add the pre-warmed (37°C) dextran working solution. Incubate the cells for 1–24 hours under growth conditions appropriate for the cell type and conduct the experiment. Replace the loading solution with fresh medium, and observe the cells using a fluorescence microscope fitted with the correct filter set.

4. For suspension cells, centrifuge to obtain a cell pellet and aspirate the supernatant. Resuspend the cells gently in pre-warmed (37°C) dextran-containing medium. Incubate the cells for 1–24 hours under growth conditions appropriate for the cell type. Re-pellet the cells by centrifugation and resuspend in fresh pre-warmed medium. Observe the cells using an EVOS imaging system fitted with the correct filter set.

Figure 11. Labeling of U2OS cells with LysoTracker, MitoTracker, and Tubulin Tracker reagents. U2OS cells labeled using Invitrogen™ LysoTracker™ Blue DND22 dye (Cat. No. L7525), Invitrogen™ MitoTracker™ Green FM dye (Cat. No. M7514), and Invitrogen™ Tubulin Tracker™ Deep Red dye (Cat. No. T34076) show superb multiplexing capability and staining specificity. Cells were imaged in Gibco™ HBSS buffer (Cat. No. 14025134) containing calcium and magnesium, supplemented with 1X Invitrogen™ probenecid solution (Cat. No. P36400). Images were generated using an Invitrogen™ EVOS™ imaging system.
ER-Tracker dyes for live-cell endoplasmic reticulum labeling

Introduction
Invitrogen™ ER-Tracker™ dyes are highly selective, cell-permeant live-cell endoplasmic reticulum stains. At low concentrations, these dyes have not been shown to be toxic to cells. When cells are stained using the protocol provided, the ER staining pattern is partially retained after fixation with formaldehyde.

Invitrogen™ ER-Tracker™ Blue-White DPX dye (Cat. No. E12353) is highly selective and photostable. It has an excitation of ~374 nm and is environment sensitive, resulting in an emission range from 430 nm to 640 nm. While a standard DAPI filter works best and is recommended, a UV longpass filter can be used when visualizing ER staining.

Invitrogen™ ER-Tracker™ Green (BODIPY™ FL glibenclamide, Cat. No. E34251) and Invitrogen™ ER-Tracker™ Red (BODIPY™ TR glibenclamide, Cat. No. E34250) are fluorescent sulfonylureas. These ER stains use glibenclamide, which binds to the sulfonylurea receptors of ATP-sensitive K⁺ channels that are prominent on the ER; however, they may have more disseminated tissue- and cell type–dependent distributions. It is important to note that the pharmacological activity of glibenclamide could potentially affect ER function.

Materials
• ER-Tracker dye
• DMSO
• Gibco™ Hanks’ Balanced Salt Solution with calcium and magnesium (HBSS, calcium, magnesium, no phenol red) (Cat. No. 14025-092)
  • Optional: 4% formaldehyde
  • Optional: 0.2% Triton X-100

Protocol
This protocol was optimized using bovine pulmonary artery endothelial cells and has been confirmed in other common cell lines. Recommendations for experimental protocols should be used as a starting point, and optimal labeling conditions for each cell type should be determined empirically.

This protocol is continued on page 32
ER-Tracker dyes for live-cell endoplasmic reticulum labeling, cont.

Reagent preparation
ER-Tracker Blue-White DPX dye is supplied as aliquots of a 1 mM stock solution in DMSO. Allow each vial to warm to room temperature before use, then briefly centrifuge to collect the DMSO solution at the bottom of the vial.

ER-Tracker Green and ER-Tracker Red dyes are supplied as 100 μg of lyophilized material. Prepare a 1 mM stock solution of the appropriate dye: for ER-Tracker Green dye, dissolve the contents of the vial in 128 μL of DMSO; for ER-Tracker Red dye, dissolve the contents of the vial in 110 μL of DMSO. It is recommended that the 1 mM solution then be separated into aliquots and stored frozen with desiccant.

Cell preparation and staining
1. **Prepare staining solution.** Dilute the 1 mM stock solution to the final working concentration. We recommend working concentrations of 100 nM–1 μM for ER-Tracker Blue-White DPX dye and ~1 μM for ER-Tracker Green dye and ER-Tracker Red dye. To minimize potential labeling artifacts, use the lowest dye concentrations possible. Best results are obtained when staining is performed in Hanks’ Balanced Salt Solution with calcium and magnesium (HBSS) at 37°C and 5% CO₂.

2. **Stain the cells.** For adherent cells, remove the medium from the culture dish, rinse with HBSS, and add pre-warmed staining solution. Incubate the cells for approximately 15–30 minutes at 37°C. Replace the staining solution with fresh probe-free medium and view the cells using an Invitrogen™ EVOS™ imaging system. If the stained cells are to be fixed, refer to the fixation steps below for the appropriate dye.

Fixation and permeabilization for ER-Tracker Blue-White DPX dye
1. **Fix and permeabilize cells.** The ER-Tracker Blue-White DPX dye’s signal is only partially retained after formaldehyde fixation. Fix stained cells with 4% formaldehyde for 10–20 minutes at 37°C. If additional staining will be performed, cells can be permeabilized with 0.2% Triton X-100 solution for 10 minutes.

2. **Wash and view cells.** After cells are fixed, perform two 5-minute washes in any suitable buffer and view.

This protocol is continued on page 33
Fixation for ER-Tracker Green and ER-Tracker Red Dyes

1. **Fix cells.** If stained cells are to be fixed, fixation is recommended using 4% formaldehyde for 2 minutes at 37°C.

2. **Wash and view cells.** After fixation, perform two 5-minute washes in any suitable buffer prior to mounting, viewing, or further staining. Permeabilization is not recommended; signal is not retained after permeabilization with Triton X-100 solution.

---

**Figure 12. Labeling of HeLa cells with ER-Tracker dye.** HeLa cells labeled using NucBlue™ Live ReadyProbes™ Reagent (Cat. No. R37605), ER-Tracker Green dye (Cat. No. E34251), and Tubulin Tracker Deep Red dye (Cat. No. T34076) show superb multiplexing capability and uniformity of staining compared with overexpressed fluorescent protein fusions. Cells were imaged in HBSS buffer (Cat. No. 14025134) containing calcium and magnesium, supplemented with 1X probenecid solution (Cat. No. P36400). Images were generated using an EVOS imaging system.
SelectFX Nuclear Labeling Kit
For fixed nuclear labeling, includes DAPI, SYTOX Green, 7-AAD, and TO-PRO-3 dyes

Introduction
The Invitrogen™ SelectFX™ Nuclear Labeling Kit (Cat. No. S33025) provides four spectrally distinct fluorescent dyes for staining nuclei in fixed-cell preparations: blue-fluorescent DAPI, green-fluorescent Invitrogen™ SYTOX™ Green stain, red-fluorescent 7-aminoactinomycin D (7-AAD), and far red–fluorescent Invitrogen™ TO-PRO™-3 dye. These dyes are ideal for use as counterstains in multicolor applications—simply select the stain that contrasts spectrally with other fluorescent probes applied to the sample. When used according to the protocol provided, the dyes in the SelectFX Nuclear Labeling Kit provide highly selective nuclear staining with little or no cytoplasmic labeling. The stained nuclei stand out in vivid contrast to other fluorescently labeled cell structures when observed by fluorescence microscopy. These dyes have excitation wavelengths that match the common laser lines for confocal microscopy and flow cytometry and can be used with standard filter sets on Invitrogen™ EVOS™ imaging systems and microplate readers.

Materials
- SelectFX Nuclear Labeling Kit (Cat. No. S33025)
- Phosphate-buffered saline (PBS)
- Distilled water
- 4% formaldehyde
- 0.2% Triton X-100 in PBS
- Mounting medium

Protocol
Experimental protocol for fluorescence microscopy
The protocol outlined below has been optimized for fixation in 4% formaldehyde solutions using bovine pulmonary artery epithelial cells but is compatible with other cell types. Other fixation techniques may result in nonspecific staining or abnormal cellular morphology. RNase treatment is not necessary but could improve nuclear signals over cytoplasmic RNA background with SYTOX Green or TO-PRO-3 dye under some conditions, particularly if a higher concentration of dye is needed. If the dyes are to be used as nuclear counterstains, labeling steps involving other probes should be carried out first. DAPI and TO-PRO-3 dyes provide optimum performance when prepared in phosphate-buffered saline (PBS), whereas SYTOX Green dye and 7-AAD are best prepared in distilled water. Other buffers can be used, but cytoplasmic and nonspecific background may increase.

This protocol is continued on page 35
General staining protocol

1. **Fix cells.** Fix adherent or suspension cells using 4% formaldehyde in complete medium for 15 minutes at 37°C.

2. **Wash cells.** Wash cells for 5 minutes in PBS; repeat twice.

3. **Permeabilize cells.** Permeabilize cells for 10 minutes with 0.2% Triton X-100 solution in PBS.

4. **Rinse cells.** Rinse cells well with PBS.

5. **Optional:** Label non-nuclear structures. If other stains will be used, proceed with those label and wash steps.

6. **Apply counterstain.** When ready to stain nuclei, follow the guidelines for the dye used:
   - **DAPI:** Dilute DAPI stock solution (component A) 1:300 in PBS to make a 0.2 µg/mL (600 nM) solution. Apply enough of the 600 nM solution to cover cells, then incubate for 2 minutes. Proceed to step 7.
   - **SYTOX Green stain:** Dilute SYTOX Green dye stock solution (component B) 1:300 in water to make a 0.2 µg/mL (167 nM) solution. Rinse cells in water, then apply enough of the 167 nM solution to cover cells. Incubate for 15 minutes, then rinse again with water before proceeding to the final washes in step 7.
   - **7-AAD:** Dilute 7-AAD stock solution (component C) 1:50 in water to make a 40 µg/mL (32 µM) solution. Rinse cells in water, then apply enough of the 32 µM solution to cover cells. Incubate for 45 minutes, then rinse again with distilled water before proceeding to the final washes in step 7.
   - **TO-PRO-3 dye:** Dilute TO-PRO-3 dye stock solution (component D) 1:300 in PBS to make a 0.7 µg/mL (1 µM) solution. Apply enough of the 1 µM solution to cover cells, then incubate for 15 minutes. Proceed to step 7.

7. **Wash cells.** Wash cells for 5 minutes in PBS; repeat twice.

8. **Prepare for viewing.** Mount the coverslip in an appropriate antifade medium such as Invitrogen™ ProLong™ Gold antifade reagent (Cat. No. P36930, P36934) or Invitrogen™ Slowfade™ Gold antifade reagent (Cat. No. S36936, S36937).
Staining with Hoechst dyes
Nuclear counterstain for fluorescence microscopy

Introduction
Invitrogen™ Hoechst™ 33342 nucleic acid stain is a popular cell-permeant nuclear counterstain that emits blue fluorescence when bound to dsDNA. This dye is often used to distinguish condensed pycnotic nuclei in apoptotic cells and for cell cycle studies in combination with BrdU. It is also available as a solution (Cat. No. H3570).

Materials
- Cells growing in culture
- Hoechst 33342, trihydrochloride, trihydrate (Cat. No. H1399)
- Phosphate-buffered saline (PBS)
- Invitrogen™ EVOS™ imaging system

Protocol
Preparing Hoechst dye stock solution
1. Prepare the Hoechst dye stock solution by dissolving the contents of one vial (100 mg) in 10 mL of deionized water (diH₂O) to create a 10 mg/mL (16.23 mM) solution.

Labeling cells
1. Culture cells in an appropriate medium and vessel for fluorescence microscopy.

2. Prepare the Hoechst staining solution by diluting the Hoechst stock solution 1:2,000 in PBS.

3. Remove the medium.

4. Add sufficient staining solution to cover the cells.

5. Incubate for 5–10 minutes, protected from light.

6. Optional: You may image directly in the staining solution, if you wish.

Note for step 1
Hoechst dye has poor solubility in water, so sonicate as necessary to dissolve.

Note for step 1
The 10 mg/mL Hoechst stock solution may be stored at 2–6°C for up to 6 months, or at or below –20°C for longer periods.

This protocol is continued on page 37
7. Remove the staining solution.

8. Wash the cells 3 times in PBS.

9. Image the cells.

<table>
<thead>
<tr>
<th>Spectral information and storage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Invitrogen™ dye</strong></td>
</tr>
<tr>
<td><strong>Excitation/emission</strong></td>
</tr>
<tr>
<td><strong>Standard filter set</strong></td>
</tr>
<tr>
<td><strong>EVOS Light Cube</strong></td>
</tr>
<tr>
<td><strong>Storage conditions</strong></td>
</tr>
</tbody>
</table>

**Figure 13. Multiplex imaging with Click-iT RNA assays.** NIH3T3 cells were incubated with 1 mM EU for 1 hr, then formaldehyde fixed and permeabilized with Triton X-100. EU incorporated into newly synthesized RNA (red) in some cells was detected using the Click-iT RNA Alexa Fluor 594 Imaging Kit (Cat. No. C10330). Tubulin (green) was detected with mouse anti-tubulin IgG (Cat. No. A1126) and visualized with Alexa Fluor 488 goat anti–mouse IgG (Cat. No. A11001). Nuclei (blue) were stained with Hoechst 33342 (Cat. No. H3570).
Labeling with SYTO 9 stain

Introduction
Invitrogen™ SYTO™ 9 green fluorescent nucleic acid stain (Cat. No. S34854) has been shown to stain live and dead gram-positive and gram-negative bacteria, and it is a component of the Invitrogen™ LIVE/DEAD™ BacLight™ Bacterial Viability Kits (Cat. No. L7007, L7012, and L13152).

Materials
- SYTO 9 green fluorescent nucleic acid stain (Cat. No. S34854)
- Non-phosphate buffer such as Hanks’ Balanced Salt Solution (Cat. No. 14025092)

Protocol
Adherent cells in culture may be stained in situ on coverslips.

1. Pellet cells in suspension by centrifugation and resuspend in buffered salt solution or water.

2. Add the SYTO 9 stain using the concentrations and staining conditions listed in table below as a guide.

3. Image the cells.

Suggested conditions for staining with SYTO green-fluorescent nucleic acid stains

<table>
<thead>
<tr>
<th>Application</th>
<th>SYTO 9 dye concentration</th>
<th>Staining conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial cells</td>
<td>50 nM–20 µM</td>
<td>Vortex to mix, then incubate for 1–30 minutes</td>
</tr>
<tr>
<td>Eukaryotic cells</td>
<td>10 nM–5 µM</td>
<td>Incubate for 10–120 minutes</td>
</tr>
</tbody>
</table>

Spectral information and storage for SYTO 9 stain

<table>
<thead>
<tr>
<th>Excitation/emission</th>
<th>485/498 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard filter set</td>
<td>FITC</td>
</tr>
<tr>
<td>EVOS Light Cube</td>
<td>GFP</td>
</tr>
<tr>
<td>Storage conditions</td>
<td>&lt;-20°C</td>
</tr>
</tbody>
</table>

Protocol guidelines
- We suggest broad ranges of staining concentrations, based on our laboratory experience or published methods, to provide a starting point for experiments. These conditions require adjustment for each cell type and experimental system.
- Use plastic tubes when diluting any SYTO stain, because the diluted stain adheres to glass.
- In general, the best results are obtained in buffers that do not contain phosphate, such as Hanks’ Balanced Salt Solution (Cat. No. 14025092).
- When preparing other solutions, note that residual detergent on plastic or glassware may also affect real or apparent staining of many cells or organisms, causing brightly stained material to appear in solutions with or without cells present. Wash all labware in mild detergent and rinse with hot tap water followed by several rinses with deionized water (Cat. No. 751-610).

Note
In initial experiments, it may be best to try several dye concentrations over the entire suggested range to determine the concentration that yields optimal staining. Be aware that growth medium, cell density, the presence of other cell types, and other factors may influence staining. Stained eukaryotic cells generally show diffuse cytoplasmic staining as well as nuclear staining. Particularly intense staining of intranuclear bodies is frequently observed. Because these dyes are cell-permeant and contain a net positive charge at neutral pH, they may also stain mitochondria. Staining of live yeast is primarily mitochondrial.
Labeling with SYTO 59 stain
Nuclear stain for eukaryotic and prokaryotic cells

Introduction
The cell-permeant Invitrogen™ SYTO™ 59 Red Fluorescent Nucleic Acid Stain exhibits bright red fluorescence upon binding to nucleic acids. In both live and dead eukaryotic cells, SYTO 59 stain generally shows cytoplasmic or mitochondrial as well as nuclear staining. In addition, SYTO 59 stain will stain most live and permeabilized bacteria.

Materials
- Cells growing in culture
- SYTO 59 Red Fluorescent Nucleic Acid Stain (Cat. No. S11341)
- EVOS imaging system

Protocol
1. Culture cells in an appropriate medium and vessel for fluorescence microscopy.
2. Remove the medium.
3. Wash the cells 1–3 times in a phosphate-free buffer to remove the medium.
4. Prepare the SYTO 59 staining solution by diluting the stock solution 1:1,000 (5 µM) in a phosphate-free buffer.
5. Add sufficient staining solution to cover the cells.
6. Incubate for 30 minutes, protected from light.
7. Remove the staining solution.
8. Wash the cells 3 times in a phosphate-free buffer.
9. Image the cells.

Critical notes
This protocol can be used for:
- Nucleic acid (nuclear) staining in fluorescence microscopy
This protocol should not be used for:
- Flow cytometry

Protocol tips
- Warm to room temperature and briefly centrifuge the DMSO solution to the bottom of the vial each time before use
- Try multiple dye concentrations in the range of 100 nM–5 µM to determine the optimal concentration.
- In general, the best results are obtained in buffers that do not contain phosphate, such as Hanks’ Balanced Salt Solution (Cat. No. 14025092).
- Treat all nucleic acid–binding dyes as potential mutagens and handle with care.

Spectral information and storage for SYTO 59 stain

<table>
<thead>
<tr>
<th>Excitation/emission</th>
<th>622/645 nm</th>
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</thead>
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<tr>
<td>Standard filter set</td>
<td>Cy³.5</td>
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<tr>
<td>EVOS Light Cube</td>
<td>Texas Red</td>
</tr>
<tr>
<td>Storage conditions</td>
<td>≤-20°C</td>
</tr>
</tbody>
</table>

Figure 14. Nuclear staining of BPAECs. BPAECs were cultured, stained with SYTO 59 dye (5 µM for 5 min), and then imaged.
**Introduction**

Healthy mitochondrial membranes maintain a difference in electrical potential between the interior and exterior of the organelle, referred to as a membrane potential. Invitrogen™ Tetramethylrhodamine, methyl ester (TMRM), is a cell-permeant dye that accumulates in active mitochondria with intact membrane potentials. If the cells are healthy and have functioning mitochondria, the signal is bright. Upon loss of the mitochondrial membrane potential, TMRM accumulation ceases and the signal dims or disappears.

**Materials**

- Invitrogen™ Image-iT™ TMRM Reagent (Cat. No. I34361) or Tetramethylrhodamine, methyl ester (TMRM) (Cat. No. T668)
- DMSO
- Cell growth medium
- Phosphate-buffered saline (PBS)

**Protocol**

**Prepare stock solutions**

Image-iT™ TMRM Reagent (Cat. No. I34361) is provided as a 1,000X concentrated stock solution at a concentration of 100 μM in DMSO. To use it, simply dilute the stock solution 1:1,000 in cell growth or imaging medium. Tetramethylrhodamine, methyl ester (TMRM) (Cat. No. T668) is provided as 25 mg lyophilized powder. To prepare the stock solution, first dissolve the entire 25 mg lyophilized powder in 5 mL of DMSO to make a 10 mM solution. To prepare a stock solution at 100 μM, add 10 μL of the 10 mM solution to 990 μL of DMSO. Both the 10 mM and 100 μM solutions can be stored for 6 months in the freezer at –5°C to –30°C.

**Prepare staining solution**

A 100 μM TMRM stock solution is at 1,000X concentration. To prepare a 1X staining solution at 100 nM concentration, add 10 μL of the 100 μM stock solution to 10 mL of cell growth medium. For different applications and cell types, this concentration can be adjusted between 20 nM and 250 nM. Prepare and use the staining solution fresh for best results.
Cell staining protocol

1. Grow the cells.

2. Remove the cell growth medium.

3. Add cell staining solution to the cells.

4. Incubate for 30 minutes at 37°C.

5. Optional: For increased sensitivity, wash with PBS or similar buffer.

6. Analyze the cells.

Note
For fluorescence microscopy or high-content analysis, use TRITC/RFP filter settings.

Figure 15. U2OS cells stained with 100 nM Image-iT TMRM (Cat. No I34361, red) and Hoechst 33342 stain (Cat. No. H3570, blue) for 30 minutes.
Introduction

Invitrogen™ CellEvent™ Caspase-3/7 Green Detection Reagent is a fluorogenic substrate for activated caspases 3 and 7. The reagent consists of a four amino acid peptide (DEVD) conjugated to a nucleic acid–binding dye. This cell-permeant substrate is intrinsically nonfluorescent, because the DEVD peptide inhibits the ability of the dye to bind to DNA. After activation of caspase-3 or caspase-7 in apoptotic cells, the DEVD peptide is cleaved, enabling the dye to bind to DNA and produce a bright, fluorogenic response with excitation/emission maxima of ~502/530 nm.

Materials

- CellEvent Caspase-3/7 Green Detection Reagent (Cat. No. C10423 and C10723)
- Cells
- Fetal Bovine Serum, certified, heat inactivated (Cat. No. 10082147)
- DPBS, with calcium and magnesium (Cat. No. 14040133)
- Optional: Complete medium (as a diluent for CellEvent Caspase-3/7 reagent)
- Optional: Fixative (e.g., 3.7% formaldehyde in PBS)
- Optional: Invitrogen™ ProLong™ Diamond Antifade Mountant (Cat. No. P36970) or Invitrogen™ SlowFade™ Diamond Antifade Mountant (Cat. No. S36972)

Protocol

The following protocols were developed using HeLa and U2OS cells with an optimized CellEvent Caspase-3/7 Green Detection Reagent concentration of 5 μM, but they can be adapted for any cell type. Growth medium, cell density, cell type variations, and other factors may influence labeling. In initial experiments, we recommend testing a range of concentrations for the CellEvent Caspase-3/7 Green Detection Reagent to determine the optimal conditions for your model.

Assay choice

The protocol for the endpoint apoptotic assay is provided below. However, you can also perform kinetic or dynamic measurement of the induction of apoptosis by treating the cells in complete medium with the CellEvent Caspase-3/7 Green Detection Reagent and incubating them for extended periods of time.
Endpoint assay

1. Treat cells with the appropriate apoptotic inducer for the desired time.

2. Dilute the CellEvent Caspase-3/7 Green Detection Reagent in PBS with 5% FBS (Cat. No. 14040133 and 10082147) or complete medium, to a final concentration of 2–8 μM.

3. Optional: You can stain the cells with a cell-permeant nuclear stain at this step.

4. Remove the medium from the cells, then add the diluted reagent (prepared in step 2) to the cells. For example, if you are performing staining in a 96-well plate, add 100 μL of the reagent solution to each well.

5. Incubate the cells at 37°C for at least 30 minutes.

6. Optional: You can preserve the cells with a formaldehyde-based fixative at this stage. Fixation with 3.7% formaldehyde for 15 minutes is recommended, but this can be altered based on the cell type.

7. Optional: You can stain the cells with a nuclear stain or counterstain at this step if they have not been previously counterstained.

8. Optional: To stabilize and prolong the signal, you can use ProLong Diamond Antifade Mountant (Cat. No. P36970) for ultimate overnight mounting. For quick mounting, you can use SlowFade Diamond Antifade Mountant (Cat. No. S36972).

9. Image the cells using the appropriate instrument filter sets such as those used for FITC and the Alexa Fluor™ 488 dye. The excitation/emission maxima for the CellEvent Caspase-3/7 Green Detection Reagent are 502/530 nm.

Note

For best results, dilute the CellEvent™ Caspase-3/7 Reagent in PBS with 5% FBS. You can dilute the reagent in complete medium; however, this can result in high fluorescence background. We recommend that you perform optimization, if the preferred diluent is complete medium. We recommend initial testing with 2–10 μM of CellEvent™ Caspase-3/7 Green Detection Reagent. However, the optimal concentration may be more or less than this range depending on the model.

Note

It is best to determine the incubation time for the cell type of choice; in HeLa cells, 30 minutes is sufficient.
Kinetic assay

1. Dilute the CellEvent Caspase-3/7 Green Detection Reagent in complete medium to a final concentration of 2–10 μM. Note: We recommend initial testing with 2–10 μM of CellEvent Caspase-3/7 Green Detection Reagent. However, the optimal concentration may vary depending on the experimental conditions.

2. Prepare the apoptotic inducer.

3. Add the CellEvent Caspase-3/7 Green Detection Reagent prepared in complete medium (step 1) directly to the cells in complete medium.

4. Add the apoptotic inducer to the cells treated with the CellEvent Caspase-3/7 Green Detection Reagent and return the cells to the incubator.

5. At the desired time points, remove the cells from the incubator and visualize the progression of apoptosis using FITC/Alexa Fluor™ 488 filter settings.

Figure 16. Mouse embryonic hippocampal neurons cultured 14 DIV (days in vitro) grown in Neurobasal Plus and Gibco™ B-27™ Plus supplement with 25 μM glutamate and 1X Gibco™ GlutaMAX™ Supplement. Cells were treated in half-log intervals with cadmium chloride for 18 hours and subsequently stained using NucBlue Live Cell Stain, CellEvent Green Caspase-3/7, Image-iT TMRM, and Tubulin Tracker Deep Red. Images were taken with an EVOS imaging system.
Introduction
Invitrogen™ CellROX™ Oxidative Stress Reagents are fluorogenic probes designed to reliably measure reactive oxygen species (ROS) in live cells. The cell-permeant reagents are nonfluorescent or very weakly fluorescent while in a reduced state and upon oxidation exhibit strong fluorogenic signal. CellROX Green Reagent is a DNA dye, and upon oxidation, it binds to DNA; thus, its signal is localized primarily in the nucleus and mitochondria. In contrast, the signals from CellROX Deep Red and CellROX Orange Reagents are localized in the cytoplasm. The fluorescence resulting from CellROX Oxidative Stress Reagents can be measured using traditional fluorescence microscopy, high-content imaging and analysis, microplate fluorometry, or flow cytometry.

Materials
- CellROX Oxidative Stress Reagent (Cat. No. C10422, C10443, C10444, C10448)
- Cells and culture medium
- Phosphate-buffered saline (PBS, pH 7.2–7.6)
- Optional: Fixative (i.e., 3.7% formaldehyde in PBS)
- Optional: Permeabilization solution (i.e., 0.5% Triton X-100)

Protocol
1. Treat the cells with the test compound or drug.
2. Add the CellROX Reagent at a final concentration of 5 μM.
3. Incubate the cells for 30 minutes at 37°C.
4. Remove medium and wash the cells 3 times with PBS.
5. Optional: If using CellROX Deep Red or CellROX Green, you may fix the cells with 3.7% formaldehyde for 15 minutes.
6. Optional: You may stain the cells with NucBlue™ Live Cell Stain, a nuclear counterstain, or another counterstain at this time.
7. Optional: If using CellROX Green, you may permeabilize the cells with 0.5% Triton X-100 for 10 minutes, if multiplexing with another reagent is desired.
8. Analyze the cells.
**Introduction**

Invitrogen™ CellTracker™ fluorescent probes are excellent tools for monitoring cell movement, location, proliferation, migration, chemotaxis, and invasion. The CellTracker fluorescent probes have been designed to freely pass through cell membranes; however, once inside the cell they are transformed into cell-impermeant reaction products. After conversion to impermeant versions, the CellTracker fluorescent probes are well retained in living cells through several generations. The probes are transferred to daughter cells but are not transferred to adjacent cells in a population. Cells loaded with the CellTracker fluorescent probes display fluorescence for at least 72 hours and exhibit ideal tracking dye properties—they are stable, nontoxic at working concentrations, well retained in cells, and brightly fluorescent at physiological pH. Additionally, several CellTracker fluorescent probes with various excitation and emission spectra are available, allowing for multiplexing.

**Materials**

- CellTracker dye (see the table under “EVOS microscopy” on page 49)
- Anhydrous dimethylsulfoxide (DMSO)
- Phosphate-buffered saline (PBS)

**Protocol**

**Prepare cells**

Grow cells in an appropriate culture medium. Adherent cells can be grown on coverslips inside a petri dish filled with culture medium.

**Experimental protocols**

The following protocol describes introducing the reagent into cultured cells and imaging the stained cells by fluorescence microscopy.

The optimal concentration of the probe for staining varies depending upon the application. We recommend testing at least a 10-fold range of concentrations. In general, long-term staining (more than about 3 days) or the use of rapidly dividing cells requires 5–25 µM dye. Less dye (0.5–5 µM) is needed for shorter experiments, such as viability assays. Due to the high fluorescent signal resulting from staining with CellTracker™ Deep Red dye, the optimal concentration range for this dye is 250 nM–1 µM. To maintain normal cellular physiology and reduce potential artifacts, keep the dye concentration as low as possible.

This protocol is continued on page 48
Prepare working dye solution

Before opening the dye vial, allow the product to warm to room temperature. Dissolve the lyophilized product in high-quality DMSO to a final concentration of 10 mM. For CellTracker™ Deep Red dye, add 20 µL of DMSO per vial to make a 1 mM (1,000X) solution. Dilute the stock solution to a final working concentration of 0.5–25 µM in serum-free medium. Warm the working solution to 37°C.

Staining protocol for cells in suspension

1. Harvest cells by centrifugation and aspirate the supernatant. Resuspend the cells gently in the pre-warmed working solution of CellTracker dye (see “Prepare working dye solution”).

2. Incubate 15–45 minutes under growth conditions appropriate for the particular cell type.

3. Centrifuge the cells and remove the supernatant.

4. Add a culture medium of choice and dispense the labeled cell suspension onto a slide or into a culture vessel of choice.

5. Image using the appropriate emission and excitation filters for the CellTracker™ probe (see table on page 49).

Staining protocol for adherent cells

1. Remove culture media.

2. Gently add the pre-warmed working solution of CellTracker dye (see “Prepare working dye solution” above).

3. Incubate 15–45 minutes under growth conditions appropriate for the particular cell type.

4. Remove the solution.
5. Add a culture medium of choice.

6. Image using the appropriate emission and excitation filters for the CellTracker™ probe; refer to the table below.

EVOS microscopy

The CellTracker™ probes can be used on a wide range of epifluorescence microscopes with standard optics and video enhancement. Select optical filters according to the dye. The table below summarizes the spectral characteristics of the CellTracker™ probes.

<table>
<thead>
<tr>
<th>Cat. No.</th>
<th>CellTracker™ probe</th>
<th>MW</th>
<th>Ex (nm)</th>
<th>Em (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2110</td>
<td>CellTracker™ Blue CMAC Dye (7-amino-4-chloromethylcoumarin)</td>
<td>209.6</td>
<td>353</td>
<td>466</td>
</tr>
<tr>
<td>C12881</td>
<td>CellTracker™ Blue CMF,HC Dye (4-chloromethyl-6,8-difluoro-7-hydroxycoumarin)</td>
<td>246.6</td>
<td>371</td>
<td>464</td>
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<tr>
<td>C2111</td>
<td>CellTracker™ Blue CMHC Dye (4-chloromethyl-7-hydroxycoumarin)</td>
<td>210.6</td>
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<td>470</td>
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<tr>
<td>C10094</td>
<td>CellTracker™ Violet BMQC Dye (2,3,6,7-tetrahydro-9-bromomethyl-1H,5H-quinolizino(9,1-gh) coumarin)</td>
<td>334.2</td>
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<td>516</td>
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<tr>
<td>C2925, C7025</td>
<td>CellTracker™ Green CMFDA Dye (5-chloromethylfluorescein diacetate)</td>
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<tr>
<td>C2102</td>
<td>CellTracker™ Green BODIPY™ Dye (8-chloromethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-S-indacene)</td>
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<tr>
<td>C2927</td>
<td>CellTracker™ Orange CMTMR Dye (5-(and-6)-((4-chloromethyl)benzoyl)amino) tetramethylrhodamine</td>
<td>554.0</td>
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<td>CellTracker™ Red CMTPX Dye</td>
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<td>C34565</td>
<td>CellTracker™ Deep Red Dye</td>
<td>698.3</td>
<td>630</td>
<td>660</td>
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</table>

1 Absorption and fluorescence emission maxima, determined in aqueous buffer or methanol; values may vary somewhat in cellular environments.
2 CMFDA is colorless and nonfluorescent until the acetate groups are cleaved by intracellular esterases; hydrolysis of the acetates yields a product with the indicated spectral properties.

MW = molecular weight
Cellular biology

Immunolabeling
Introduction
Immunolabeling or immunocytochemistry (ICC) is a technique for fluorescently labeling a specific biological target within a sample using an antibody. This protocol provides general instructions for immunolabeling fixed and permeabilized cells with primary antibodies that are directly labeled with fluorescent dyes. Immunolabeling with direct labeled primary antibodies can be used for high-abundance targets that do not require signal amplification with secondary detection methods.

Materials
- Phosphate-buffered saline (PBS)
- Fixative such as 4% formaldehyde in PBS
- Permeabilization reagent such as 0.2% Triton X-100 in PBS
- Optional: Image-iT FX Signal Enhancer (Cat. No. I36933)
- Blocking reagent such as 3–6% bovine serum albumin/5% normal goat serum/PBS or BlockAid™ Blocking Solution (Cat. No. B10710)
- Labeled primary antibodies. Search our extensive portfolio of high-quality antibodies at thermofisher.com/antibodies
- Optional: Counterstain such as DAPI (Cat. No. D1306)
- Mounting medium such as Invitrogen™ ProLong™ Glass Antifade Mountant (Cat. No. P36980)

Protocol
Primary antibodies of different species, or conjugated primary antibodies, may be mixed together in one solution. Some primary antibodies may require antigen retrieval. All steps are usually carried out at room temperature, unless otherwise noted.

1. Remove culture medium and fix cells (a common fixative is 4% formaldehyde in PBS, for 15 minutes).
2. Wash well in PBS (3 x 5 minutes is typical).
3. Permeabilize the cells for at least 30 minutes (a common permeabilization reagent is 0.2% Triton X-100 in PBS).

This protocol is continued on page 52
4. Wash well in PBS (typically 3 x 10 minutes).


6. Block for nonspecific antibody binding at least 60 minutes (a common blocking solution would be 3–6% bovine serum albumin/5% normal goat serum/PBS, or commercial blocking reagents such as BlockAid™ Blocking Solution (Cat. No. B10710)).

7. Incubate in primary antibody for at least 30 minutes, in blocking solution (antibody concentrations vary, but are usually 0.5–10 µg/mL).

8. Wash well in PBS, 3 x 10 minutes.

9. Counterstain as needed (such as with DAPI (Cat. No. D1306)).

10. Mount in appropriate mounting medium (for fluorescent primaries, a good antifade solution is best, such as ProLong Glass Antifade Mountant (Cat. No. P36980)).
Immunolabeling or immunocytochemistry (ICC) is a technique for fluorescently labeling a specific biological target within a sample using an antibody. This protocol provides general instructions for immunolabeling fixed and permeabilized cells with unlabeled primary antibodies, followed by secondary antibodies that are directly labeled with fluorescent dyes. Indirect immunolabeling with primary and secondary antibodies provides signal amplification since multiple secondary antibodies can bind to the primary antibody.

Materials
- Phosphate-buffered saline (PBS)
- Fixative such as 4% formaldehyde in PBS
- Permeabilization reagent such as 0.2% Triton X-100 in PBS
- Optional: Invitrogen™ Image-iT™ FX Signal Enhancer (Cat. No. I36933)
- Blocking reagent such as 3–6% bovine serum albumin/5% normal goat serum/PBS or Invitrogen™ BlockAid™ Blocking Solution (Cat. No. B10710)
- Primary and secondary antibodies. Search our extensive portfolio of high-quality antibodies at thermofisher.com/antibodies
- Optional: Counterstains such as DAPI (Cat. No. D1306)
- Mounting medium such as Invitrogen™ ProLong™ Glass Antifade Mountant (Cat. No. P36980)

Protocol
Adherent cells may be labeled by immersing them in stain solutions in multi-well plates, inverting onto label solutions on Parafilm™ laboratory film, or by placing the staining or labeling solution onto the coverslip in humidity chambers. Cells in suspension would be labeled in a tube, with spin-downs between washes and staining steps. Primary antibodies of different species, or conjugated primary antibodies, may be mixed together in one solution. Secondary antibodies recognizing different species or isotypes of primary may be mixed together into one solution. All steps are usually carried out at room temperature.

This protocol is continued on page 54
1. Remove culture medium and fix cells (a common fixative is 4% formaldehyde in PBS) for 15 minutes.

2. Wash well in PBS (3 x 5 minutes is typical).

3. Permeabilize the cells (a common permeabilization reagent is 0.2% Triton X-100 in PBS) for 30 minutes.

4. Wash well in PBS.


6. Block for nonspecific antibody binding 30–60 minutes (a common blocking solution would be 3–6% bovine serum albumin/5% normal goat serum/PBS, or commercial blocking reagents such as BlockAid™ Blocking Solution (Cat. No. B10710)).

7. Incubate in primary antibody for 30–60 minutes, in blocking solution (antibody concentrations vary, but are usually 0.5–10 µg/mL).

8. Wash well in PBS.

9. Incubate in secondary antibody for 30–60 minutes, in 3–6% bovine serum albumin/PBS (a good starting antibody concentration is 5 µg/mL).

10. Wash well in PBS.

11. Counterstain as needed (such as with DAPI (Cat. No. D1306)).

12. Mount in appropriate mounting medium (for fluorescent secondaries, a good antifade solution is best, such as ProLong Glass Antifade Mountant (Cat. No. P36980)).
Introduction
For improved detection sensitivity, streptavidin-based amplification techniques are widely used in fluorescence imaging to detect biotinylated biomolecules such as primary and secondary antibodies. Streptavidin-based detection provides signal amplification for medium- and low-abundance targets with a simple workflow. This protocol provides general instructions for performing biotin/streptavidin amplification on fixed cells.

Materials
- Phosphate-buffered saline (PBS)
- Fixative such as 4% formaldehyde in PBS
- Permeabilization reagent such as 0.2% Triton X-100 in PBS
- Invitrogen™ Endogenous Biotin-Blocking Kit (Cat. No. E21390)
- Optional: Invitrogen™ Image-iT™ FX Signal Enhancer (Cat. No. I36933)
- Blocking reagent such as 3–6% bovine serum albumin/5% normal goat serum/PBS or Invitrogen™ BlockAid™ Blocking Solution (Cat. No. B10710)
- Biotinylated primary or secondary antibody
- Labeled streptavidin
- Optional: Counterstains such as DAPI (Cat. No. D1306)
- Mounting medium such as Invitrogen™ ProLong™ Glass Antifade Mountant (Cat. No. P36980)

Protocol
Adherent cells may be labeled by immersing them in stain solutions in multi-well plates, inverting onto label solutions on Parafilm™ laboratory film, or by placing the staining or labeling solution onto the coverslip in humidity chambers. Cells in suspension would be labeled in a tube, with spin-downs between washes and staining steps. All steps are usually carried out at room temperature.

1. Remove culture medium and fix cells (a common fixative is 4% formaldehyde in PBS) for 15 minutes.

2. Wash well in PBS (3 x 5 minutes is typical).

This protocol is continued on page 56
3. Permeabilize the cells (a common permeabilization reagent is 0.2% Triton X-100 in PBS) for 30 minutes.

4. Wash well in PBS.

5. Block endogenous biotin using the Endogenous Biotin-Blocking Kit protocol (Cat. No. E21390, which involves two ~20-minute steps plus washes).


7. Block for nonspecific antibody binding 30–60 minutes. A common blocking solution would be 3–6% bovine serum albumin/5% normal goat serum/PBS, or commercial blocking reagents such as BlockAid Blocking Solution (Cat. No. B10710).

8. Incubate in primary antibody for 30–60 minutes, in blocking solution (antibody concentrations vary, but usually between 0.5–10 µg/mL).

9. Wash well in PBS.

10. (If primary is not already biotinylated) Incubate in biotinylated secondary antibody for 30–60 minutes, in 3–6% bovine serum albumin/PBS (a good starting antibody concentration is 5 µg/mL).

11. Wash well in PBS.

12. Label with conjugated streptavidin 30 minutes (2–5 µg/mL in 3–6% bovine serum albumin/PBS).

13. Wash well in PBS.

14. Counterstain as needed (such as with DAPI (Cat. No. D1306)).

15. Mount in appropriate mounting medium. For fluorescent streptavidins, a good antifade solution is best, such as ProLong Glass Antifade Mountant (Cat. No. P36980).
Introduction
Immunolabeling or immunohistochemistry (IHC) is a technique for fluorescently labeling a specific biological target within a sample using an antibody. This protocol provides general instructions for immunolabeling fixed paraffin tissue sections with unlabeled primary antibodies followed by secondary antibodies that are directly labeled with fluorescent dyes. Indirect immunolabeling with primary and secondary antibodies provides signal amplification since multiple secondary antibodies can bind to the primary antibody.

Materials
- Solvent for deparaffinization such as Histo-Clear (Cat. No. 50-329-51) or other citric solvent
- Ethanol
- Phosphate-buffered saline (PBS)
- Fixative such as 4% formaldehyde in PBS
- Permeabilization reagent such as 0.2% Triton X-100 in PBS
- Optional: Invitrogen™ ReadyProbes™ Tissue Autofluorescence Quenching Kit (Cat. No. R37630)
- PBT (phosphate-buffered saline (PBS) with 0.1% Triton X-100 and 0.1% bovine serum albumin (BSA))
- Optional: Invitrogen™ Image-iT™ FX Signal Enhancer (Cat. No. I36933)
- Blocking reagent such as 3–6% bovine serum albumin/5% normal goat serum/PBS or Invitrogen™ BlockAid™ Blocking Solution (Cat. No. B10710)
- Primary and secondary antibodies. Search our extensive portfolio of high-quality antibodies at thermofisher.com/antibodies
- Optional: Counterstains such as DAPI (Cat. No. D1306)
- Mounting medium such as Invitrogen™ ProLong™ Glass Antifade Mountant (Cat. No. P36980)

Protocol
1. Deparaffinize in solvent. Histo-Clear or other citric solvent will have less autofluorescence generation than xylenes.
2. Rehydrate through a graded ethanol series back down to PBS.

This protocol is continued on page 58
IHC indirect with secondaries on paraffin tissue, cont.

General labeling protocol for secondary detection for fixed paraffin tissue sections

3. Permeabilize the section for at least 30 minutes (a common permeabilization reagent is 0.2% Triton X-100 in PBS).


5. Wash very well 3 x 10 minutes PBT.

6. Optional: Perform antigen retrieval method of choice; not all primary antibodies require this, and it can increase autofluorescence.

7. Wash well in PBT (typically 3 x 10 minutes).


9. Block for nonspecific antibody binding at least 60 minutes. A common blocking solution would be 3–6% bovine serum albumin/5% normal goat serum/PBS, or commercial blocking reagents such as BlockAid Blocking Solution (Cat. No. B10710).

10. Incubate in primary antibody for at least 2 hours, in blocking solution (antibody concentrations vary, but are usually 0.5–10 µg/mL). Very commonly, incubation will go overnight at 4ºC.

11. Wash well in PBT.

12. Incubate in secondary antibody for at least 2 hours, in 3–6% bovine serum albumin/PBS (a good starting antibody concentration is 5 µg/mL).

13. Wash well in PBS.

14. Counterstain as needed, such as with DAPI (Cat. No. D1306).

15. Mount in appropriate mounting medium. For fluorescent secondaries, a good antifade solution is best, such as ProLong Glass Antifade Mountant (Cat. No. P36980).

Figure 19. Mouse intestinal ileum 8 µm FFPE section labeled with NucBlue Live Cell Stain and goat anti-rabbit Alexa Fluor Plus 555 secondary to detect a rabbit pan-actin primary antibody. Imaged on an EVOS M7000 Imaging System using an Olympus 10x Super apochromat objective.
IHC indirect with secondaries on cryosection tissue

General labeling protocol for secondary detection for fixed tissue sections

Introduction
Immunolabeling or immunohistochemistry (IHC) is a technique for fluorescently labeling a specific biological target within a sample using an antibody. This protocol provides general instructions for immunolabeling cryosectioned tissue with unlabeled primary antibodies followed by secondary antibodies that are directly labeled with fluorescent dyes. Indirect immunolabeling with primary and secondary antibodies provides signal amplification since multiple secondary antibodies can bind to the primary antibody.

Materials
- Phosphate-buffered saline (PBS)
- Permeabilization reagent such as 0.2% Triton X-100 in PBS
- PBT (phosphate-buffered saline (PBS) with 0.1% Triton X-100 and 0.1% bovine serum albumin (BSA))
- Optional: Invitrogen™ Image-iT™ FX Signal Enhancer (Cat. No. I36933)
- Blocking reagent such as 3–6% bovine serum albumin/5% normal goat serum/PBS or Invitrogen™ BlockAid™ Blocking Solution (Cat. No. B10710)
- Primary and secondary antibodies—search our extensive portfolio of high-quality antibodies at thermofisher.com/antibodies
- Optional: Counterstains such as DAPI (Cat. No. D1306)
- Mounting medium such as Invitrogen™ ProLong™ Glass Antifade Mountant (Cat. No. P36980)

Protocol
1. Permeabilize the section for at least 30 minutes (a common permeabilization reagent is 0.2% Triton X-100 in PBS).

2. Wash well in PBT (typically 3 x 10 minutes).


4. Block for nonspecific antibody binding at least 60 minutes. A common blocking solution would be 3–6% bovine serum albumin/5% normal goat serum/PBS, or commercial blocking reagents such as BlockAid Blocking Solution, (Cat. No. B10710).

This protocol is continued on page 60
IHC indirect with secondaries on cryosection tissue, cont.

General labeling protocol for secondary detection for fixed tissue sections

5. Incubate in primary antibody for at least 2 hours, in blocking solution (antibody concentrations vary, but are usually 0.5–10 µg/mL). Very commonly, incubation will go overnight at 4°C.

6. Wash well in PBT.

7. Incubate in secondary antibody for at least 2 hours, in 3–6% bovine serum albumin/PBS (a good starting antibody concentration is 5 µg/mL).

8. Wash well in PBS.

9. Counterstain as needed, such as with DAPI (Cat. No. D1306).

10. Mount in an appropriate mounting medium. For fluorescent secondaries, a good antifade solution is best, such as ProLong Glass Antifade Mountant (Cat. No. P36980).

Figure 20. Human cerebellum cryopreserved tissue section. The tissue was labeled with NucBlue™ Fixed Cell ReadyProbes™ Reagent (Cat. No. R37606), Mouse Anti–Beta-Tubulin Antibody (Cat. No. A32723), Alexa Fluor™ Plus 555 Phalloidin (Cat. No. A30106), and a rabbit anti-GFAP antibody detected with Alexa Fluor™ Plus 647 Goat Anti-Rabbit Secondary Antibody (Cat. No. 32733). The tissue was mounted in ProLong™ Glass Antifade Mountant (Cat. No. P36980) and images acquired using an EVOS™ M7000 automated microscope (Cat. No. AMF7000) with DAPI (Cat. No. AMEP4950), GFP (Cat. No. AMEP4951), RFP (Cat. No. AMEP4952), and Cy5 (Cat. No. AMEP4956) EVOS™ Light Cubes. The image is a stitched composite of 272 individual fields of view.
IHC biotin/streptavidin amplification on fixed tissue

General labeling protocol for streptavidin detection for fixed tissue sections

Introduction
For improved detection sensitivity, streptavidin-based amplification techniques are widely used in fluorescence imaging to detect biotinylated biomolecules such as primary and secondary antibodies. Streptavidin-based detection provides signal amplification for medium- and low-abundance targets with a simple workflow. This protocol provides general instructions for performing biotin/streptavidin amplification on fixed tissue sections.

Materials

- Phosphate-buffered saline (PBS)
- Permeabilization reagent such as 0.2% Triton X-100 in PBS
- PBT (phosphate-buffered saline (PBS) with 0.1% Triton X-100 and 0.1% bovine serum albumin (BSA))
- Optional: Image-iT FX Signal Enhancer (Cat. No. I36933)
- Blocking reagent such as 3–6% bovine serum albumin/5% normal goat serum/PBS or Invitrogen™ BlockAid™ Blocking Solution (Cat. No. B10710)
- Biotinylated primary or secondary antibody—search our extensive portfolio of high-quality antibodies at thermofisher.com/antibodies
- Labeled streptavidin
- Optional: Counterstains such as DAPI (Cat. No. D1306)
- Mounting medium such as Invitrogen™ ProLong™ Glass Antifade Mountant (Cat. No. P36980)

Protocol

1. Permeabilize the section for at least 30 minutes (a common permeabilization reagent is 0.2% Triton X-100 in PBS).

2. Wash well in PBT (typically 3 x 10 minutes).


This protocol is continued on page 62
4. Block for nonspecific antibody binding at least 60 minutes. A common blocking solution would be 3–6% bovine serum albumin/5% normal goat serum/PBS, or commercial blocking reagents such as BlockAid Blocking Solution (Cat. No. B10710).

5. Incubate in primary antibody for at least 2 hours, in blocking solution (antibody concentrations vary, but are usually 0.5–10 µg/mL). Very commonly, incubation will go overnight at 4ºC.

6. Wash well in PBT.

7. (Steps 7 and 8 can be skipped if the primary is biotinylated.) Incubate in biotinylated secondary antibody for at least 2 hours, in 3–6% bovine serum albumin/PBT (a good starting antibody concentration is 5 µg/mL).

8. Wash well in PBT.

9. Label with conjugated streptavidin 1–2 hours (2–5 µg/mL in 3–6% bovine serum albumin/PBT).

10. Wash well in PBS.

11. Counterstain as needed, such as with DAPI (Cat. No. D1306).

12. Mount in appropriate mounting medium. For fluorescent secondaries, a good antifade solution is best, such as ProLong Glass Antifade Mountant (Cat. No. P36980).
Introduction
Invitrogen™ Tyramide SuperBoost™ signal amplification is a highly sensitive method for the detection of low-abundance targets in multiplexable fluorescence ICC/IHC/ISH experiments. Tyramide SuperBoost technology combines the brightness of Invitrogen™ Alexa Fluor™ dyes with poly-HRP–mediated tyramide signal amplification to distinguish signal from noise. The technology yields precision and sensitivity 10–200 times greater than standard ICC/IHC/ISH and 2–10 times that of other tyramide amplification techniques like TSA™. Tyramide signal amplification used in the Invitrogen™ Tyramide SuperBoost™ kits utilizes the catalytic activity of horseradish peroxidase (HRP) for high-density labeling of a target protein or nucleic acid sequence in situ. Typical ICC/IHC/ISH experiments using the Tyramide SuperBoost kits require 10–100 times less primary antibody than standard ICC/IHC/ISH experiments to achieve the same signal intensity. Since the Tyramide SuperBoost kits greatly enhance specific signal intensity over background, they can be easily optimized to detect specific signals in samples where high endogenous autofluorescence is observed.

Tyramide SuperBoost kits are simple to use and easily adapted to standard ICC, IHC, or FISH experimental protocols, using any cell or tissue type. Cells labeled using a Tyramide SuperBoost kit can be imaged using any type of microscope, producing high-resolution multiplex images. In tissue samples, it is possible to use the primary antibodies from the same host species for easier multiplexing.

Materials
- Tyramide SuperBoost kits with Alexa Fluor™ tyramides; for selection guide visit thermofisher.com/tyramide
- Cells or tissue; use positive and negative controls as needed
- Slides, coverslips, and containers
- Primary or secondary antibodies, as needed. Search our extensive portfolio of high-quality Invitrogen™ antibodies at thermofisher.com/antibodies
- Gibco™ PBS (phosphate-buffered saline), pH 7.4, without calcium, magnesium, or phenol red (Cat. No. 10010031)
- 95% ethanol
- Gibco™ Distilled Water, highly purified (Cat. No. 15230-147)
- Invitrogen™ ReadyProbes™ Hydrophobic Barrier Pap Pen (Cat. No. R3777)
- Invitrogen™ Image-iT™ Fixation/Permeabilization Kit (Cat. No. R37602)

Figure 21. Sequential labeling and detection of three different rabbit primary antibodies using the Tyramide SuperBoost kits. A formalin-fixed, paraffin-embedded (FFPE) rat intestinal section was labeled sequentially with rabbit primary antibodies against H2B, actin, and Ki-67. Primary antibody detection was performed using three different Invitrogen Alexa Fluor Tyramide SuperBoost kits. Briefly, tissue samples underwent heat-induced antigen retrieval in citrate buffer, pH 6 (10 min on high setting in a pressure cooker) and were then sequentially labeled with rabbit anti-H2B antibody (detected with the Alexa Fluor 647 Tyramide SuperBoost Kit (green)), rabbit anti–smooth muscle actin antibody (detected with the Alexa Fluor 488 Tyramide SuperBoost Kit (red)), and rabbit anti-Ki67 antibody (detected with the Alexa Fluor 594 Tyramide SuperBoost Kit (blue)). In between each antibody labeling, tissue samples were microwaved in citrate buffer, pH 6, on high power until boiling (~2 min), then microwaved for 15 min at 20% power, and finally allowed to cool to room temperature before subsequent labeling with the next rabbit antibody.

This protocol is continued on page 64
ICC and IHC tyramide signal amplification, cont.

Using Tyramide SuperBoost kits

- Invitrogen™ Endogenous Biotin-Blocking Kit (Cat. No. E21390)
- Thermo Scientific™ Citrate Buffer (pH 6.0), Concentrate (Cat. No. 005000)
- Invitrogen™ ProLong™ Diamond Antifade Mountant or Invitrogen™ SlowFade™ Diamond Antifade Mountant (Cat. No. P36961 or Cat. No. S36963)

Protocol

Prepare reagents

1. **100X tyramide stock solution**: Dissolve the Alexa Fluor tyramide reagent (component C1) in 150 μL (for 150 slides) or 50 μL (for 50 slides) of DMSO (component E). Invert the vial several times to dissolve any tyramide that might coat its sides. You can store the 100X tyramide stock solution at 2–8°C for up to 6 months in a sealed vial. Store the vial away from moisture, if possible.

2. **100X H₂O₂ solution**: Add 1 drop (approximately 50 µL) of hydrogen peroxide solution (component C2) to 1 mL of distilled water.

3. **1X reaction buffer**: Add 1 drop (approximately 50 µL) of 20X reaction buffer to 1 mL of distilled water.

4. **Reaction Stop Reagent stock solution**: Add 1.45 mL of 95% ethanol to one vial of Reaction Stop Reagent (component D). Vortex the vial to dissolve any Reaction Stop Reagent coating the sides of the bottle. The Reaction Stop Reagent stock solution will be diluted 1:11 in PBS before use to prepare a working solution. The unused portion of the stock solution can be stored at −20°C for 6 months.

5. **Reaction Stop Reagent working solution**: Dilute the Reaction Stop Reagent stock solution (prepared in step 4) 1:11 in PBS. Note: Prepare the Reaction Stop Reagent working solution fresh on the day of use.

Prepare cells (fixation and permeabilization)

Fix and permeabilize cells according to standard fixation and permeabilization protocols. If fluorescent proteins (GFP/RFP) are present, we recommend using the Image-iT Fixation/Permeabilization Kit (Cat. No. R37602) to prepare your cells.

Note

- You can use a hydrophobic barrier pen (wax pen) to hold liquid reagents on the sample slide or coverslip.
- Do not let the cells or tissue samples dry out.
- For longer incubations, we recommend using a humidified chamber (for example, a covered box with damp paper towel).

Critical note

Allow vials to warm to room temperature before opening.

Note

Prepare the 100X H₂O₂ solution fresh on the day of use.

Prepare the 1X reaction buffer fresh on the day of use. Tris buffer at pH 7.4 can be substituted for reaction buffer for similar performance. Other HRP enzyme–compatible buffers are possible replacements for the reaction buffer but have not been tested.
Prepare tissues
The Tyramide SuperBoost system is compatible with all types of tissues that can be labeled with standard IHC/FISH techniques. Deparaffinize and dehydrate the tissue according to standard IHC protocols before treating it for endogenous peroxidase activity in step 1.

Peroxidase labeling

1. **Optional:** If needed, quench the endogenous peroxidase activity of the sample by adding enough drops of 3% hydrogen peroxide solution (component C2) to cover the sample and incubate for 60 minutes at room temperature.

2. Rinse the cells or tissue three times with 1X PBS at room temperature.

3. **Optional:** If using HRP-conjugated streptavidin, block endogenous biotin in the sample with the Endogenous Biotin-Blocking Kit (Cat. No. E21390) as recommended by the manufacturer. Rinse the cells or tissue three times with 1X PBS at room temperature before proceeding to the next step.

4. Add 2–3 drops (approximately 100–150 µL) of Blocking buffer (component A) to the sample and incubate for 60 minutes at room temperature.

5. Label the cells or tissue with primary antibody with mouse or rabbit as the host. If using a SuperBoost kit with streptavidin, use a biotin-conjugated primary antibody or other ligand. Dilute the antibody or biotin-conjugated ligand in blocking buffer (10% goat serum) or another compatible blocking solution such as 2% BSA or BlockAid Blocking Solution (Cat. No. B10710), and incubate with the cells or tissue for 60 minutes at room temperature or overnight at 2–8°C. For FISH, incubate the DNA/RNA probes according to the manufacturer’s protocol.

6. Rinse the cells or tissue for 10 minutes with PBS at room temperature. Repeat this step three times.

7. Add 2–3 drops (approximately 100–150 µL) of poly-HRP–conjugated secondary antibody or HRP-conjugated streptavidin (component B) to the cells or tissue and incubate for 60 minutes at room temperature or overnight at 2–8°C.

8. Rinse the cells or tissue for 10 minutes with PBS at room temperature. Repeat this step three times.

This protocol is continued on page 66

**Note**
If you observe nonspecific signal, you can shorten this incubation period in step 7.
Tyramide labeling

1. Prepare a tyramide working solution according to the table below.

<table>
<thead>
<tr>
<th>Tyramide working solution</th>
<th>Number of coverslips (18 mm × 18 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>100X Tyramide stock solution (step 1)</td>
<td>5 µL</td>
</tr>
<tr>
<td>100X H₂O₂ solution (step 2)</td>
<td>5 µL</td>
</tr>
<tr>
<td>1X reaction buffer (step 3)</td>
<td>500 µL</td>
</tr>
</tbody>
</table>

2. Apply 100 µL of the tyramide working solution to the cells or tissue and incubate for 2–10 minutes at room temperature.

3. Apply 100 µL of Reaction Stop Reagent that was prepared in step 5.

4. Rinse the cells or tissue three times with PBS.

5. **Tyramide SuperBoost kits containing Biotin-XX tyramide only**: If using a kit containing Biotin-XX tyramide, then use the conjugated streptavidin as recommended by the manufacturer. Some of the recommended streptavidin conjugates are listed in the table below.

<table>
<thead>
<tr>
<th>Invitrogen™ Streptavidin conjugates recommended for the detection of Biotin-XX</th>
<th>Ex/Em (nm)</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptavidin, Alexa Fluor® 350 conjugate</td>
<td>346/442</td>
<td>S11249</td>
</tr>
<tr>
<td>Streptavidin, Alexa Fluor® 405 conjugate</td>
<td>402/421</td>
<td>S32351</td>
</tr>
<tr>
<td>Streptavidin, Alexa Fluor® 488 conjugate</td>
<td>495/519</td>
<td>S11223</td>
</tr>
<tr>
<td>Streptavidin, Alexa Fluor® 555 conjugate</td>
<td>555/565</td>
<td>S21381</td>
</tr>
<tr>
<td>Streptavidin, Alexa Fluor® 594 conjugate</td>
<td>590/617</td>
<td>S11227</td>
</tr>
<tr>
<td>Streptavidin, Alexa Fluor® 647 conjugate</td>
<td>650/668</td>
<td>S21374</td>
</tr>
</tbody>
</table>

**Multiplex with primary antibodies from a different species**

After step 4 or 5, cells or tissue samples can be multiplexed with another Tyramide SuperBoost kit or using standard IHC/ICC protocols.

When multiplexing, use a primary antibody from a host that is different than the one used in step 5, and a fluorescent label that is spectrally compatible with the first fluorescent label.

*This protocol is continued on page 67*
Multiplex with primary antibodies from the same species in IHC

For tissue samples (IHC), Tyramide SuperBoost kits are compatible with the method described by Toth and Mezey [1].

In summary, dilute Citrate Buffer (pH 6.0), Concentrate (Cat. No. 005000) 1:20 in distilled water. After step 4 or 5, place the tissue in the diluted citrate buffer (pH 6.0) and heat in a microwave oven on 100% power until boiling (1–2.5 minutes). Reduce the power to 20% and keep microwaving for an additional 15 minutes. Let the tissue sample cool to room temperature while keeping it in the citrate buffer. Wash the sample twice with 1X PBS and repeat steps 1 from Peroxidase labeling to step 5 from tyramide labeling with a primary antibody of the same species, if desired. Use a tyramide that is spectrally compatible with the tyramide used in the first round.

Counterstain and detect

1. Counterstain the cells or tissue as needed using standard protocols. Some of the reagents recommended for counterstaining are listed in the table below.

<table>
<thead>
<tr>
<th>Invitrogen™ products recommended for counterstain</th>
<th>Product</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus</td>
<td>NucBlue™ Fixed Cell ReadyProbes™ Reagent (DAPI)</td>
<td>R37606</td>
</tr>
<tr>
<td></td>
<td>NucGreen™ Dead 488 ReadyProbes™ Reagent (SYTOX™ Green)</td>
<td>R37109</td>
</tr>
<tr>
<td></td>
<td>NucRed™ Dead 647 ReadyProbes™ Reagent (TO-PRO-3 iodide)</td>
<td>R37113</td>
</tr>
<tr>
<td>Actin cytoskeleton</td>
<td>ActinGreen™ 488 ReadyProbes™ Reagent (AlexaFluor™ 488 phalloidin)</td>
<td>R37110</td>
</tr>
<tr>
<td></td>
<td>ActinRed™ 555 ReadyProbes™ Reagent (Rhodamine phalloidin)</td>
<td>R37112</td>
</tr>
<tr>
<td>Cell membrane</td>
<td>Wheat Germ Agglutinin, Alexa Fluor™ 488 Conjugate</td>
<td>W11261</td>
</tr>
<tr>
<td></td>
<td>Wheat Germ Agglutinin, Alexa Fluor™ 594 Conjugate</td>
<td>W11262</td>
</tr>
<tr>
<td></td>
<td>Wheat Germ Agglutinin, Alexa Fluor™ 647 Conjugate</td>
<td>W32466</td>
</tr>
</tbody>
</table>

2. Mount the coverslips using a mountant with antifade properties such as the ProLong Diamond Antifade Mountant (Cat. No. P36961) or the SlowFade Diamond Antifade Mountant (Cat. No. S36963). For optimal results, follow the instructions provided with the mountant.

3. Analyze the cells or tissue using a compatible imaging instrument. The Tyramide SuperBoost system is compatible with all types of Invitrogen™ EVOS™ cell imaging systems equipped with compatible fluorescent filters. High-content analyzers also have been successfully used to analyze the cells and tissues on slides and plates.

Reference

Introduction

Cell painting was invented by Anne Carpenter and colleagues from the Broad Institute to provide an image-based profiling tool amenable to the development of drug discovery assays and scalable to multiparameter screening campaigns. As originally described by Carpenter and colleagues [1,2], cellular, organelle, and morphological measurements can be extracted from each cell based on changes in size, shape, texture, and fluorescence intensity to detect subtle changes in biological phenotype. The power of cell painting as a phenotypic measuring tool is best characterized by its comprehensive ability to extract the data-rich information gained from studying multiparametric cellular biology at the cytoskeletal, plasma membrane, and organelle levels. This contrasts with conventional high-throughput screening applications that evaluate only a few measurements for the purposes of scale, while having limited breadth to detect diverse sets of phenotypic changes caused by compound exposure. While morphological changes to the cellular structure are central to cell painting, several organelle-based measurements are also evaluated, including the nucleus, nucleolus, endoplasmic reticulum, Golgi apparatus, and mitochondria. Single cell measurements can be extracted from this imaging-based technology, enabling the identification of those cells most sensitive to compound exposure relative to the overall population. While originally adopted for drug discovery applications [3,4], the technology has since expanded to drug safety [5], environmental toxicology [6], and multi-omics applications, including the prediction of lung cancer variants [7]. Cell painting can help provide hits to initial discovery screens and provide mechanistic insights unavailable to traditional high-throughput assays.

Cell painting methods

Multiparameter overview of cell painting

Cell painting consists of a multiplexed panel of eight organelle and morphological markers. The markers include Invitrogen™ Hoechst 34580 to label the nucleus, Concanavalin A, Alexa Fluor™ 488 Conjugate for the endoplasmic reticulum, SYTO 14™ Green Fluorescent Nucleic Acid Stain that labels both the nucleoli and cytoplasmic RNA, Wheat Germ Agglutinin (WGA), Alexa Fluor™ 555 Conjugate that labels both the Golgi apparatus and plasma membrane, Alexa Fluor™ 568 Phalloidin to label the actin cytoskeleton, and Mitotracker™ Deep Red FM Dye to label the mitochondria and measure the mitochondrial membrane potential proportional to intensity (Figure 22). Collectively, this panel requires an image acquisition configuration of at least five fluorophores and fluorescence channels to capture the eight markers.

Figure 22. Cell painting screening of U2OS cells. The cells were labeled using the Image-iT Cell Painting Kit, and analyzed on the CellInsight CX7 LZR Pro HCS Platform with a 20X 0.7 NA objective to screen for nuclei (channel 1), endoplasmic reticulum and cytoplasmic RNA and nucleoli (channel 2), Golgi apparatus and plasma membrane (channel 3), mitochondria (channel 4), and actin (channel 5), resulting in a red, green, blue (RGB) image by combining all 5 channels with pseudocoloring.

This protocol is continued on page 70
Cell painting, cont.

Protocol

Cell painting labeling with the Image-iT Cell Painting Kit (Cat No. I64000)
To help accelerate development of the cell painting assay, we have recently released the Thermo Scientific™ Image-iT™ Cell Painting Kit in conjunction with the cell painting assay in our high-content Thermo Scientific™ CellInsight™ CX7 Pro HCS platforms.

Prepare stock solutions

1. Dissolve 5 mg of Hoechst 34580 in 5 mL of ddH₂O to make a 1 mg/mL solution (2,000X).

2. Dissolve 5 mg of Concanavalin A, Alexa Fluor 488 Conjugate in 1 mL of 0.1 M sodium bicarbonate (~pH 8.3) to make a 5 mg/mL solution (50X).

3. Dissolve 5 mg of WGA, Alexa Fluor 555 Conjugate in 5 mL of ddH₂O to make a 1 mg/mL solution (666X).

4. Dissolve 300 units of Alexa Fluor 568 Phalloidin in 150 μL of DMSO to make a 66 μM solution (400X).

5. Dissolve the contents of one vial of MitoTracker Deep Red FM Dye in 91.98 μL of DMSO to make 1 mM solution (2,000X).

Cell painting labeling

1. Seed cells into a 384-well plate in growth medium (40 μL cell suspension/well) at a density of 1,000 cells/well. For 96-well plates, use 100 μL cell suspension/well at a density of 4,000 cells/well.

2. Incubate for 60 minutes at room temperature, followed by incubation for ~20 hours at 37°C, 5% CO₂, and ~95% relative humidity to allow overnight recovery and growth of the plated cells.

Note

SYTO 14 Green Fluorescent Nucleic Acid Stain is supplied as a 5 mM solution (1,666X) and does not require preparation.

Note

These conditions are adopted for U2OS cells. For other cell types, the conditions can be modified for optimal results.

This protocol is continued on page 71

For Research Use Only. Not for use in diagnostic procedures.
3. On the following day, prepare 10X test compounds in DMSO and dispense at 1:10 dilution to each well to achieve 10 μM assay concentration, then incubate the plates for 24–48 hours at 37°C, 5% CO₂ and ~95% relative humidity. To avoid DMSO vehicle effects, be sure to keep the final DMSO concentration below 0.5%.

4. Prepare a 10X staining solution of MitoTracker Deep Red FM Dye by adding 50 μL of stock solution (1 mM), to 10 mL of complete medium. Dispense this medium at a 1:10 dilution to each well to achieve a final concentration of 500 nM.

5. Incubate for 30 minutes at 37°C, 5% CO₂, and 95% relative humidity.

6. Prepare an 8% paraformaldehyde (PFA) fixation solution (without methanol) and dispense at 1:2 dilution to each well to achieve a final concentration of 4% PFA.

7. Incubate for 15 minutes at room temperature.

8. Remove the supernatant and replace with 0.1% Triton™ X-100 solution.

9. Incubate for 15 minutes at room temperature.

10. Remove the supernatant and replace with the staining solution of Alexa Fluor 568 Phalloidin, Concanavalin A Alexa Fluor 488, Hoechst 34580, WGA Alexa Fluor 555, and SYTO 14 Green Fluorescent Stain diluted in 1X HBSS + 1% BSA according to the following table.

<table>
<thead>
<tr>
<th>Dye (stock concentration)</th>
<th>Hoechst 34580 (1 mg/mL)</th>
<th>Concanavalin A, Alexa Fluor 488 Conjugate (5 mg/mL)</th>
<th>SYTO 14 stain (5 mM)</th>
<th>Wheat Germ Agglutinin Alexa Fluor 555 Conjugate (1 mg/mL)</th>
<th>Alexa Fluor 568 Phalloidin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution from stock solution</td>
<td>1:2,000</td>
<td>1:50</td>
<td>1:1,666</td>
<td>1:666</td>
<td>1:400</td>
</tr>
<tr>
<td>Final concentration in well</td>
<td>1 μg/mL</td>
<td>0.1 mg/mL</td>
<td>3 μM</td>
<td>1.5 μg/mL</td>
<td>165 nM</td>
</tr>
<tr>
<td>Diluent buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1X HBSS + 1% BSA</td>
</tr>
</tbody>
</table>

11. Incubate for 30 minutes at room temperature.

Critical note

Do not remove the medium.

This protocol is continued on page 72
12. Discard solution and wash twice with 1X HBSS.

13. Fill the wells with 1X HBSS/0.05% sodium azide to prevent bacterial growth.

14. Tightly seal the plates with adhesive seals. Plates are now ready for screening.

The CellInsight CX7 LZR Pro HCS Platform and configuration of the cell painting application

The cell painting application has been developed for the Thermo Scientific™ CellInsight™ CX7 Pro series instruments, including the LED-based Thermo Scientific™ CellInsight™ CX7 Pro platform and the laser-based Thermo Scientific™ CellInsight™ CX7 LZR Pro platform. Image acquisition on either instrument is performed using the back-illuminated scientific CMOS camera for optimum detection capability of the 8-target screen. Note, the CellInsight CX7 LZR Pro platform will enable superior fluorescence specificity due to the narrow excitation band spectra of the laser fluorescence compared to an LED. Scanning is completed in an automated format, including laser-based autofocusing performed on every well. Either instrument also leverages HCS Studio real-time analysis capabilities that enable simultaneous image acquisition and analysis by using this real-time analysis functionality; sufficient fields are acquired using the 20x objective so that a desired number of cells is individually analyzed. Additional cells can be monitored by increasing the “intra well stop” criterion to the desired number.

Cell painting results in U2OS cells

The resulting data generated from the cell painting application can be evaluated at the well population level as mean averages, or the single cell measurements of every validated U2OS cell can be evaluated. For additional quality control considerations, the cell level cutouts of each cell displayed on the scatterplot can be shown. Phenotypic changes in cell painting responses in untreated versus pharmacological controls are shown in Figure 23.

References
