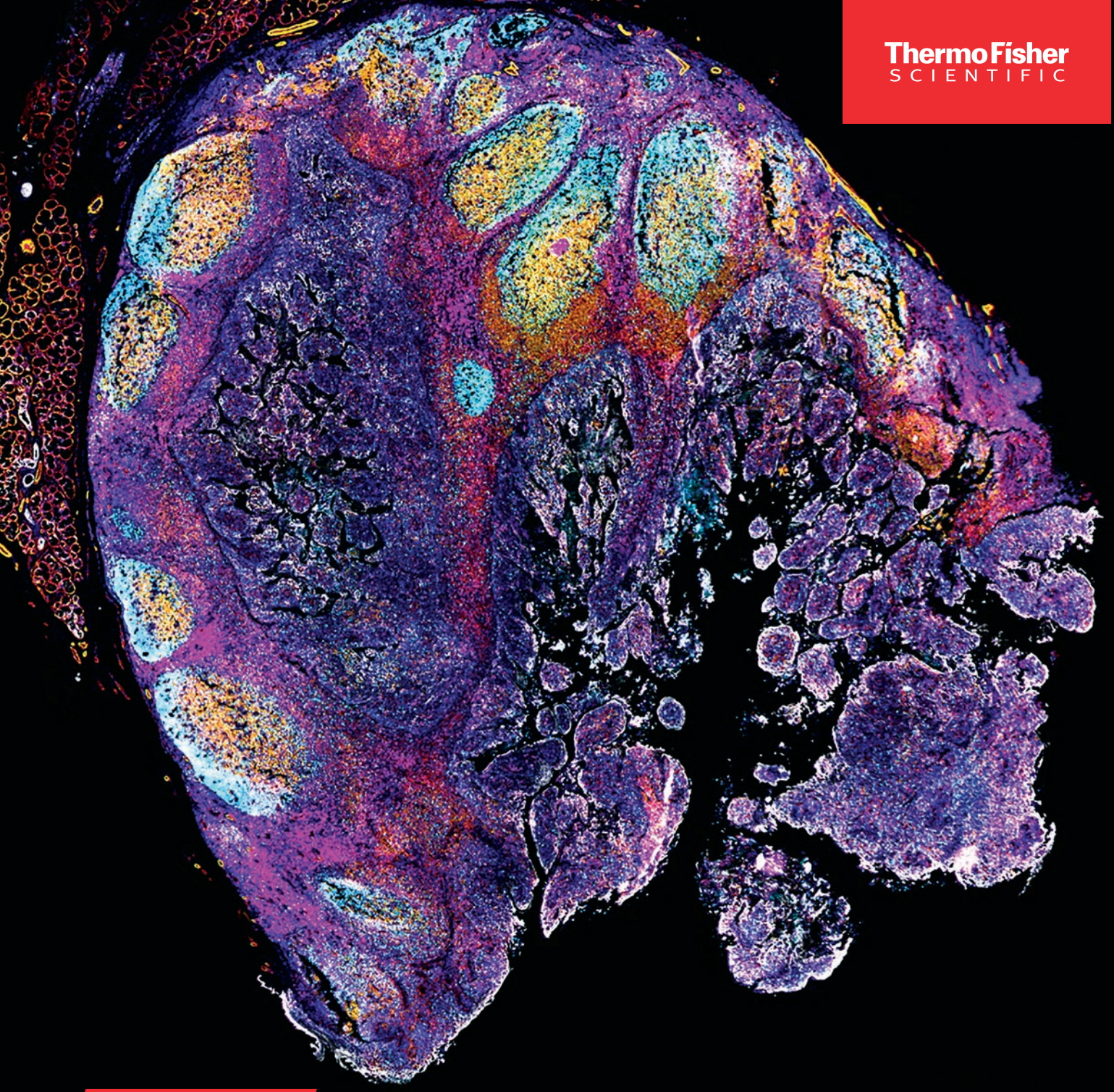

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Cellular analysis

Imaging protocol handbook

Second edition of trusted imaging protocols

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Introduction

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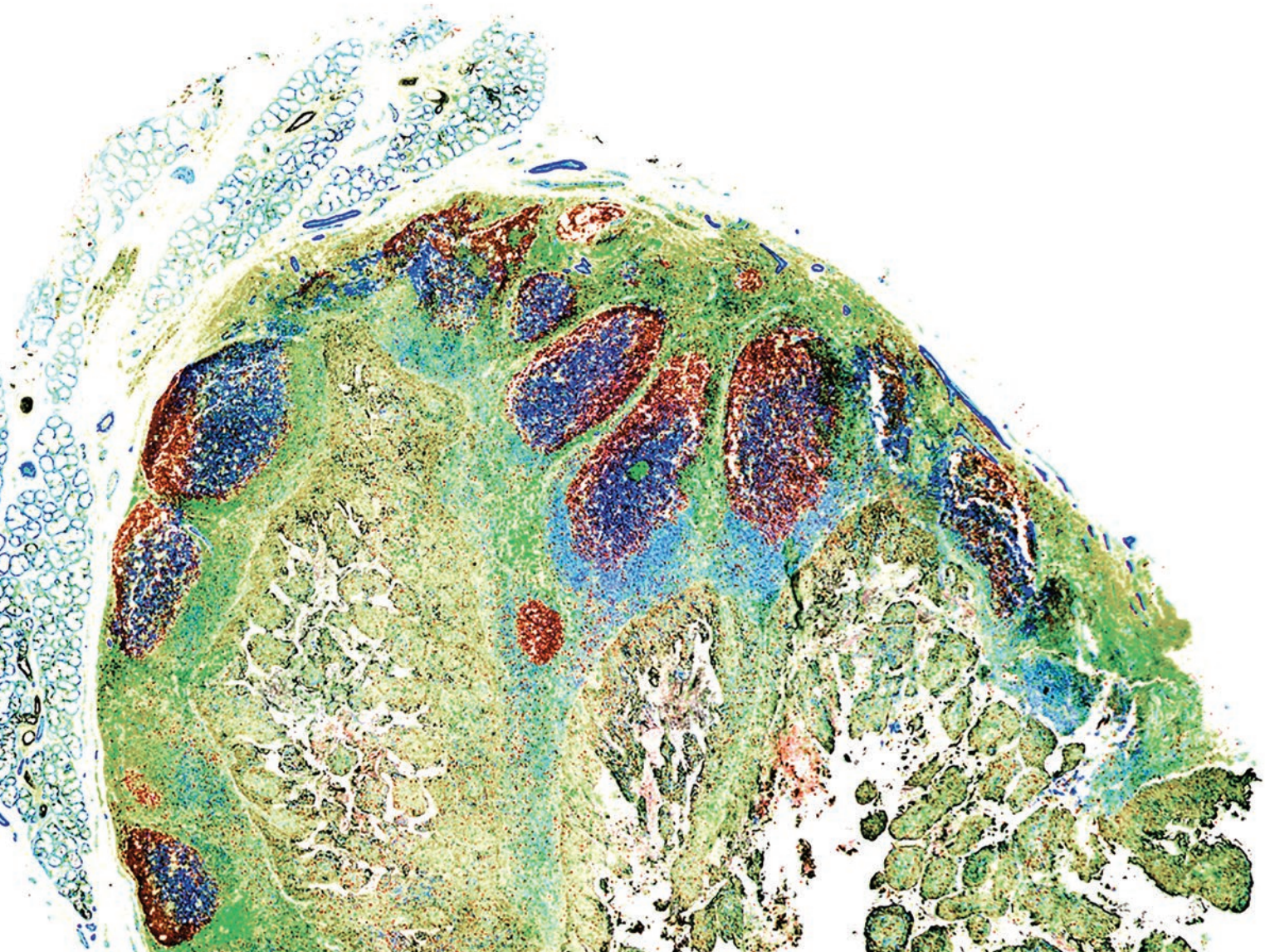
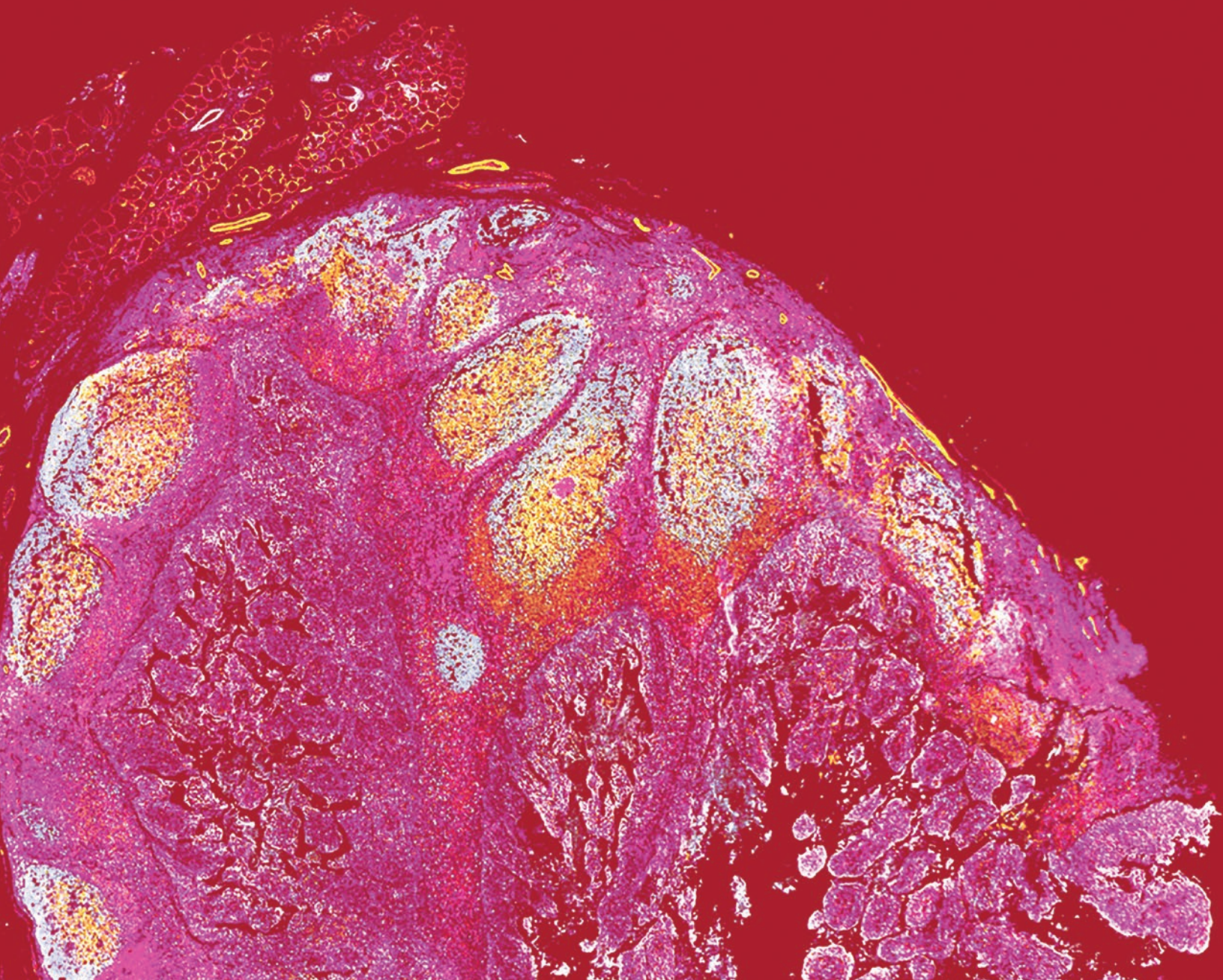


Contents

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

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

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.

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 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.

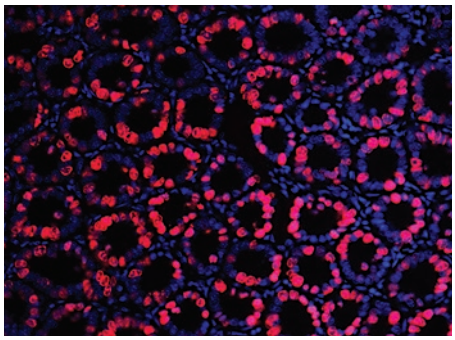


Figure 1. Eliminate long detection procedures with Click-iT EdU. A rat ileum tissue section was detected using the red-fluorescent Click-iT EdU Alexa Fluor 594 Imaging Kit (Cat. No. C10084). EdU staining is completed in 80 min., while BrdU protocols require harsh permeabilization and overnight anti-BrdU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

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.

This protocol is continued on page 3

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.

	Number of coverslips						
Reaction component*	**1**	**2**	**4**	**5**	**10**	**25**	**50**
1X Click-iT EdU reaction buffer	430 µL	860 µL	1.8 mL	2.2 mL	4.3 mL	10.7 mL	21.4 mL
$CuSO_4$ (component E)	20 µL	40 µL	80 µL	100 µL	200 µL	500 µL	1 mL
Alexa Fluor azide	1.2 µL	2.5 µL	5 µL	6 µL	12.5 µL	31 µL	62 µL
1X Click-iT EdU buffer additive	50 µL	100 µL	200 µL	250 µL	500 µL	1.25 mL	2.5 mL
Total volume	500 µL	1 mL	2 mL	2.5 mL	5 mL	12.5 mL	25 mL

* 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.

Invitrogen™ dye	Hoechst™ 33342	Alexa Fluor™ 488	Alexa Fluor™ 555	Alexa Fluor™ 594	Alexa Fluor™ 647
Excitation/emission (nm)	350/461	495/519	555/615	590/615	650/670
Standard filter set	DAPI	FITC	RFP	Texas Red	Cy®5

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.

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

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 × 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.

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.

Note

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

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.

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.

	Number of coverslips						
Reaction component*	**1**	**2**	**4**	**5**	**10**	**25**	**50**
1X Click-iT reaction buffer	440 µL	880 µL	1.84 mL	2.25 mL	4.4 mL	10.9 mL	21.9 mL
Copper protectant	10 µL	20 µL	40 µL	50 µL	100 µL	250 µL	500 µL
Alexa Fluor picolyl azide (component B)	1.2 µL	2.5 µL	5 µL	6 µL	12.5 µL	31 µL	62 µL
1X Click-iT EdU buffer additive	50 µL	100 µL	200 µL	250 µL	500 µL	1.25 mL	2.5 mL
Total volume	500 µL	1 mL	2 mL	2.5 mL	5 mL	12.5 mL	25 mL

* 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 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.

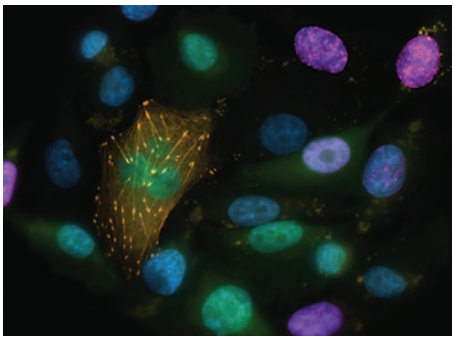


Figure 2. Multicolor imaging with the Click-iT Plus EdU Alexa Fluor 647 compatible with GFP.

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.

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.

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.

Invitrogen™ dye	Hoechst™ 33342	Alexa Fluor™ 488	Alexa Fluor™ 555	Alexa Fluor™ 594	Alexa Fluor™ 647
Excitation/emission (nm)	350/461	495/519	555/615	590/615	650/670
Standard filter set	DAPI	FITC	RFP	Texas Red	Cy®5

Click-iT EdU Colorimetric IHC Detection Kit

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% H_2O_2 in PBS
- 18 × 18 mm coverslips (for standard microscopy)
- *Optional:* Invitrogen™ EdU (Cat. No. A10044, E10187, E10415)

Note

The kit includes sufficient reagents for labeling 50 tissue sections using a 500 µL reaction volume per test. Larger amounts of EdU may be purchased separately, if needed.

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.

Critical note

Allow vials to warm to room temperature before opening.

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.

Note

If treating 4 or fewer slides, you can use the wash chamber (provided in the kit) filled with 24 mL of Wash Buffer.

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.

Species	Reference*
Nematode (*C. elegans*)	Dorsett M, Westlund B, Schedl T (2009) *Genetics* 183: 233–247.
Flatworm (marine)	*BioProbes* 61
Cricket	Bando T, Mito T, Maeda Y et al. (2009) *Development* 136: 2235–2245.
Mouse	Salic A (2008) *Proc Natl Acad Sci USA* 105: 2415–2420. Bonaguidi MA, Peng CY, McGuire T et al. (2008) *J Neurosci* 28: 9194–9204. Kharas MG, Janes MR, Scarfone VM et al. (2008) *J Clin Invest* 118: 3038–3050. Zeng C et al. (2010) *Brain Res* 1319: 21–32.
Rat	Scientific poster, ASCB 2007
Zebrafish larva	*BioProbes* 57
Zebra finch	Scientific poster, ASCB 2007
Human-derived stem cells	McCord AM, Jamal M, Williams ES et al. (2009) *Clin Cancer Res* 15: 5145–5153. Momcilovic O, Choi S, Varum S et al. (2009) *Stem Cells* 27: 1822–1835.

* 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.

Tissue deparaffinization procedure	
Solution	**Incubation time**
Xylene	5 minutes
Xylene	5 minutes
100% EtOH	5 minutes
100% EtOH	3 minutes
95% EtOH	3 minutes
85% EtOH	3 minutes
75% EtOH	3 minutes
50% EtOH	3 minutes
1X PBS	5 minutes

2. To quench endogenous peroxidase enzymes, immerse the slides in a solution of 3% H_2O_2 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.

Recommended Trypsin-EDTA treatment for various tissues			
Species	**Tissue type**	**Incubation time**	**Temperature**
Mouse, embryonic	Cardiac	0–5 minutes	Room temperature
Mouse, adult	Cardiac	20–30 minutes	Room temperature
Rat	Mammary	10 minutes	Room temperature
Rat	Intestine	20–30 minutes	Room temperature
Rat	Uterine	30 minutes	37°C
Zebra fish, adult	Caudal fin	0–5 minutes	Room temperature

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.

This protocol is continued on page 12

Click-iT EdU Colorimetric IHC Detection Kit, cont.

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.

	Number of coverslips				
Reaction component*	**1**	**2**	**4**	**5**	**10**
1X Click-iT Reaction Buffer (from "Prepare stock solutions" step 2)	439 µL	878 µL	1.8 mL	2.2 mL	4.4 mL
$CuSO_4$ (component C)	10 µL	20 µL	40 µL	50 µL	100 µL
Biotin azide (from "Prepare stock solutions" step 4)	1.2 µL	2.5 µL	5 µL	6 µL	12.5 µL
1X Click-iT™ EdU Reaction Buffer Additive (from "Edu detection" step 1)	50 µL	100 µL	200 µL	250 µL	500 µL
Total volume (approximate)	500 µL	1 mL	2 mL	2.5 mL	5 mL

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

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.

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.

We recommend using the wash chamber included in the kit.

This protocol is continued on page 13

5. Wash the tissue sections 3 times for 2 minutes each with 1X PBS at room temperature.

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

7. 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.

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

9. 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.

10. 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.

11. 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.

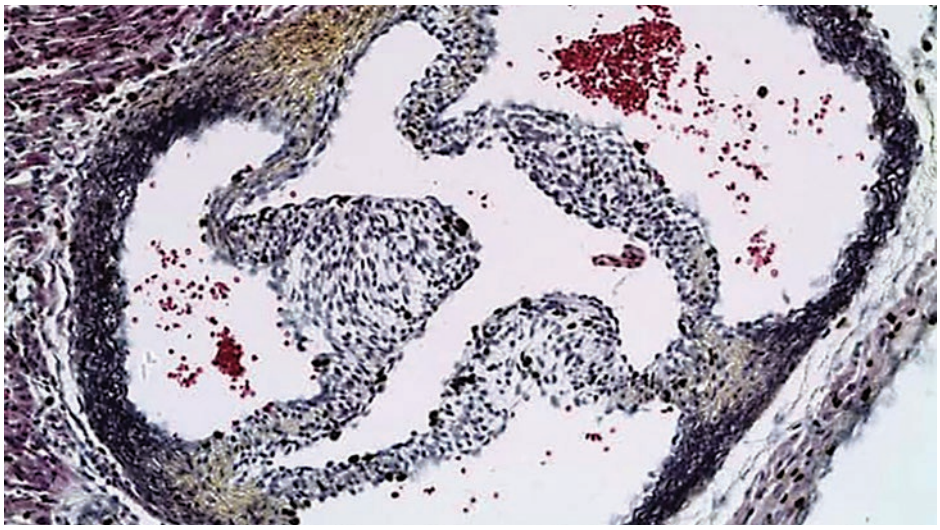


Figure 3. Labeling of proliferating cells in mouse cardiac tissue using the Click-iT EdU Colorimetric kit and Movat's pentachrome stain.

Click-iT EdU labeling *in vivo* cell proliferation protocol

Introduction

In this protocol, we will discuss how to label cell proliferation *in vivo* with Invitrogen Click-iT EdU Imaging Kit. Click-iT cell proliferation assays can be used *in vivo* following EdU administration. EdU is cell permeable and can be delivered *in vivo* through methods including injection (intraperitoneal, intramuscular, subcutaneous), in drinking water, or direct incubation in certain organisms (e.g., *Drosophila* and zebrafish larvae) where EdU becomes incorporated into actively dividing cells. The Click-iT technology is compatible with immunohistochemical, immunocytochemical, and fluorescent dyes that are fixation tolerant or designed for fixed cell labeling. Fluorescent signal accumulates in the nuclei of cells where DNA has been synthesized during the EdU incubation period for easy quantitation.

Critical notes

This protocol can be used for:

- Detecting DNA synthesis using a fluorescence microscope

This protocol should not be used for:

- Flow cytometry; for a flow cytometry protocol, see Click-iT EdU Protocol for Flow Cytometry

Materials

- Invitrogen Image-iT Fixative Solutions 4% Formaldehyde Fixative Solution in PBS (methanol-free) (Cat. No. R37814)
- Thermo Scientific Chemicals Xylenes, 99%, for biochemistry and histology (Cat. No. 447240010)
- Gibco Phosphate buffered saline (e.g., Gibco PBS (10X), pH 7.4, Cat. No. 70011044)
- Thermo Scientific Absolute Ethanol, 200 proof, Molecular Biology Grade (Cat. No. T038181000CS)
- Invitrogen EdU (5-ethynyl-2'-deoxyuridine) (Cat. No. E10187)
- Click-iT EdU Imaging Kit (Cat. Nos. C10338, C10340, C10337, C10339)
- Invitrogen Image-iT Fixation/Permeabilization Kit (e.g., 0.5% Triton X-100 in PBS, Cat. No. R37602)
- 3% bovine serum albumin (BSA) in PBS (3% BSA in PBS), pH 7.4
- For dual pulse 5-Bromo-2'-deoxyuridine (BrdU), 99%, Thermo Scientific Chemicals (Cat. No. H27260.06)
- Invitrogen BrdU Monoclonal Antibody (MoBU-1) (e.g., Cat. No. B35128)

This protocol is continued on page 15

Protocol

EdU labeling in FFPE tissue

Examples of EdU concentrations and incorporation into animal model systems.

Species	Cell or Tissue type	Cell or Tissue Preparation	EdU Conc	EdU incubation time	Administration method	Detection	Reference
Mouse	Colon	Frozen, fixed tissue	1 mg/mouse	4–144 hours	IP	Alexa Fluor 488	J Immunol 188(5):2427–2436 (2012).
	Small intestine and brain	FFPE (brain and small intestine), fresh, fixed (small intestine) tissue	100–200 µg	24–96 hours	IP	Alexa Fluor 568 and TMR	Proc Natl Acad Sci USA 105(7): 2415–2420 (2008).
	Retina (dissociated cells)	Fixed cells	25 µg/g	4 hours	IP	Alexa Fluor 647	Mol Biol Cell 23(22):4362–72 (2012).
	Embryonic brain	Cryosection tissue	5 µg/g	24–72 hours	IP (pregnant dams)	Alexa Fluor 594	Proc Natl Acad Sci USA 19; 110(8):3113–8 (2013).
	Organ of Corti (cochlear neurosensory epithelia)	Whole mount tissue	50 mg/kg	4 hours	Subcutaneous injection	Alexa Fluor 488	J Neurosci 31(24):8883–93 (2011).
	Optic nerves	Cryosection, fixed tissue	0.2 mg/mL	2–8 weeks (water exchanged every 72 hours)	Drinking water	Alexa Fluor 594	J Neurosci 32(36):12528–42 (2012).
Rat	Lung	Cryosection, fixed tissue	50 mg/kg	24 hours	IP	Alexa Fluor 488	Anal Cell Pathol (Amst) 2015:326385 (2015).
	Olfactory Epithelium	Cryosection, fixed tissue	50 mg/kg	2 hours	Subcutaneous	Alexa Fluor 594	J Neurosci 38(21):5022–5037 (2018).
	Bone (HSPCs from bone marrow)	Fresh and fixed cells	60 mg/kg	24 hours	IP (pregnant dams)	Alexa Fluor 647, Alexa Fluor 488 for flow	Nat Commun 13(1):1327 (2022).
Zebrafish	Larval intestine	FFPE tissue	100 µg/mL	16 hours	Soaking	Alexa Fluor 488	Proc Natl Acad Sci USA 108 Suppl 1(Suppl 1):4570–4577 (2011).
	Larval jaws	Fixed tissue	400 µM	24 hours	Soaking	Click-iT EdU	FASEB J 35(11):e22002 (2021).
	Cardiac tissue	Cryosection, fixed tissue	10 mM	3 days	IP	Click-iT EdU	Elife 10:e66079 (2021).
Drosophila	Larval brain, eye discs and trachea	Fixed tissue	20–100 µM	10–60 minutes	Soaking	Alexa Fluor 594	Development 138(23):5201–5212 (2011).
	Larval imaginal wing discs	Fixed tissue	10 µM	30 minutes	Soaking	Alexa Fluor 555	Genes Dev 26(18):2027–2037 (2012).
Maize	Anther	Fixed tissue	20 µg/mL	6 hours	N6 culture medium	Alexa Fluor 488	Plant Physiol 176(2):1610–1626 (2018).
Xenopus (tadpoles)	Intestine	FFPE tissue	10 mg/mL	30–60 min	IP	Alexa Fluor 594	Cold Spring Harb Protoc 2017(9):pdb. prot097717 (2017).
Chicken	Cochlea	Fixed whole mount tissue	50 mg/kg	4–8 hours	Subcutaneous injection	Alexa Fluor 488 and Alexa Fluor 594	Laryngoscope 119(9):1770–1775 (2009).
Nematode (*C. elegans*)	Germ line cells	Fixed cells	20 µM	3–4 hours	Fed through EdU labeled *E. coli* plates	Click-iT EdU	Genetics 183(1):233–247 (2009).

This protocol is continued on page 16

Click-iT EdU labeling *in vivo* cell proliferation protocol, cont.

A generalized example protocol for EdU detection in FFPE tissue is provided below.

1. After incorporation of EdU, isolate the target tissue, fix in formalin, embed in paraffin, section, and mount the tissue on slides using a standard FFPE protocol.

2. Deparaffinize the tissue using a standard deparaffinization rehydration protocol. Slides can be placed in a rack and washed in a Coplin jar using the sequential steps below.

Step	Solution	Incubation time
1	Xylene	5 minutes
2	Xylene	5 minutes
3	100% EtOH	5 minutes
4	100% EtOH	3 minutes
5	95% EtOH	3 minutes
6	85% EtOH	3 minutes
7	75% EtOH	3 minutes
8	50% EtOH	3 minutes
9	1X PBS	5 minutes

3. Wash tissue in 3% BSA in PBS.

4. Detect EdU by the click reaction according to the Click-iT EdU or Click-iT Plus EdU Cell Proliferation Kit. Incubate tissue sections in the Click-iT reaction cocktail for 30 minutes at room temperature, protected from light.

5. Wash tissue in 3% BSA in PBS.

6. Stain the tissue with Hoechst 33342 (from the Click-iT EdU Cell Proliferation Kit) or other appropriate counterstains. Wash the tissue 3X in PBS.

7. Prepare stained tissue in mounting media (such as ProLong antifade mountants) and image slide.

Optional

Stain the tissue with primary and secondary antibodies for non-EdU protein detection. Wash 3X in 3% BSA in PBS.

This protocol is continued on page 17

Protocol for dual-labeled EdU and BrdU FFPE tissue

A generalized protocol for dual pulse EdU-BrdU labeling in FFPE tissue is provided below.

Examples of dual BrdU and EdU concentrations and incorporation into animal model systems.

Species	Cell or Tissue type	Cell or Tissue Preparation	EdU/BrdU Conc	EdU/BrdU incubation time	Administration method	Detection	Reference
Mouse	Brain (embryonic)	Frozen tissue	EdU: 20 mg/kg body weight BrdU: 200 mg/kg body weight	1–2 days	IP (pregnant dams)	Alexa Fluor 488	J Neurosci 31(17):6440–6448 (2011).
	Brain	Fixed tissue	EdU: 7.5 mg/mL, 0.1 mL/10 g mouse BrdU: 7.5 mg/mL, 0.1 mL/10 g mouse	EdU 0–20 hours then BrdU at 24–44 hours	IP	Alexa Fluor 488	Mol Biol Cell 22(12):1960–1970 (2011).
	Skin tumors	Cryosections (fixed) tissue	6 × 50 µg (both)	EdU: 4 weeks BrdU: 2 hours	IP	Click-iT EdU	Proc Natl Acad Sci USA 109(52):21468–21473 (2012).
Zebra finch	Brain, liver, intestine	Cryosections (fixed) tissue	50 mg/kg BrdU and 41 mg/kg EdU	2–8 hours	IM	Alexa Fluor 488	Biology (Basel) 9(11):356 (2020).

1. After incorporation of EdU followed by BrdU *in vivo*, isolate the target tissue, fix in formalin, embed in paraffin, section, and mount the tissue on slides using a standard FFPE protocol.

2. Use a standard deparaffinization rehydration protocol to deparaffinize the tissue (see step 2, page 16).

3. Perform antigen retrieval for BrdU detection. A common method is pH 6 citrate-based heat-induced epitope retrieval (HIER), but other methods can be used.

4. Perform DNA denaturation for BrdU detection. A common method is incubation in 1–2.5 M HCl at room temperature, but other methods such as treatment with nucleases can be used.

This protocol is continued on page 18

Click-iT EdU labeling *in vivo* cell proliferation protocol, cont.

5. Neutralize the tissue in 0.1 M sodium borate buffer (pH 8.5) for 10 minutes at room temperature.

6. Wash the tissue 3X in 3% BSA in PBS.

7. Detect EdU by the click reaction according to the Click-iT EdU or Click-iT Plus EdU Cell Proliferation Kit. Incubate tissue sections in the Click-iT reaction cocktail for 30 minutes at room temperature, protected from light.

8. Wash the tissue in 3% BSA in PBS.

9. Incubate the tissue with an anti-BrdU primary antibody that does not cross react with EdU (e.g., clone MoBU-1).

10. Wash the tissue 3X in 3% BSA in PBS.

11. Incubate with appropriate secondary antibody.

12. Wash the tissue 3X in 3% BSA in PBS.

13. Stain the tissue with Hoechst 33342 (from the Click-iT EdU Cell Proliferation Imaging Kit) or other appropriate counterstains. Wash the tissue 3X in PBS.

14. Prepare stained tissue in mounting media (such as ProLong antifade mountants) and image slide.

Appropriate filters for Click-iT EdU Imaging Kits

Example filters that can be used with Click-iT EdU Imaging Kits.

Invitrogen™ dye	Hoechst™ 33342	Alexa Fluor™ 488	Alexa Fluor™ 555	Alexa Fluor™ 594	Alexa Fluor™ 647
Excitation/emission (nm)	350/461	495/519	555/615	590/615	650/670
Standard filter set	DAPI	FITC	RFP	Texas Red	Cy®5

Click-iT EdU labeling is compatible with most fixation protocols.

This protocol is continued on page 19

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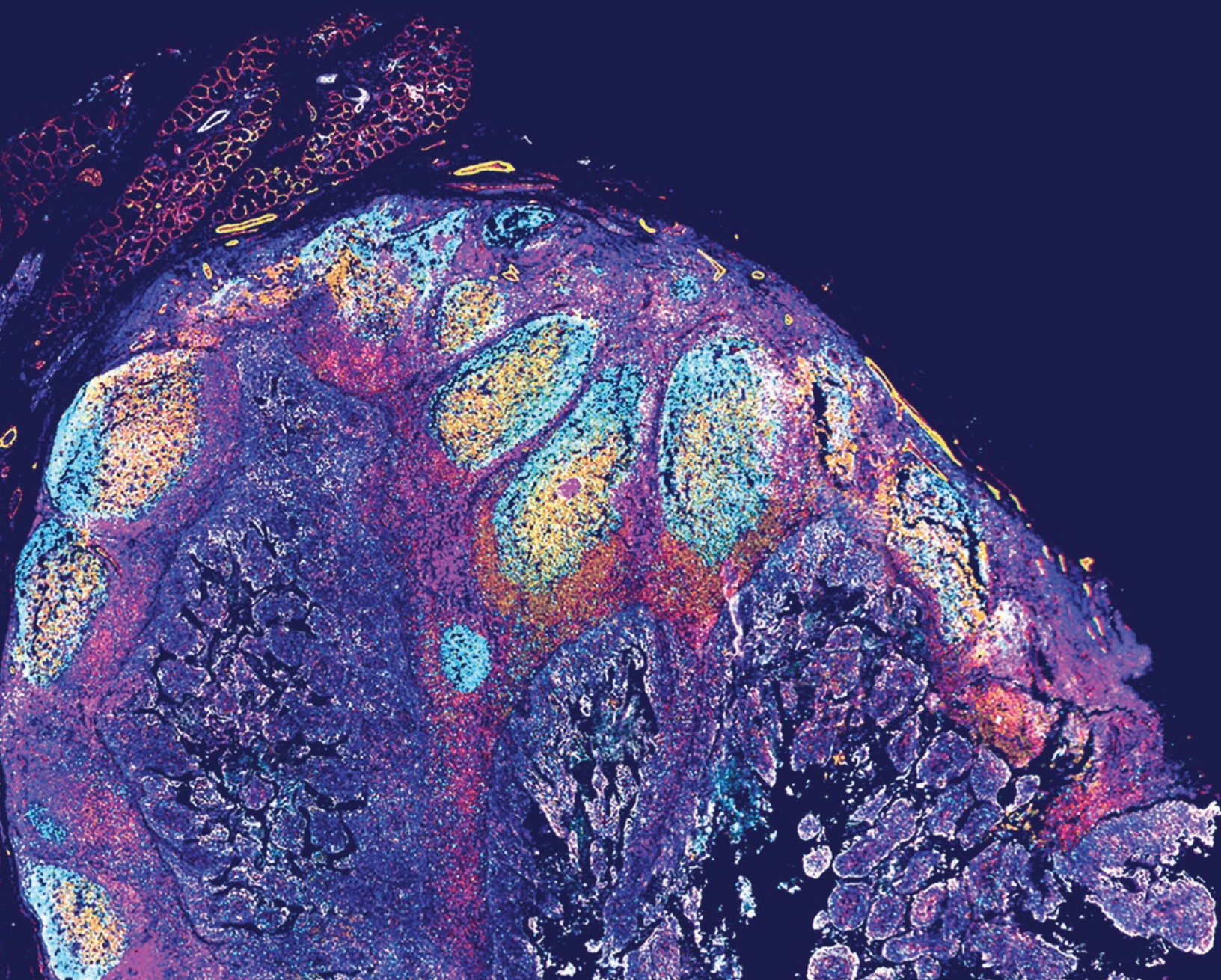
Notes

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.

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

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.

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.

Spectral information and storage

Invitrogen™ dye	Calcein AM	Ethidium homodimer-1
Excitation/emission	494/517 nm	528/617 nm
Standard filter set	FITC or GFP	RFP
EVOS Light Cube	GFP	RFP
Storage conditions	–20°C	–20°C

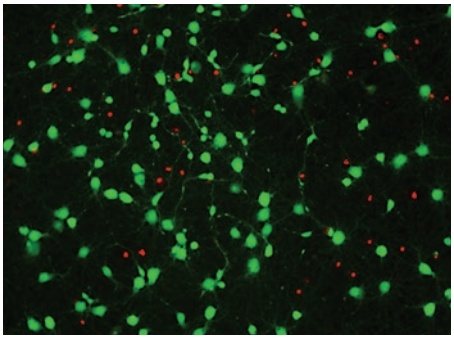


Figure 4. Cell viability staining performed with calcein AM and ethidium homodimer-1 from the LIVE/DEAD Viability/Cytotoxicity Kit.

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		
Invitrogen™ dye	**NucBlue Live ReadyProbes Reagent**	**Propidium iodide**
Excitation/emission	360/460 nm	528/617 nm
Standard filter set	DAPI	TRITC or RFP
EVOS Light Cube	DAPI	TRITC or RFP
Storage conditions	Room temperature	Room temperature

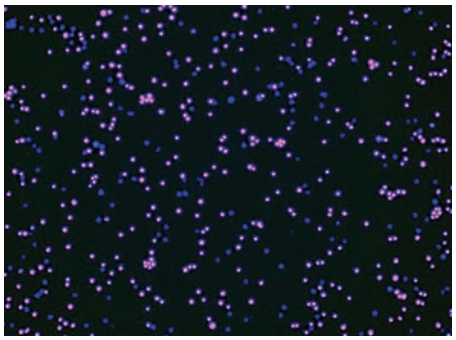


Figure 5. Jurkat cell viability determination using the ReadyProbes Cell Viability Imaging Kit.

Cell structure

MitoTracker mitochondrion-selective probes

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.

Critical note

Allow vials to warm to room temperature before opening.

General guidelines

Use working concentrations of 25–500 nM. For staining cells that are to be fixed and permeabilized (see "Fixation and permeabilization after staining"), use a working concentration of 100–500 nM. To reduce potential artifacts and mitochondrial toxicity from overloading, keep the concentration of dye as low as possible. For the MitoTracker Green FM probes, use a slightly lower concentration (20–200 nM). At higher concentrations, these probes tend to stain other cellular structures.

This protocol is continued on page 24

MitoTracker mitochondrion-selective probes, cont.

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.

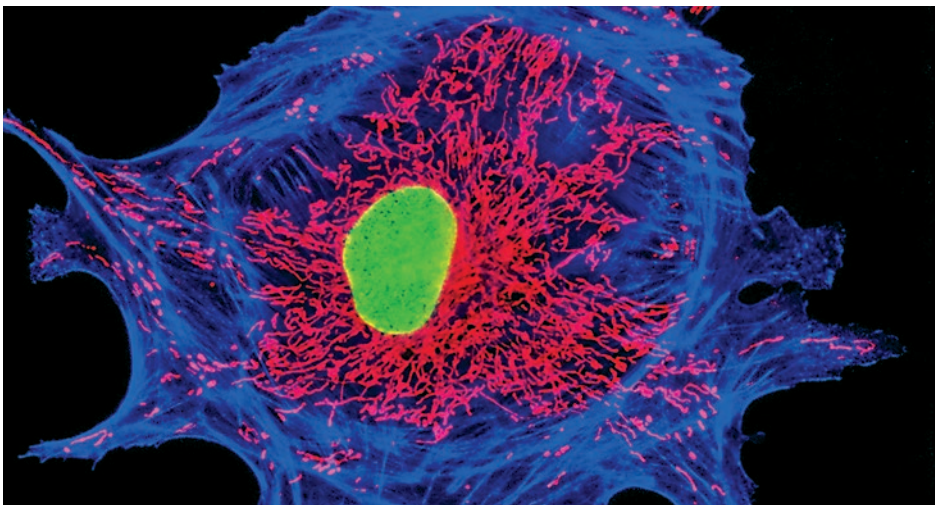


Figure 6. Multicolor staining of bovine pulmonary artery endothelial (BPAE) cells. Cells were stained with Alexa Fluor 488 NPCP (Ms), MitoTracker Red CMXRos, and Alexa Fluor 350 phalloidin.

This protocol is continued on page 25

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)

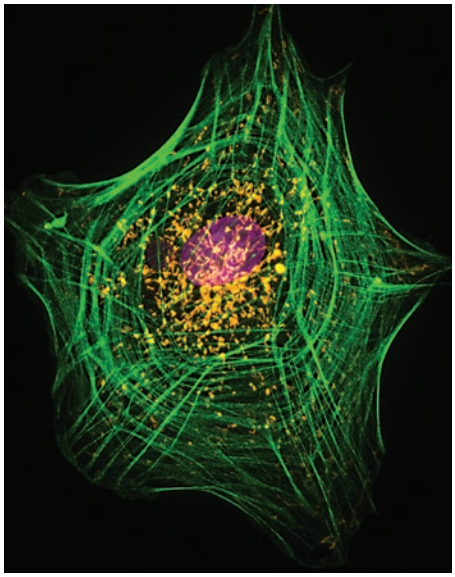


Figure 7. Fixed, permeabilized, and labeled muntjac skin fibroblast. Mitochondria were labeled with mouse anti–OxPhos Complex V inhibitor protein antibody and visualized using orange-fluorescent Invitrogen™ Alexa Fluor™ 555 goat anti–mouse IgG antibody (Cat. No. A21422). F-actin was labeled with green-fluorescent Invitrogen™ Alexa Fluor™ 488 phalloidin (Cat. No. A12379), and the nucleus was stained with Invitrogen™ TO-PRO™-3 iodide (Cat. No. T3605, pseudocolored magenta).

This protocol is continued on page 27

Cat. No.	Amount	Conjugate	Excitation[1]	Emission[1]	Approximate MW
A22281	300 U	Alexa Fluor™ 350 Phalloidin	346	442	1,100
A30104	300 U	Alexa Fluor™ Plus 405 Phalloidin	405	450	1,010
A12379	300 U	Alexa Fluor™ 488 Phalloidin	495	518	1,320
F432	300 U	Fluorescein Phalloidin	496	516	1,175
O7466	300 U	Oregon Green™ 488 Phalloidin	496	520	1,180
O7465	300 U	Oregon Green™ 514 Phalloidin	511	528	1,281
A22282	300 U	Alexa Fluor™ 532 Phalloidin	531	554	1,350
R415	300 U	Rhodamine Phalloidin	540	565	1,250
A22283	300 U	Alexa Fluor™ 546 Phalloidin	556	570	1,800
A34055	300 U	Alexa Fluor™ 555 Phalloidin	555	565	1,910
A30106	300 U	Alexa Fluor™ Plus 555 Phalloidin	555	565	1,488
B3475	300 U	BODIPY™ 558/568 Phalloidin	558	569	1,115
A12380	300 U	Alexa Fluor™ 568 Phalloidin	578	600	1,590
A12381	300 U	Alexa Fluor™ 594 Phalloidin	581	609	1,620
T7471	300 U	Texas Red™ X Phalloidin	591	608	1,490
A22284	300 U	Alexa Fluor™ 633 Phalloidin	632	647	1,900
A34054	300 U	Alexa Fluor™ 635 Phalloidin	633	647	1,850
A22287	300 U	Alexa Fluor™ 647 Phalloidin	650	668	1,950
A30107	300 U	Alexa Fluor™ Plus 647 Phalloidin	650	668	1,514
A22284	300 U	Alexa Fluor™ 660 Phalloidin	663	690	1,750
A22286	300 U	Alexa Fluor™ 680 Phalloidin	679	702	1,850
A30105	300 U	Alexa Fluor™ Plus 750 Phalloidin	758	784	2,122
B7474	50 U	Biotin-XX Phalloidin	NA	NA	1,300
P3457	1 mg	Phalloidin (unlabeled)	NA	NA	790

[1] Approximate fluorescence excitation and emission maxima, in nm. Go to thermofisher.com for complete spectra information.

Protocol

Prepare fluorescent phalloidins

Prepare a stock solution of fluorescent phalloidins using one of the following methods.

- **(Recommended) DMSO stock solution:** Dissolve the vial contents in 150 µL of anhydrous DMSO to yield a 400X stock solution at a concentration of approximately 66 µM, which provides for 2,000 assays/mL.

The DMSO stock solution is stable for at least one year, when stored at ≤–20°C. This solution has been tested for stability for 5 freeze/thaw cycles. Aliquot the stock solution if additional freeze/thaw cycles are needed.

Note

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.

This protocol is continued on page 28

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.

Note

One unit/assay of Biotin-XX Phalloidin is equivalent to 10 µL of the methanolic stock solution.

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).

This protocol is continued on page 29

8. Prepare the staining solution as indicated in table below.

For…	Do this…
Fluorescent phalloidin staining solution	1. Dilute the stock solution. • **For a DMSO stock solution**—dilute 0.5 µL of the 400X 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.

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.

9. 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.

10. *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.

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

12. 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.

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 30

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.

Note

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.

CellLight BacMam 2.0 reagents

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.

CellLight fluorescent protein selection guide					
Excitation wavelength range (nm)	GFP 488–510		RFP 555–584		CFP 435–485
Actin	C10582	C10506	C10583	C10502	—
Cytoplasm	B10383	—	—	—	—
Endoplasmic reticulum	C10590	—	C10591	—	—
Early endosomes	C10586	—	C10587	—	—
Late endosomes	C10588	—	C10589	—	—
Golgi	C10592	—	C10593	—	—
Histones (histone 2B)	C10594	—	—	—	—
Lysosomes	C10596	C10507	C10597	C10504	—
Mitochondria	C10600	C10508	C10601	C10505	—
Nucleus	C10602	—	C10603	—	—
Peroxisomes	C10604	—	—	—	—
Plasma membrane	C10607	—	C10608	—	C10606
Talin	C10611	—	C10612	—	—
Tubulin	C10613	C10509	C10614	C10503	—
BacMam 2.0 transduction control	B10383	—	—	—	—
Unconjugated					
Null virus (control)			C10615		

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 32

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.

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

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 x 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.

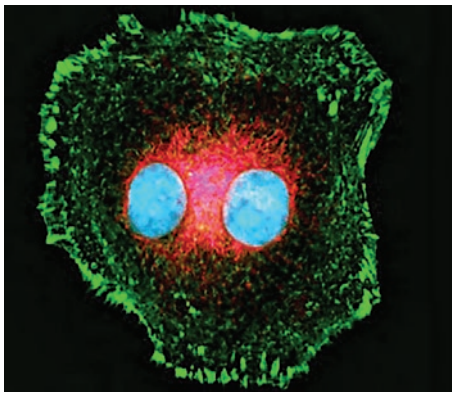


Figure 8. Labeling of HeLa cells with CellLight reagents. HeLa cells were transduced with Invitrogen™ CellLight™ Mitochondria-RFP reagent (Cat. No. C10505) and Invitrogen™ CellLight™ Talin-GFP reagent (Cat. No. C10611) for 24 hours, then labeled with Invitrogen™ NucBlue™ Live ReadyProbes™ Reagent (Cat. 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.

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.

Tubulin Tracker reagents

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 34

Procedural guidelines

- Recommendations for experimental protocols should be used as a starting point, and optimal labeling conditions for each cell type should be determined empirically.
- The Pluronic F-127 solution enhances the loading of Tubulin Tracker™ Green reagent in live cells, but does not seems to enhance the loading of Tubulin Tracker™ Deep Red reagent. For best results, vortex well solutions that contain Pluronic F-127 before use.
- Probenecid prevents efflux of fluorescent reagents in many live cell types. To minimize off-target effects on other cellular functions, we recommend using probenecid at 1X concentration while loading and imaging with Tubulin Tracker Green and Tubulin Tracker Deep Red reagents. Do not incubate in probenecid for more than a few hours.

Note

Before you begin:

- Make a 4,000X stock solution of Tubulin Tracker Green reagent by dissolving the contents of the vial in 15 µL (Cat. No. T34078) or 75 µL (Cat. No. T34075) of anhydrous DMSO. This stock solution is stable for at least 3 months when stored at ≤–20°C.
- For Tubulin Tracker Green reagent only: add an equal volume of Pluronic F-127 (20% solution in DMSO) to the Tubulin Tracker™ Green stock solution. This 2,000X intermediate stock solution is stable for 14 days when stored at ≤–20°C.
- Make a 1,000X stock solution of Tubulin Tracker Deep Red reagent by dissolving the contents of the vial in 60 µL of anhydrous DMSO. This stock solution is stable for at least 3 months when stored at ≤–20°C.
- Dissolve probenecid in 1 mL of live cell–compatible buffer to make a 100X stock solution. This solution is stable for 6 months when stored at ≤–20°C.

Tubulin Tracker reagents, cont.

4. Incubate for 30 minutes at 37°C and 5% CO_2. *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% CO_2. *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.

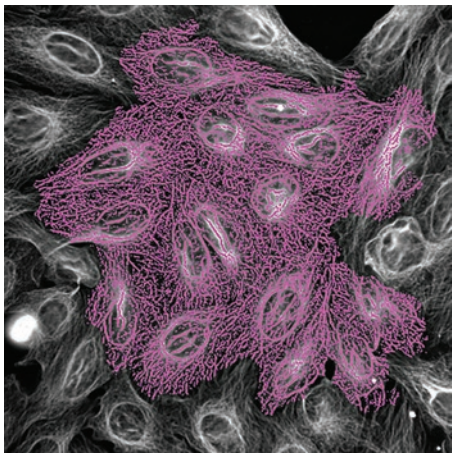


Figure 9. Cell treated with Tublin Tracker Deep Red reagent.

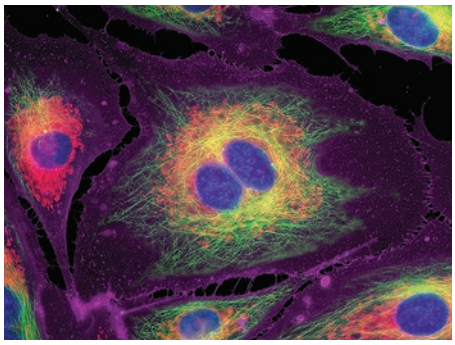


Figure 10. Live-cell labeling using Invitrogen™ CellMask™ Deep Red plasma membrane stain. Live bovine pulmonary arterial endothelial cells were labeled with CellMask Deep Red plasma membrane stain (Cat. No. C10046), tubulin-selective Tubulin Tracker Green dye (Oregon Green 488 Taxol, bis-acetate (Cat. 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 36

LysoTracker and LysoSensor probes, cont.

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.

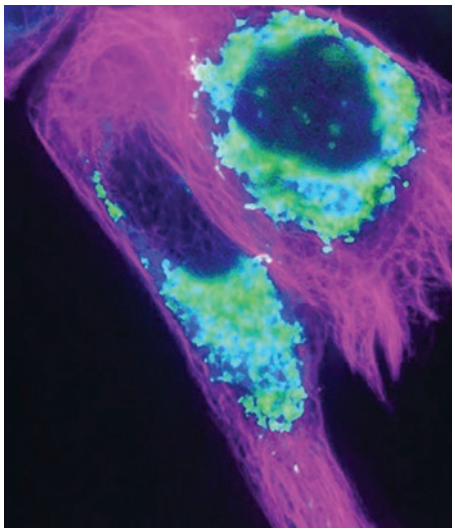


Figure 11. Labeling of U2OS cells with LysoTracker, MitoTracker, and Tubulin Tracker reagents. U2OS cells labeled using Invitrogen™ LysoTracker™ Blue DND22 dye (Cat. No. L7525), Invitrogen™ MitoTracker™ Green FM dye (Cat. No. M7514), and Invitrogen™ Tubulin Tracker™ Deep Red dye (Cat. No. T34076) show 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 38

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% CO_2.

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 39

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.

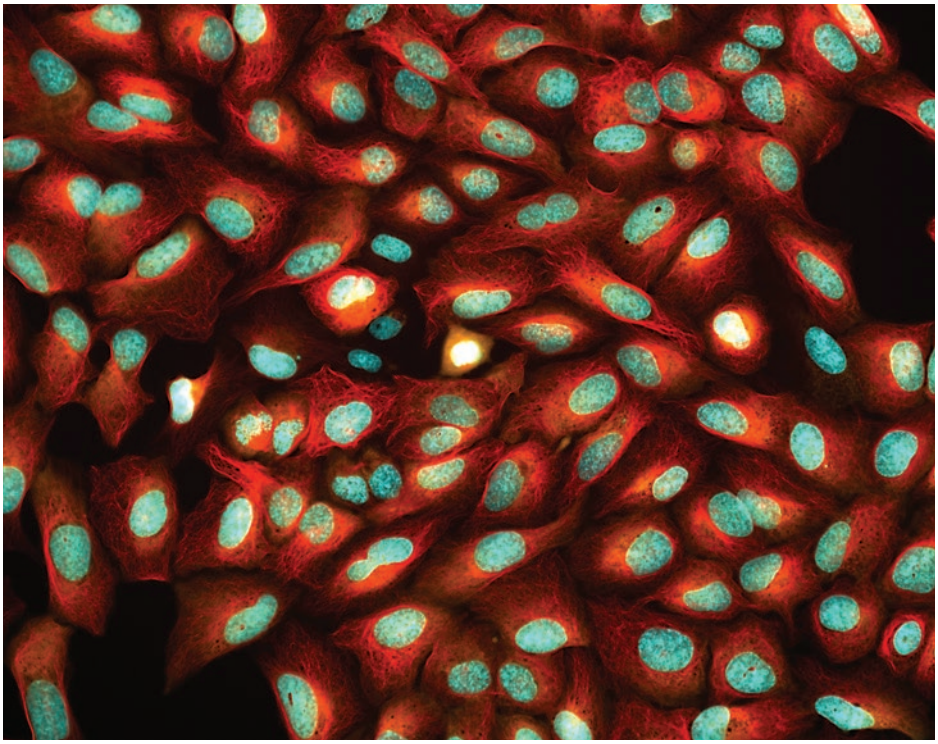


Figure 12. Labeling of HeLa cells with ER-Tracker dye. HeLa cells labeled using NucBlue™ Live ReadyProbes™ Reagent (Cat. No. R37605), ER-Tracker Green dye (Cat. No. E34251), and Tubulin Tracker Deep Red dye (Cat. No. T34076) show superb multiplexing capability and uniformity of staining compared with 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 41

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 (diH_2O) 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.

This protocol is continued on page 43

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

- Hoechst dye is a known mutagen and should be handled with care.
- Dissolving Hoechst dye in PBS is not recommended, but phosphate-containing buffers may be used with dilute solutions of the dye.
- Unbound Hoechst dye has a maximum emission in the 510–540 nm range; a green haze may be observed if too much dye is applied.
- The fluorescence signal from Hoechst dye is quenched by BrdU.

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.

7. Remove the staining solution.

8. Wash the cells 3 times in PBS.

9. Image the cells.

Spectral information and storage	
Invitrogen™ dye	Hoechst 33342
Excitation/emission	350/461 nm
Standard filter set	DAPI
EVOS Light Cube	DAPI
Storage conditions	2–6°C or ≤–20°C

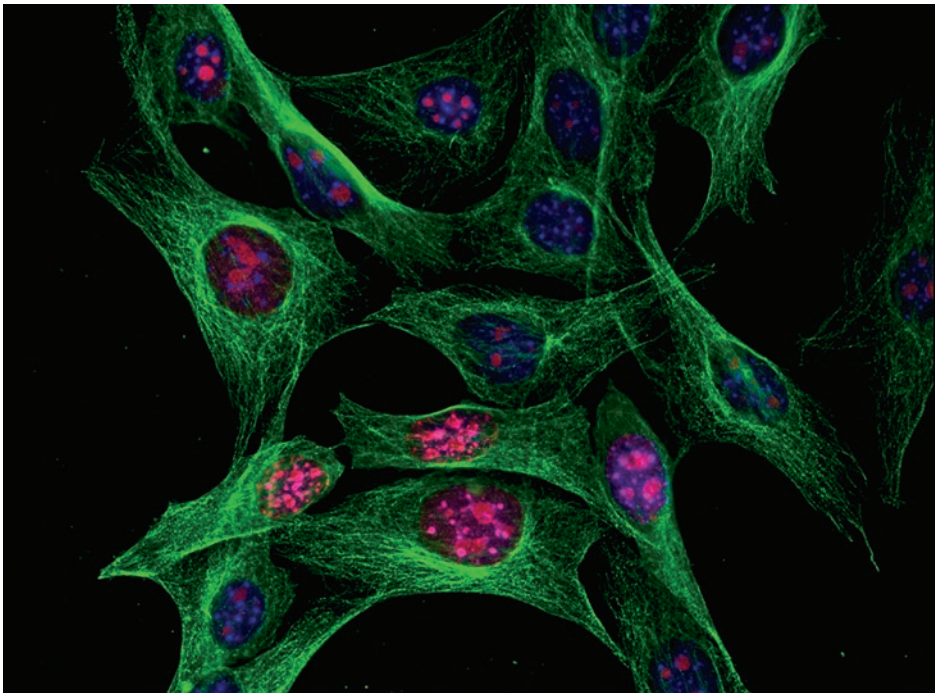


Figure 13. Multiplex imaging with Click-iT RNA assays. NIH3T3 cells were incubated with 1 mM EU for 1 hr, then formaldehyde fixed and permeabilized with Triton X-100. EU incorporated into newly synthesized RNA (red) in some cells was detected using the Click-iT RNA Alexa Fluor 594 Imaging Kit (Cat. No. C10330). Tubulin (green) was detected with mouse anti-tubulin IgG (Cat. No. A11126) 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™ *Bac*Light™ 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		
Application	**SYTO 9 dye concentration**	**Staining conditions**
Bacterial cells	50 nM–20 µM	Vortex to mix, then incubate for 1–30 minutes
Eukaryotic cells	10 nM–5 µM	Incubate for 10–120 minutes

Spectral information and storage for SYTO 9 stain	
Excitation/emission	485/498 nm
Standard filter set	FITC
EVOS Light Cube	GFP
Storage conditions	<–20°C

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.

Critical notes

This protocol can be used for:

- Nucleic acid (nuclear) staining in fluorescence microscopy

This protocol should not be used for:

- Flow cytometry

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.

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	
Excitation/emission	622/645 nm
Standard filter set	Cy®3.5
EVOS Light Cube	Texas Red
Storage conditions	≤–20°C

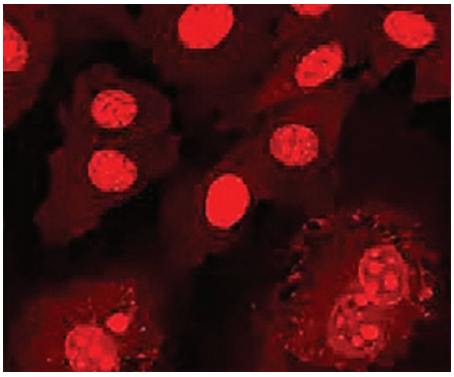


Figure 14. Nuclear staining of BPAECs. BPAECs were cultured, stained with SYTO 59 dye (5 µM for 5 min), and then imaged.

Notes

Live-cell assays

Tetramethylrhodamine (TMRM)

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.

This protocol is continued on page 48

Tetramethylrhodamine (TMRM), cont.

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.

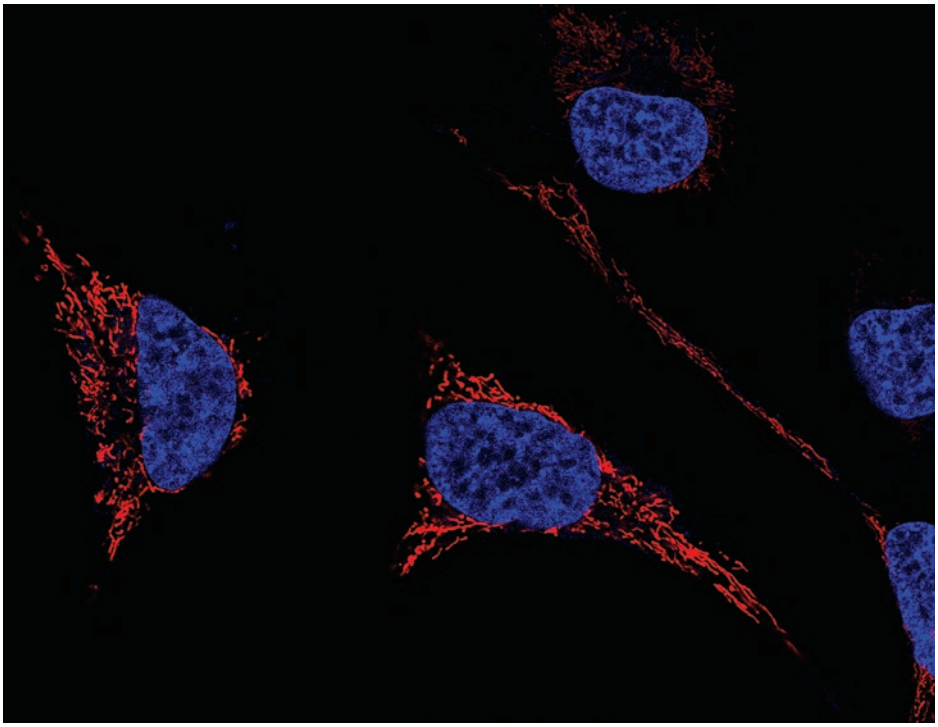


Figure 15. U2OS cells stained with 100 nM Image-iT TMRM (Cat. No I34361, red) and Hoechst 33342 stain (Cat. No. H3570, blue) for 30 minutes.

CellEvent Caspase-3/7 Green Detection Reagent

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.

This protocol is continued on page 50

CellEvent Caspase-3/7 Green Detection Reagent, cont.

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.

This protocol is continued on page 51

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.

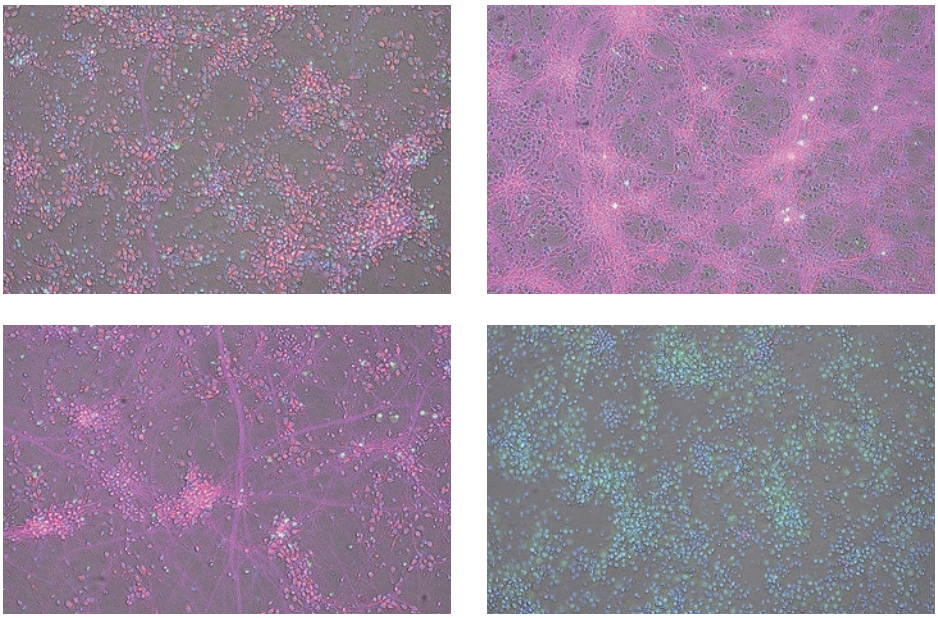


Figure 16. Mouse embryonic hippocampal neurons cultured 14 DIV (days *in vitro*) grown in Neurobasal Plus and Gibco™ B-27™ Plus supplement with 25 µM glutamate and 1X Gibco™ GlutaMAX™ Supplement . Cells were treated in half-log intervals with cadmium chloride for 18 hours and subsequently stained using 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.

CellROX Oxidative Stress Reagents

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.

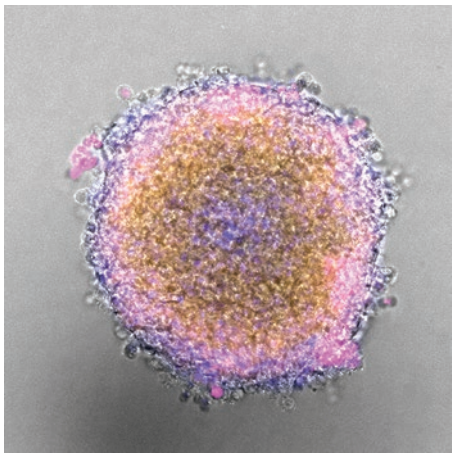


Figure 17. A549 spheroid grown in Nunclon Sphera (Cat. No. 174925) plates, labeled with NucBlue Live Cell Stain (Cat. No. R37605), MitoTracker Orange CMTMRos (Cat. No. M7510), and CellROX Deep Red (Cat. No. C10422). Image was acquired with an EVOS imaging system.

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.

CellTracker Fluorescent Probes

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)

Critical note

Avoid amine- and thiol-containing buffers.

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.

Note

For the molecular weight of CellTracker™ reagents, see the table under “EVOS microscopy” on page 49.

This protocol is continued on page 54

CellTracker Fluorescent Probes, cont.

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.

This protocol is continued on page 55

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.

Cat. No.	CellTracker™ probe	MW	Ex (nm)[1]	Em (nm)[1]
C2110	CellTracker™ Blue CMAC Dye (7-amino-4-chloromethylcoumarin)	209.6	353	466
C12881	CellTracker™ Blue CMF_2HC Dye (4-chloromethyl-6,8-difluoro-7-hydroxycoumarin)	246.6	371	464
C2111	CellTracker™ Blue CMHC Dye (4-chloromethyl-7-hydroxycoumarin)	210.6	372	470
C10094	CellTracker™ Violet BMQC Dye (2,3,6,7-tetrahydro-9-bromomethyl-1*H*,5*H*-quinolizino(9,1-*gh*) coumarin)	334.2	415	516
C2925, C7025	CellTracker™ Green CMFDA Dye (5-chloromethylfluorescein diacetate)	464.9	492[2]	517[2]
C2102	CellTracker™ Green BODIPY™ Dye (8-chloromethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-S-indacene)	296.6	522	529
C2927	CellTracker™ Orange CMTMR Dye (5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine	554.0	541	565
C34551	CellTracker™ Orange CMRA Dye	550.4	548	576
C34552	CellTracker™ Red CMTPX Dye	686.3	577	602
C34565	CellTracker™ Deep Red Dye	698.3	630	660

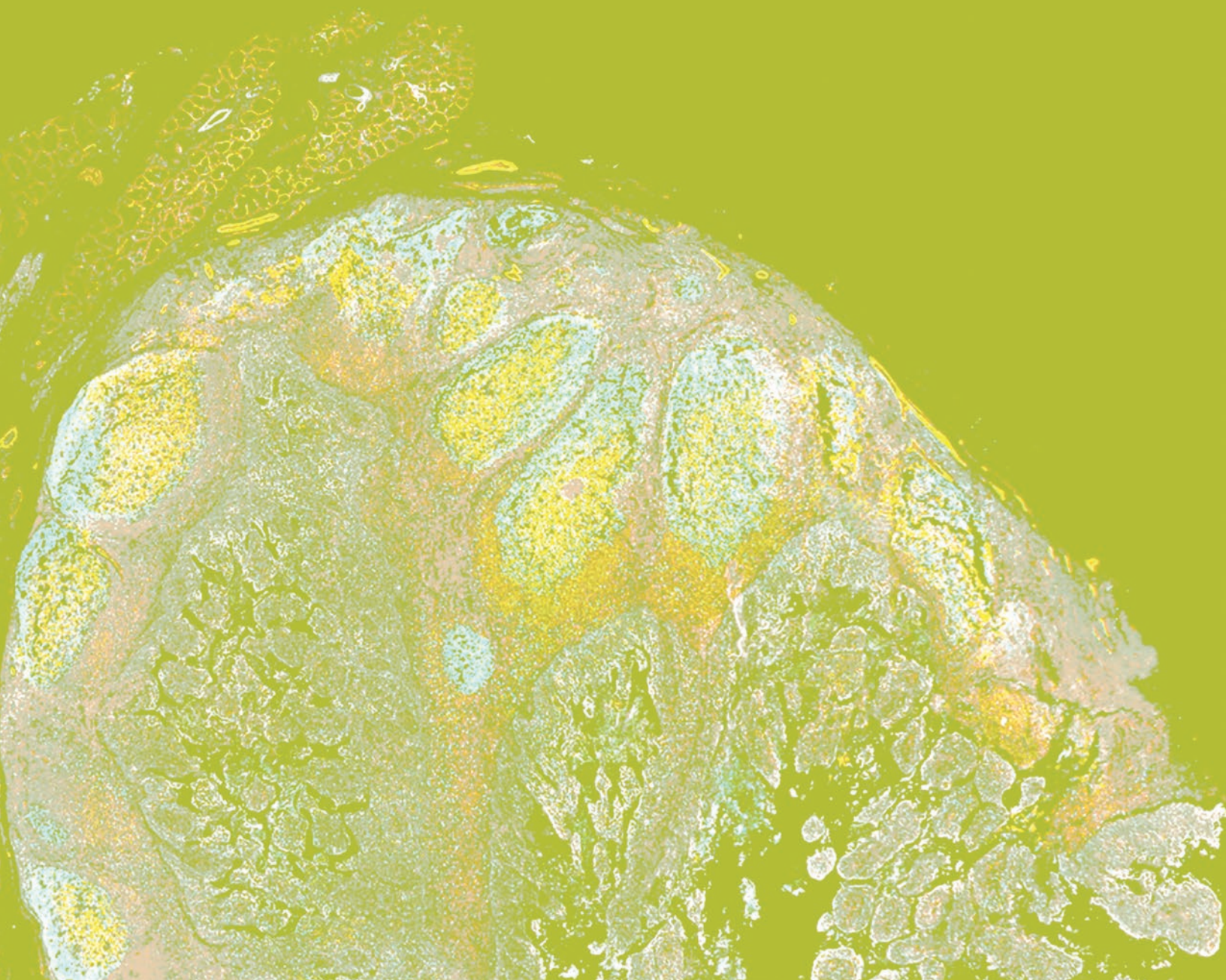
[1] Absorption and fluorescence emission maxima, determined in aqueous buffer or methanol; values may vary somewhat in cellular environments.

[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

Notes

Immunolabeling



ICC formaldehyde fixed, permeabilized cells

Direct with labeled primary antibodies

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)

Note

For a complete list of primary antibodies, please visit thermofisher.com/us/en/home/life-science/antibodies/primary-antibodies.html

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 58

ICC formaldehyde fixed, permeabilized cells, cont.

Direct with labeled primary antibodies

4. Wash well in PBS (typically 3 x 10 minutes).

5. *Optional:* Block for nonspecific dye binding using the Image-iT FX Signal Enhancer (Cat. No. I36933).

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)).

ICC formaldehyde fixed, permeabilized cells

Indirect with secondaries

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 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)

Note

For a complete list of secondary antibodies, visit thermofisher.com/us/en/home/life-science/antibodies/secondary-antibodies.html

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 60

ICC formaldehyde fixed, permeabilized cells, cont.

Indirect with secondaries

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.
5. *Optional:* Block for nonspecific dye binding using the Image-iT FX Signal Enhancer (Cat. No. I36933).
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)).

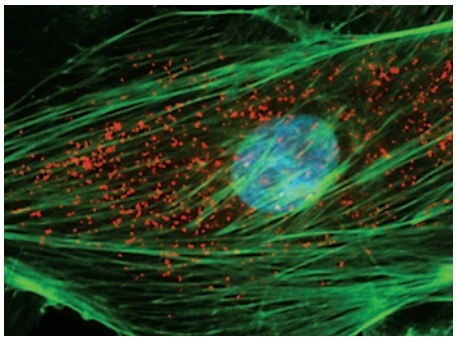


Figure 18. Three-color imaging of actin (green), nuclei (blue), and peroxisomes (red) in fixed BPAE cells. BPAE cells were fixed, permeabilized, and blocked using the Image-iT Fixation/Permeabilization Kit (Cat. No. R37602). Peroxisomes were labeled using rabbit anti-PMP70 antibody (Cat. No. 718300) followed by detection with Alexa Fluor 594 goat anti–rabbit IgG secondary antibody (ReadyProbes reagent (Cat. No. R37117)). Actin was stained using ActinGreen 488 ReadyProbes reagent (Cat. No. R37110), and nuclei were counterstained with NucBlue Fixed Cell ReadyProbes reagent (Cat. No. R37606).

ICC biotin/streptavidin amplification

General labeling protocol for secondary detection for cultured cells—streptavidin

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 62

ICC biotin/streptavidin amplification, cont.

General labeling protocol for secondary detection for cultured cells—streptavidin

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).

6. *Optional:* Block for nonspecific dye binding using the Image-iT FX Signal Enhancer (Cat. No. I36933).

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).

IHC indirect with secondaries on paraffin tissue

General labeling protocol for secondary detection for fixed paraffin 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 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.

Protocol guidelines

- Very thick sections may require longer wash and incubation times.
- Tissue sections, particularly in paraffin, may have autofluorescence issues that can lower the signal-to-background ratio necessary to detect the antigen.
- 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. Some primary antibodies may require antigen retrieval. All steps are usually carried out at room temperature, unless otherwise noted.

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 64

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).

4. *Optional:* Reduce autofluorescence using the ReadyProbes Tissue Autofluorescence Quenching Kit (Cat. No. R37630).

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).

8. *Optional:* Block for nonspecific dye binding using the Image-iT FX Signal Enhancer (Cat. No. I36933).

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).

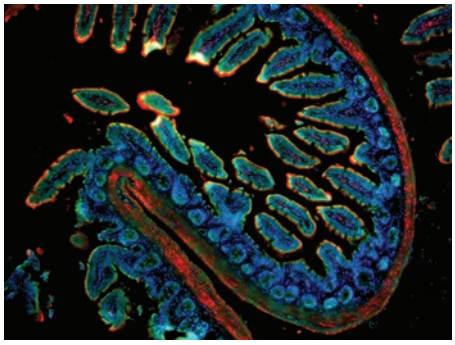


Figure 19. Mouse intestinal ileum 8 µm FFPE section labeled with NucBlue Live Cell Stain and goat anti-rabbit Alexa Fluor Plus 555 secondary to detect a rabbit pan-actin primary antibody. Imaged on an EVOS M7000 Imaging System using an Olympus 10x Super apochromat objective.

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).
3. *Optional:* Block for nonspecific dye binding using the Image-iT FX Signal Enhancer (Cat. No. I36933).
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 66

Protocol guidelines

- Cryosections may be labeled free-floating or mounted onto slides and should be rehydrated in PBS prior to proceeding with step 1.
- Very thick sections may require longer wash and incubation times. Tissue sections, particularly paraffin, may have autofluorescence issues that can lower the signal-to-background ratio necessary to detect the antigen.
- 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. Some primary antibodies may require antigen retrieval.
- All steps are usually carried out at room temperature, unless otherwise noted.

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 4ºC.

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. 32-2600) detected with Alexa Fluor™ Plus 488 Goat Anti-Mouse Secondary 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).
3. *Optional:* Block for nonspecific dye binding using the Image-iT FX Signal Enhancer (Cat. No. I36933).

Protocol guidelines

- Cryosections may be labeled free-floating or mounted onto slides and should be rehydrated in PBS prior to proceeding with step 1. Paraffin sections will likely be mounted onto slides and will need to be deparaffinized (such as in xylene or commercial citrus solvent) and run through a graded ethanol series down to buffer before proceeding with step 1.
- Very thick sections may require longer wash and incubation times. Tissue sections, particularly paraffin, may have autofluorescence issues that can lower the signal-to-background ratio necessary to detect the antigen.
- 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. Some primary antibodies may require antigen retrieval.
- All steps are usually at room temperature, unless otherwise noted.

This protocol is continued on page 68

IHC biotin/streptavidin amplification on fixed tissue, cont.

General labeling protocol for streptavidin detection for fixed tissue sections

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.

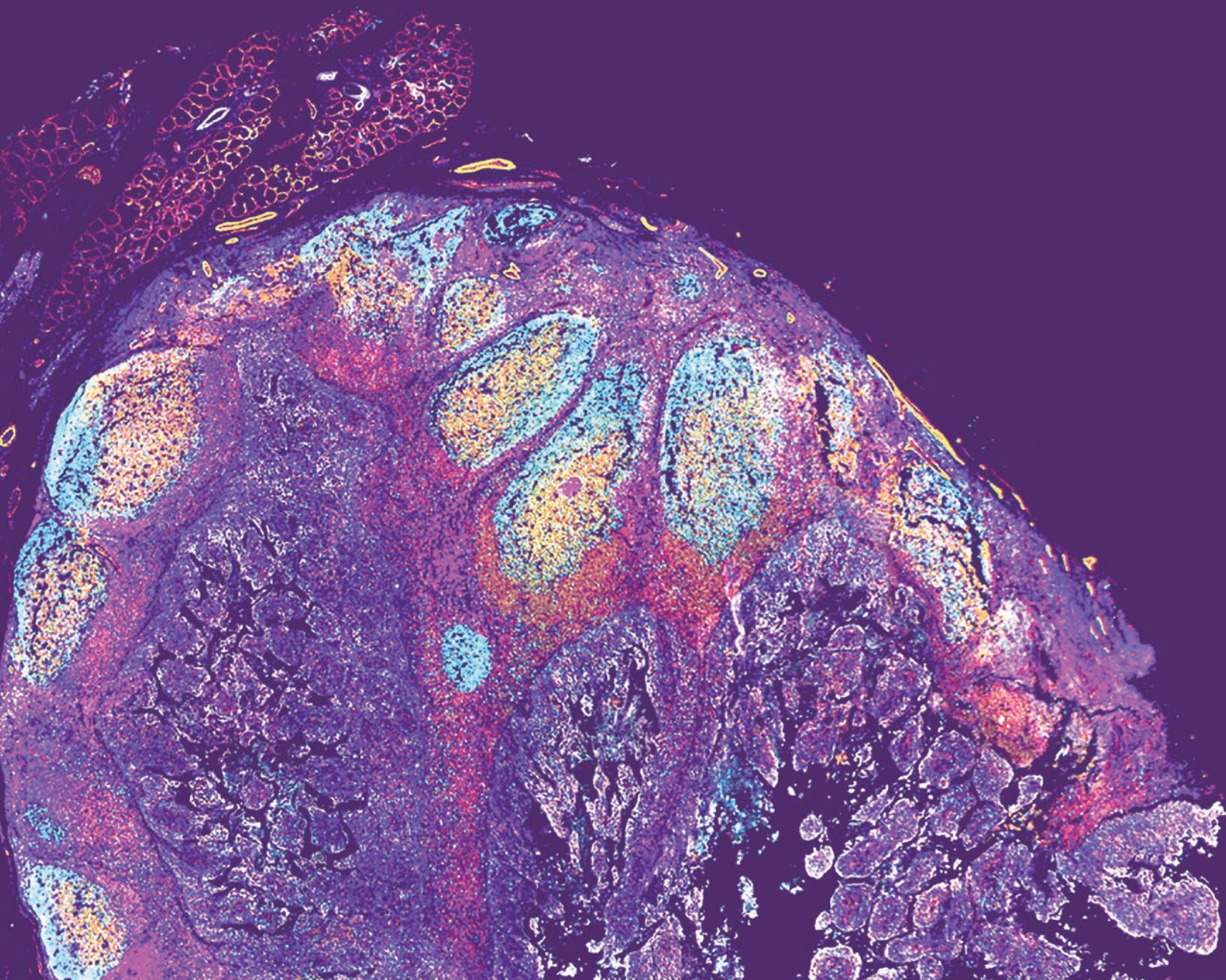
9. Label with conjugated streptavidin 1–2 hours (2–5 µg/mL in 3–6% bovine serum albumin/PBT).

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).

Spatial biology and organoid labeling



Staining 3D cell cultures for imaging with antibodies and cellular reagents and dyes

Introduction

3D cell cultures and the study of organoids and spheroids require new approaches to be visualized with fluorescence microscopy. Unlike 2D cell cultures which can easily be visualized by light transmitted through the sample, 3D cultures may be too thick for light to effectively pass through. Clearing reagents are recommended when imaging fixed 3D cell cultures of various thickness, including organoids and spheroids. Clearing 3D culture samples enable optimal clarity for sharp, bright fluorescent images.

Materials

- 4% paraformaldehyde (e.g., Image-iT Fixative Solution, Cat. No. R37814)
- Nunclon Sphera 96-well plates (Cat. No. 174925)
- Nunclon 96-well optical-bottom plates (Cat. No. 164588)
- Microscope coverslips
- Phosphate buffer saline (e.g., Gibco PBS (10X), pH 7.4, Cat. No. 70011044)
- Primary antibody (e.g., Invitrogen Antibodies)
- Non-curing glass slide mountant (e.g., SlowFade Glass Soft-set Antifade Mountant, Cat. No. S36917)
- Clearing reagents (CytoVista 3D Cell Culture Clearing/Staining Kit, Cat. No. V11325). Kit includes:
  - Tissue penetration buffer (CytoVista Antibody Penetration Buffer, Cat. No. V11310)
  - Antibody dilution buffer (CytoVista Antibody Dilution Buffer, Cat. No. V11305)
  - Blocking buffer (CytoVista Blocking Buffer, Cat. No. V11306)
  - Wash buffer (CytoVista Wash Buffer, Cat. No. V11312)
- DAPI counterstain (e.g., NucBlue Fixed Cell ReadyProbes Reagent (DAPI), Cat. No. R37606)

We strongly recommend you optimize the primary antibody before using the kit. Positive and negative samples should be stained with a serial dilution including 1:10, 1:100, 1:500, 1:1000. Thicker spheroids may need more antibody.

Protocol tips

- We recommend that you cut your pipet tips to widen the openings. This will help to prevent shearing of the spheroids.
- With suspension spheroids/samples it is nearly impossible to remove all supernatant without also removing or damaging the spheroid. We recommend pipeting out the supernatent.
- This protocol is recommended for spheroids cultured up to 500 microns in thickness.
- Read instructions with fluorescent dyes and reagents—not all of them can be fixed and many need to be used pre-fixation.

Critical note

We strongly recommend you optimize the primary antibody before using the kit. Positive and negative samples should be stained with a serial dilution including 1:10, 1:100, 1:500, 1:1000. Thicker spheroids may need more antibody.

This protocol is continued on page 70

Staining 3D cell cultures for imaging with antibodies and cellular reagents and dyes, cont.

Protocol

Spheroid fixation and permeabilization

1. Centrifuge samples 500 x g for 5 min. Gently remove cell culture media.
2. Wash cells once with cold PBS. Centrifuge at 500 x g for 5 min. Remove supernatant.
3. Add 4% paraformaldehyde at a volume that is approximately 2X the volume of the tissue. Ensure that the spheroids are submerged in the fixative. Fix for 1 h at room temperature with gentle agitation.
4. Wash cells once with cold PBS. Centrifuge at 500 x g for 5 min. Remove supernatant.
5. Permeabilize the spheres for antibody access by incubating spheres for 15 min at room temperature with gentle agitation. Suitable permeabilization reagents include CytoVista Antibody Penetration buffer.
6. Centrifuge at 500 x g for 5 min. Remove supernatant.
7. Wash samples twice with 1% fetal bovine serum in PBS.
8. Centrifuge at 500 x g for 5 min. Remove supernatant.
9. Block the samples with blocking buffer such as the CytoVista Blocking Buffer for 2 h at 37°C with gentle agitation.

Critical note

If you have delicate spheroids, we recommend that you skip steps 1 and 2 and add equal volume of 8% PFA directly to the samples in plate.

Optional

Add fluorescent cell reagents for viability before fixation. We recommend using LIVE/DEAD fixable dye for viability staining.

Note

Spheroids can be fixed within the tissue culture plate or transferred into a microcentrifuge tube for fixation.

Labeling

1. Add primary antibodies prepared in CytoVista Antibody Dilution Buffer.
2. Incubate samples overnight at room temperature with gentle agitation.

Protocol tip

Titrate the primary antibody concentration for optimal results. Too much or too little primary antibody can result in sub-optimal or no labeling. Antibody concentration can vary depending on the thickness of the tissue.

This protocol is continued on page 71

3. Wash samples 3X with CytoVista Wash Buffer to remove any unbound excess antibody solution. Centrifuge at 500 x g for 5 min. Remove supernatant.

4. Add secondary antibodies prepared in CytoVista Antibody Dilution Buffer.

5. Incubate samples overnight at room temperature with gentle agitation.

6. Add 1 drop of counterstain, NucBlue per mL of sample or 300 nM DAPI.

7. Incubate sample for 30 min at room temperature with gentle agitation.

8. Centrifuge at 500 x g for 5 min. Remove supernatant.

9. Wash sample 3X with CytoVista Wash Buffer. Centrifuge at 500 x g for 5 min. Remove supernatant.

Note

We recommend that you titrate the antibody to find the optimal concentration.

Note

300 nm DAPI preparation: Add 2 mL of dimethylformamide (DMF) to the entire contents of the DAPI vial to make a 14.3 mM (5 mg/mL) DAPI stock solution. Add 2.1 µL of the 14.3 mM DAPI stock solution to 100 µL PBS to make a 300 µM DAPI intermediate dilution. Dilute the 300 µM DAPI intermediate dilution 1:1,000 in PBS as needed to make a 300 nM DAPI stain solution.

Mounting

1. SlowFade mountant can be pipetted to cells in glass bottom plates. Other options include pipetting spheres on a coverslip. Add 1-3 drops of SlowFade Glass mount on top of the section. Place coverslip over microscope slide and image.

Protocol tip

Allow the SlowFade Antifade Mountants to warm to room temperature. If the vials are refrigerated or frozen, thaw for 1 hour at room temperature. Avoid shaking the bottle to prevent air bubbles.

Imaging

We recommend using a microscope with Z stack capabilities such as the Invitrogen EVOS M5000, EVOS M7000, or CellInsight CX7 high-content platform. Celleste 6 Image Analysis Software has 2D/3D-deconvolution features that can be used for sharper images.

This protocol is continued on page 72

Staining 3D cell cultures for imaging with antibodies and cellular reagents and dyes, cont.

Cell reagents for spheroid imaging

Perform cell viability assay

1. Add 5 µL component A and 20 µL component B to 10 mL DPBS to create staining solution.
2. Add 100–200 µL of the staining solution directly to cells in medium.
3. Incubate for 2 hours at room temperature with gentle agitation.

Protocol tip

We recommend the LIVE/DEAD Viability/Cytotoxicity Kit (Cat. No. L3224). **Perform this assay before fixation.**

Note

Protect cells from light during this incubation.

Measure reactive oxygen species (ROS) in live spheroid cells

1. Add CellROX Green dye to media for a final concentration of 5 µM (e.g., 1 µL per 500 µL of media).
2. Incubate for 2 hours at room temperature with gentle agitation. Protect cells light.
3. Wash with PBS. Centrifuge at 500 x g for 5 min. Gently remove supernatant. Repeat 3X.
4. Follow with fixation. Compatible with antibody staining and counter staining with NucBlue Live ReadyProbes Reagent (Cat. No. R37605).

Protocol tip

We recommend CellROX Green Reagent (Cat. No. C10444). **Perform this measurement before fixation.** Only CellROX Green and Deep Red can be fixed and permeabilized.

Protocol tip

Generate control with spheroids treated with 100 µM Menadione (e.g., Fisher Chemicals Cat. No. ICN10225925).

Cell proliferation assay with Click-iT Plus EdU Cell Prolife ration Kit for Imaging

Materials

- Click-iT Plus EdU Cell Proliferation Kit for Imaging, Alexa Fluor 488 (Cat. No. C10337)
- Click-iT Plus EdU Cell Proliferation Kit for Imaging, Alexa Fluor 555 (Cat. No. C10638)
- Click-iT Plus EdU Cell Proliferation Kit for Imaging, Alexa Fluor 594 (Cat. No. C10639)
- Click-iT Plus EdU Cell Proliferation Kit for Imaging, Alexa Fluor 647 (Cat. No. C10640)

This protocol is continued on page 73

Protocol

1. Optimize cell culture so spheroid size increases. The reagent will be incorporated during S-phase of the cell cycle.

2. Add 2 mL 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.

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. Add fresh media containing 20 µM EdU and incubate overnight at 37°C. Proliferating cells will incorporate EdU at this stage.

6. Proceed to fixation.

Note

Use when growing cells in culture.

Note

Store any remaining solution at 2–8°C.

References

1. Sherman H, Gitschier HJ, Rossi AE. A Novel Three-Dimensional Immune Oncology Model for High-Throughput Testing of Tumoricidal Activity. Front Immunol. 2018;9:857. Published 2018 Apr 23. PMID: 29740450
2. Mittler F, Obeïd P, Rulina AV, Haguet V, Gidrol X, Balakirev MY. High-Content Monitoring of Drug Effects in a 3D Spheroid Model. Front Oncol. 2017;7:293. Published 2017 Dec 11. PMID: 29322028

Multiplexed IHC staining with primary antibodies conjugated to fluorophores

Introduction

The following protocol provides a step-by-step guide for performing multiplexed staining of a tissue sample using a combination of primary antibodies linked to fluorophores that target specific markers. Additionally, this protocol includes instructions on how to create single color controls that are essential for spectral unmixing of a multiplexed sample containing fluorophores with emission spectra that highly overlap.

Materials

- Primary antibodies to your targets specific for IHC
- Xylene (Cat. No. 396931000) (FFPE only)
- 100% Ethanol (Cat. No. T038181000) (FFPE only)
- Phosphate buffered saline (10X) pH 7.4, RNase-free (Cat. No. AM9624)
- Tissue Tek Staining Dishes (3) (Cat. No. NC9507198)
- Tissue Tek Clearing Agent Dishes (2) (Cat. No. NC9012500)
- Tween-20 (Cat. No. 85113)
- Mounting medium
  - ProLong Glass Antifade Mountant (Cat. No. P36984)
  - SlowFade Glass Soft-set Antifade Mountant (Cat. No. S36917)

Optional Materials

- ReadyProbes Hydrophobic Barrier Pap Pen, (Cat. No. R3777)
- Antigen Retrieval Reagents:
  - Citrate Buffer (pH 6.0), Concentrate (Cat. No. 005000) or eBioscience IHC Antigen Retrieval Solution — Low pH (10X) (Cat. No. 00-4955-58) or eBioscience IHC Antigen Retrieval Solution— High pH (10X) (Cat. No. 00-4956-58)
- Hydrogen peroxide, 30% w/v (Example: Cat. No. H325-4)
- Sodium Hydroxide Solution (50% w/w/Certified) (Example: Cat. No. SS254500)
- NucBlue™ Fixed Cell ReadyProbes™ Reagent (Cat. No. R37606)

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Protocol

Prepare Controls

The following negative and single-color controls are required for spectral unmixing of the multiplex. Please prepare each of the controls including:

Single-color controls	Protocol
Single-color control: Make one for each primary antibody conjugate found in multiplex staining	Dilute the primary antibody conjugate in 3% BSA blocking buffer to the same concentration as was used in the multiplex sample.
	Add to sample and incubate 1 hour at RT in a humidified chamber in the dark.
	Wash 3x with 1X PBST (0.05% Tween-20)
	Proceed to mounting step. Do not use any counter stain.
Nuclear counter stain only control	Add nuclear counter stain such as NucBlue or DAPI at the same concentration as was used in the multiplex sample.
	Wash 3x with 1X PBST (0.05% Tween-20).
	Proceed to mounting step.
Unstained tissue sample	No counter stain or any fluorophore conjugates.
	Proceed to mounting step.

We strongly recommend you optimize the primary antibody before doing the multiplex staining. Stain tissues with a serial dilution including 1:10, 1:50, 1:100, 1:500 of the primary antibody conjugated to a fluorophore.

Protocol tips

- Make sure to prepare enough tissue samples for the multiplex sample and the necessary single-color control samples.
- Do not let the tissue sample dry out. Dried out tissue will result in incorrect or no signal. Ensure that there is enough solution to completely cover the tissue during incubation and wash steps. We recommend using a humidified chamber (for example, a covered box with damp paper towel).
- Crisp staining results require optimizing primary antibody dilution.

This protocol is continued on page 76

Multiplexed IHC staining with primary antibodies conjugated to fluorophores, cont.

Tissue preparation

Deparaffinize the tissue using a standard deparaffinization rehydration protocol.

Step	Solution	Incubation time
1	Xylene	5 minutes
2	Xylene	5 minutes
3	1:1 solution of Xylene : 100% EtOH	5 minutes
4	100% EtOH	3 minutes
5	95% EtOH	3 minutes
6	85% EtOH	3 minutes
7	75% EtOH	3 minutes
8	50% EtOH	3 minutes
9	dH_2O	Rinse
10	1X PBS	5 minutes
11	1X PBS	5 minutes
12	1X PBS	5 minutes

Antigen retrieval

Perform heat-induced epitope retrieval (HIER) using either Citrate Buffer (pH 6.0) or EDTA (pH 9) using a microwave or pressure cooker according to standard antigen retrieval protocols.

Note

An automated slide stainer can be used for the dewaxing/deparaffinization and HIER steps.

1. Depressurize and crack pressure cooker lid for 5 minutes to allow sample cooling.
2. Remove the slide rack with forceps, submerge it into a clear staining dish containing 200 mL of ddH_2O, and wash for 1 minute with frequent agitation.
3. Repeat the wash one more time with fresh ddH_2O.
4. Transfer the slide rack to 1X PBS.

Note

Samples can be stored covered in 1X PBS at RT overnight.

Critical note

Do not let slides dry out from this point forward

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Permeabilization

1. Draw hydrophobic barrier with pen.
2. Allow barrier to dry at RT for 2-3 minutes.
3. Prepare 0.1% Triton/PBS
4. Remove each slide and flick excess 1X PBS, without completely drying out the sections.
5. Place the slides face up on a flat surface and immediately add 400 µL of the working permeabilization solution for 30 minutes at RT.
6. Decant the working perm buffer from the slides, and wash slides in 1X PBS thoroughly by shaking up and down for 1 minute.
7. Repeat wash with fresh 1X PBS, three times.

Optional: White Light Photobleached (autofluorescence treatment)

Perform autofluorescence reduction with white light prior to labeling (method described in Nat Cancer. 2023 Jul;4(7):1036–1052).

Prepare reagents:

Sodium hydroxide (NaOH) stock solution

Reagent	Volume
NaOH solution (50% w/w)	0.5 mL
Deionized water	9.5 mL

Working autofluorescence solution

Reagent	Volume
1 M NaOH Stock	2.4 mL
H_2O_2 (30% w/v)	4.5 mL
PBS	93.1 mL

Note

Final concentration should be 24 mM NaOH and 4.5% H_2O_2 in PBS.

This protocol is continued on page 78

Multiplexed IHC staining with primary antibodies conjugated to fluorophores, cont.

1. Rinse samples with 1X PBS at room temperature.
2. Place slides in a clear container covered with the working autofluorescence solution (4.5% hydrogen peroxide and 24 mM NaOH in PBS).
3. Illuminate with white light for 30 min.
4. Wash 3x in 1X PBST (0.05% Tween-20)

Note

We do not recommend using the UV light as it can destroy certain antigens.

Block samples for non-specific binding.

1. Block with 3% BSA for 1 hour at RT.

Multiplex labeling with primary antibodies conjugated to fluorophores

1. Create single-color controls and unstained control. Unstained control must be used with every sample block.
2. Create your multiplex master mix by diluting in each of your primary antibody conjugates to desired concentration in 3% BSA blocking buffer. We recommend having a final dilution of 1:10 (final concentration 0.02 mg/mL) if you did not optimize the dilution for the multiplex staining.
3. Add to sample and incubate 1 hour at RT in a humidified chamber in the dark.
4. Wash 3x with 1X PBST (0.05% Tween-20)
5. Add nuclear counter stain such as NucBlue or DAPI.
6. Wash 3x with 1X PBST (0.05% Tween-20).

Note

Staining can be incubated overnight at 4°C. This may increase both intensity of staining or background.

Critical note

Do not soak samples in 1X PBST longer than 30 minutes.

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7. Mount the coverslips using a mountant with antifade properties:
   - ProLong Glass Antifade Mountant (Cat. No. P36984) is recommend for hard-set mounting and long-term archiving of tissue samples.
   - SlowFade Glass Soft-set Antifade Mountant (Cat. No. S36917) is recommended for soft-set mounting if coverslip removal is required for tissue samples.

   For optimal results, follow the instructions provided with the mountant.

8. Analyze the tissue using a compatible imaging instrument.

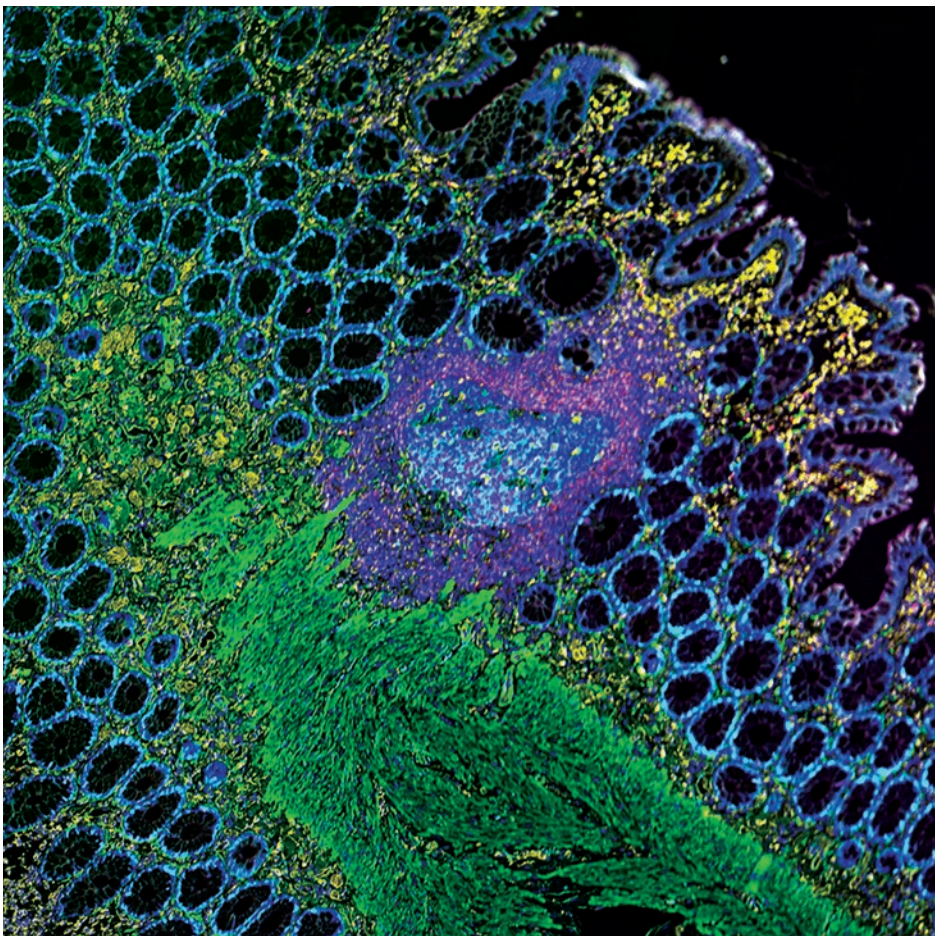

Figure 21. 7 marker multiplex IHC of a colon section.

Reference

Lin JR, Chen YA, Campton D, Cooper J, Coy S, Yapp C, Tefft JB, McCarty E, Ligon KL, Rodig SJ, Reese S, George T, Santagata S, Sorger PK. High-plex immunofluorescence imaging and traditional histology of the same tissue section for discovering image-based biomarkers. Nat Cancer. 2023 Jul;4(7):1036-1052. doi: 10.1038/s43018-023-00576-1. PMID: 37349501

Conjugating primary antibodies to fluorophores using ReadyLabel™ Antibody Labeling Kits

Introduction

The Invitrogen™ ReadyLabel™ Antibody Labeling Kits allow for fast, convenient labeling of off-the-shelf antibodies and cell culture supernatants without prior purification of the antibody solution. Each ReadyLabel™ Antibody Labeling Kit contains everything needed to perform five labeling reactions and will produce purified antibody conjugates, without the purification step.

The kits can be used to label antibodies in solutions containing BSA or other stabilizing proteins, antibodies in a primary amine-containing buffer such as Tris or glycine, or even cell culture supernatants, without prior antibody purification. The reactive dye efficiently reacts with primary amines of antibodies to form stable, covalent conjugates. For smaller amounts of antibody, the ReadyLabel™ 20 µg Antibody Labeling Kit will provide superior results; for larger quantities use the ReadyLabel™ 100 µg Antibody Labeling Kit. ReadyLabel™ Flex kits are designed for use with a variety of amine-reactive dyes.

Materials

- ReadyLabel™ spin columns (Component A)
- ReadyLabel™ Wash Buffer (Component B)
- Dimethylsulfoxide (DMSO) (Component C)
- ReadyLabel™ Labeling Buffer (Component D)
- ReadyLabel™ Neutralization Buffer (Component E)
- ReadyLabel™ Elution Buffer pH 3.3 (Component F)
- ReadyLabel™ Elution Buffer pH 2.0 (Component G)
- Reactive Dyes
- Antibody solution, or cell culture supernatant
- Benchtop centrifuge capable of up to 1,000 x g
- 1.5 or 2 mL microcentrifuge tubes
- Pipettors and disposable pipette tips

- R10701 ReadyLabel™ Flex Antibody Labeling Kit for 5 x 20 µg of antibody
- R10702 ReadyLabel™ Flex Antibody Labeling Kit for 5 x 100 µg of antibody
- R10709 ReadyLabel™ AlexaFluor™ 488 Antibody Labeling Kit for 5 x 20 µg of antibody
- R10710 ReadyLabel™ AlexaFluor™ 647 Antibody Labeling Kit for 5 x 20 µg of antibody
- R10711 ReadyLabel™ Biotin Antibody Labeling Kit for 5 x 20 µg of antibody
- R10712 ReadyLabel™ AlexaFluor™ 488 Antibody Labeling Kit for 5 x 100 µg of antibody
- R10713 ReadyLabel™ AlexaFluor™ 647 Antibody Labeling Kit for 5 x 100 µg of antibody
- R10714 ReadyLabel™ Biotin Antibody Labeling Kit for 5 x 100 µg of antibody

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Procedure for 100 µg ReadyLabel™ kits

Prepare the spin column

1. Twist to remove the bottom plug of the column (Component A). Loosen the cap but do not fully remove.
2. Place the column in a collection tube, then centrifuge the column-tube assembly at 1,000 x g for 30 seconds to remove the storage buffer. Discard the flowthrough. The cap may be discarded after this step.
3. Add 500 µL of Wash Buffer (Component B) to the column, then centrifuge at 1,000 x g for 30 seconds. Discard the flowthrough.

Protocol tips

- Do not reuse the spin columns.
- This kit is designed for IgG antibody labeling and cannot be used to label other proteins.
- Work quickly during the dye dissolution and loading steps; once dissolved the dye begins to lose reactivity, which could result in a reduced degree of labeling.

Purify and label the antibody

1. Load 100 µg of antibody solution onto the washed resin, then centrifuge at 100 x g for 2 minutes.
2. Additional spin time may be needed if residual liquid remains above the resin in the column. Do not proceed to the next step until all liquid has flowed through the resin.
3. Add 7 µL DMSO (Component C) to a vial of lyophilized dye and pipette gently to dissolve the dye.
4. Dilute the dissolved dye with 65 µL of Labeling Buffer (Component D).
5. Immediately add 60 µL of the diluted dye onto the resin and centrifuge at 100 x g for 1 minute. Discard the flowthrough.
6. Incubate at room temperature for 30 minutes.

Protocol tips

ReadyLabel™ Flex Kit Recommendations

- ReadyLabel™ Flex kits are designed for use with a variety of amine-reactive dyes.
- Use 100 µg of dye per 100 µg antibody labeling reaction or 20 µg of dye per 20 µg antibody labeling reaction.
- Dissolve reactive dye with DMSO (Component C) using less than 1/10 of the final diluted volume. Then dilute to ~1 mg/mL in labeling buffer (Component D).
- For example, dissolve a 100 µg vial of lyophilized reactive dye with 10 µL DMSO, then dilute with 90 µL labeling buffer to a final volume of 100 µL at 1 µg/µL. Use for 1 x 100 µg or 5 x 20 µg reactions.

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Conjugating primary antibodies to fluorophores using ReadyLabel™ Antibody Labeling Kits, cont.

Elute the labeled antibody

1. Add 500 µL Wash Buffer (Component B) to the column, then centrifuge at 1,000 x g for 30 seconds. Discard the flowthrough.

2. Repeat the wash step (Step 1).

3. Add 60 µL Neutralization Buffer (Component E) to a clean 1.5 mL centrifuge tube and transfer the washed column to this tube.

4. Add 240 µL Elution Buffer pH 3.3 (Component F) to the resin, then centrifuge at 300 x g for 3 minutes.

5. If a poor yield is achieved, Elution Buffer 2.0 (Component G) may be substituted for Elution Buffer 3.3, or they may be used sequentially.

Procedure for 20 µg ReadyLabel™ kits

Prepare the spin column

1. Twist to remove the bottom plug of the column (Component A). Loosen the cap but do not fully remove.

2. Place the column in a collection tube, then centrifuge the column-tube assembly at 1,000 x g for 30 seconds to remove the storage buffer. Discard the flowthrough. The cap may be discarded after this step.

3. Add 100 µL of Wash Buffer (Component B) to the column, then centrifuge at 1,000 x g for 30 seconds. Discard the flowthrough.

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Purify and label the antibody

1. Load 20 µg of antibody solution onto the washed resin, then centrifuge at 200 x g for 2 minutes.
2. Additional spin time may be needed if residual liquid remains above the resin in the column.
3. Add 2 µL DMSO (Component C) to a vial of lyophilized dye and pipette gently to dissolve the dye.
4. Dilute the dissolved dye with 18 µL of Labeling Buffer (Component D).
5. Immediately add 15 µL of the diluted dye onto the resin and centrifuge at 200 x g for 2 minutes. Discard the flowthrough.
6. Incubate at room temperature for 30 minutes.

Critical note

Do not proceed to the next step until all liquid has flowed through the resin.

Elute the labeled antibody

1. Add 500 µL Wash Buffer (Component B) to the column, then centrifuge at 1,000 x g for 30 seconds. Discard the flowthrough.
2. Repeat the wash step (Step 1).
3. Add 60 µL Neutralization Buffer (Component E) to a clean 1.5 mL centrifuge tube and transfer the washed column to this tube.
4. Add 240 µL Elution Buffer pH 3.3 (Component F) to the resin, then centrifuge at 300 x g for 3 minutes.

Note

If a poor yield is achieved, Elution Buffer 2.0 (Component G) may be substituted for Elution Buffer 3.3, or they may be used sequentially.

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Conjugating primary antibodies to fluorophores using ReadyLabel™ Antibody Labeling Kits, cont.

Optimization and troubleshooting

Working with antibody solutions containing glycerol

- The following tables include suggested guidelines for loading antibodies containing varying amounts of glycerol. These times are a suggested starting point; additional spin time may be required.
- It may be necessary to increase the speed of centrifugation if the antibody/glycerol solution does not flow through the resin in a reasonable amount of time.

Note

Increased speeds may lead to reduced antibody yield.

100 µg ReadyLabel™ Glycerol Guidelines

Percent glycerol	Antibody loading spin time (minutes)	Spin speed (x g)	Dye loading time (minutes)	Spin speed (x g)
0	2	100	2	200
10	4	100	2	200
25	6	100	3	200
50	6	100	3	200

20 µg ReadyLabel™ Glycerol Guidelines

Percent glycerol	Antibody loading spin time (minutes)	Spin speed (x g)	Dye loading time (minutes)	Spin speed (x g)
0	2	200	2	200
10	4	200	4	200
25	6	200	4	200
50	8	200	4	200

Storage and handling of conjugates

- Store the labeled antibody conjugate at 2–8°C, protected from light.
- Eluted antibody conjugates will typically be between pH 7 and pH 8.5 and should be used within two weeks for best results.
- For longer storage, add 2 mM sodium azide or buffer exchange into a solution containing 2 mM sodium azide to prevent contamination.
- For longer storage of dilute (< 1 mg/mL) purified antibody conjugates, add BSA or other stabilizing protein at 1–10 mg/mL (0.1–1% BSA).
- For long-term storage, divide the conjugate into small aliquots and freeze at ≤ –20°C. Avoid repeated freezing and thawing.
- For optimal use, centrifuge solutions of conjugates in a microcentrifuge before use; only the supernatant should then be used in the experiment. This step will remove any aggregates that may have formed during storage.

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Inefficient removal of free dye

Despite removing most free dye from antibody conjugates using the provided resin columns, it is possible that small amounts of free dye will be present in the conjugate solution. The presence of free dye, which can be determined by thin layer chromatography, will result in erroneously high calculated values for the degree of labeling. Remaining free dye can be removed by applying the conjugate to a Zeba™ Dye and Biotin Removal Spin Column (A44296) or by extensive dialysis. For typical applications that include a washing step after the antibody conjugate has been applied to cells or tissue, any residual dye will be removed by the wash step.

Inefficient removal of BSA or contaminating proteins

Despite removing most contaminating proteins from antibody conjugates using the provided resin columns, it is possible that small amounts of BSA or other protein will be present in the conjugate solution. The presence of contaminating proteins, which can be determined by SDS PAGE, will result in erroneously high calculated values for the concentration of the conjugate solution. For typical applications that involve a blocking step prior to application of the conjugate and a wash step after the application of the conjugate, contaminating protein will be removed by the wash step.

Figure 22. FFPE Human Colon Tissue stained with ReadyLabel Alexa Fluor 488 Cytokeratin, ReadyLabel Alexa Fluor 594 SMA, ReadyLabel Alexa Fluor 647 PCNA and ReadyLabel Alexa Fluor Plus 750 CD68

Multiplexed SuperBoost tissue staining with tyramides

Introduction

Multiplexing is essential for spatial biology studies as it allows for the simultaneous detection of multiple target molecules within a single tissue sample. The Tyramide SuperBoost technology is ideal for tissue multiplexing because it detects multiple proteins of interest through multiple rounds of applying and removing primary and secondary antibodies without significantly decreasing the fluorescence intensity of signal. Once antibodies are removed, the tissue can be reprobed with a primary antibody from mouse and rabbit without risk of cross-reactivity, followed by detection with another round of Tyramide SuperBoost signal amplification. By enhancing signal intensity and specificity, Tyramide SuperBoost Signal Amplification enables detection and visualization of multiple targets including low abundance proteins within tissue samples, contributing to a deeper understanding of spatial relationships and cellular interactions in biological systems.

This protocol provides guidelines for utilizing the Tyramide SuperBoost kits for multiplex detection and visualization of target molecules in tissue starting from an unlabeled FFPE tissue sample on a slide to produce a multicolored tissue sample with multiple targets labeled with fluorescent tyramides. The labeled tissue is then ready for spatial imaging on any type of fluorescence microscope including the Invitrogen EVOS M7000 Imaging System and CellInsight LED and LZR Pro systems, or spatial imaging systems such as the Akoya PhenoImager and Invitrogen EVOS S1000 Imaging System. For added convenience, these steps can be performed on an automated slide stainer such as the Leica BOND RX systems.

Materials

Materials required

- Tyramide SuperBoost Kit (Multiple Kit Options)
- Primary antibodies to your targets specific for IHC (mouse or rabbit host)
- SuperBoost secondary antibodies conjugated to Poly HRP (Included within the kit options, additional vials can be purchased Cat. No. B40951, B40961, B40962)
- Slides, coverslips, containers
- PBS (phosphate buffered saline), pH 7.4 (without calcium, magnesium, or phenol red) (Cat. No. 10010031)
- 95% ethanol (Cat. No. AC615110010)
- Distilled water, highly purified (Cat. No. 15230-147)
- Mountant:
  - ProLong Glass Antifade Mountant (Cat. No. P36984) or SlowFade Glass Soft-set Antifade Mountant (Cat. No. S36917)
- DAPI counterstain (e.g., NucBlue Fixed Cell ReadyProbes Reagent- DAPI, Cat. No. R37606)

Protocol tips

- Do not let the tissue sample dry out. Dried out tissue will result in incorrect or no signal. Ensure that there is enough solution to completely cover the tissue during incubation and wash steps. We recommend using a humidified chamber (for example, a covered box with damp paper towel).
- Crisp staining results require optimizing primary antibody dilution.
- Incubation duration for the tyramide labeling reaction is crucial for getting high resolution images with specific signal and must be optimized for specific labeling.
- If multiplexing TSA fluorophores, please optimize the heat-induced epitope (antigen) retrieval (HIER) procedure for your samples.
- Prepare reagents on day of use.

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Optional materials

- ReadyProbes Hydrophobic Barrier Pap Pen, (Cat. No. R3777)
- Antigen Retrieval Reagents (only one needed):
  - Citrate Buffer (pH 6.0), Concentrate (Cat. No. 005000) or eBioscience IHC Antigen Retrieval Solution - Low pH (10X) (Cat. No. 00-4955-58) or eBioscience IHC Antigen Retrieval Solution - High pH (10X) (Cat. No. 00-4956-58)
- Hydrogen peroxide, 30% w/v (Example: Cat. No. H325-4)
- Sodium Hydroxide Solution (50% w/w/Certified) (Example: Cat. No. SS254500)
- Image-iT FX Signal Enhancer (Cat. No. I36933)
- Endogenous Biotin-Blocking Kit (Cat. No. E21390)

Procedure

Prepare reagents

Reagents included in the Tyramide SuperBoost kit:

- Blocking buffer (10% Goat Serum) (Component A)
- Poly-HRP-conjugated secondary antibody or HRP-conjugated streptavidin (Component B)
- Alexa Fluor tyramide reagent (Component C1)
- Hydrogen Peroxide (Component C2)
- Reaction buffer (Component C3)
- Reaction Stop Reagent (Component D)
- Dimethylsulfoxide (DMSO) (Component E)

Note

Tyramide SuperBoost kits come in two sizes including 150 slides or 50 slides with or without secondary antibody conjugated to HRP.

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Multiplexed SuperBoost tissue staining with tyramides, cont.

100X tyramide stock solution

1. Dissolve the Alexa Fluor tyramide reagent (Component C1) in 150 µL (for 150 slides kit size) or 50 µL (for 50 slides kit size) of DMSO (Component E).
2. Vortex to dissolve any tyramide that might coat the sides or cap of the vial.
3. Pulse briefly in centrifuge for contents to come down.

Protocol tip

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. For longer storage, aliquot into 5–10 µL volumes and store at –20°C.

100X hydrogen peroxide solution

1. Add 1 drop (approximately 40 µL) of Hydrogen Peroxide Solution (Component C2) to 1 mL of distilled water.

Note

Prepare the 100X H_2O_2 solution fresh on the day of use.

1X reaction buffer

1. Add 1 drop (approximately 50 µL) of 20X Reaction buffer (Component C3) to 1 mL of distilled water.

Note

Prepare the 1X reaction buffer fresh on the day of use.

Reaction stop reagent (11X reaction stop reagent stock solution)

1. Add 1.45 mL of 95% ethanol to one vial of Reaction Stop Reagent (Component D).
2. Vortex the vial to dissolve any stop reagent coating the sides and cap of the vial.

Protocol tip

Unused portion of the reaction stop reagent stock solution can be stored at –20°C for 6 months.

Reaction stop reagent working solution

1. In a separate tube, prepare reaction stop reagent working solution by diluting 1:11 in PBS before use to prepare a working solution.

Note

Prepare the reaction stop reagent working solution fresh on the day of use.

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Tyramide working solution

The volumes in this table are based on 100 µL of tyramide working solution needed per 18-mm × 18-mm coverslip. This volume can be adjusted based on the size of the coverslip and tissue sample. (Optional) If performing autofluorescence reduction

Note

Prepare the tyramide working solution fresh on the day of use.

	Number of coverslips (18-mm × 18-mm)		
Component	**1**	**10**	**50**
100X tyramide stock solution	1 µL	10 µL	50 µL
100X H_2O_2 solution	1 µL	10 µL	50 µL
1X reaction buffer	100 µL	1 mL	5 mL

Prepare humid chamber

Protocol tip

Build a humidified chamber to keep samples from drying out.

1. Wet paper towels with distilled water.
2. Layer slide container with wet paper towels.
3. Layer with Parafilm to cover the wet paper towels.

Prepare controls, optimize primary antibody dilution, and setting tyramide labeling reaction

In many multiplex experiments, the primary antibody concentration can be optimized while keeping the secondary antibody staining and tyramide reaction conditions the same for all antibodies.

Critical note

We strongly recommend you optimize the primary antibody before using the kit. Positive and negative control slides should be stained with a serial dilution including 1:100, 1:500, 1:1000, 1:5,000, 1:10,000.

The incubation period for the tyramide working is crucial in getting high resolution images with specific signal. We highly recommend that you optimize the incubation period using positive and negative control slides at various incubation time points when conducting this experiment for the first time.

To optimize the incubation time for this step, perform 0, 2, 5, 7 and 10-minute incubations using positive and negative control slides.

- If non-specific signal is present in negative controls or if the signal is blurry in positive controls, decrease the incubation time.
- If dim or no signal is present in positive controls, increase the incubation time.

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Multiplexed SuperBoost tissue staining with tyramides, cont.

Control slides

- No primary negative control (primary antibody omitted).
- No primary and no secondary negative control (primary and secondary antibody omitted).

Tissue preparation

1. Dewax/deparaffinize and rehydrate the FFPE tissue according to standard IHC-P (paraffin) protocols.

2. Perform heat-induced epitope retrieval (HIER) using either Citrate Buffer (pH 6.0) or EDTA (pH 9) using a microwave or pressure cooker according to standard antigen retrieval protocols.

Note

An automated slide stainer can be used for the dewaxing/deparaffinization and HIER steps.

Optional: Perform autofluorescence reduction with white light prior to labeling (method described in Nat Cancer. 2023 Jul;4(7):1036-1052).

Prepare reagents:

Sodium hydroxide (NaOH) stock solution

Reagent	Volume
NaOH solution (50% w/w)	0.5 mL
Deionized water	9.5 mL

Working autofluorescence solution

Reagent	Volume
1 M NaOH Stock	2.4 mL
H_2O_2 (30% w/v)	4.5 mL
PBS	93.1 mL

Note

Final concentration should be 24 mM NaOH and 4.5% H_2O_2 in PBS.

3. Rinse samples with 1X PBS at room temperature.

4. Place slides in a clear container covered with the working autofluorescence solution (4.5% hydrogen peroxide and 24 mM NaOH in PBS).

5. Illuminate with white light for 60 min.

Note

We do not recommend using the UV light as it can destroy certain antigens.

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Optional: Reduce non-specific dye binding with Image-iT FX Signal Enhancer

6. Rinse samples with 1X PBS at room temperature.

7. Apply 4 drops or 200 µL of Image-iT FX Signal Enhancer to cover each coverslip or section.

8. Incubate for 30 minutes at room temperature in a humid environment.

9. Rinse samples with 1X PBS at room temperature.

Quench endogenous peroxidase and block tissue

Prepare all reagents before starting these steps. Components A–D and C1–C3 are found in the kit.

Quench the endogenous peroxidase activity of the sample.

1. Cover the sample with 3% Hydrogen Peroxide Solution (Component C2).

2. Incubate for 60 minutes at room temperature in a humid environment.

3. Rinse samples with 1X PBS at room temperature.

4. Apply one or two drops of the streptavidin reagent (Component A from the Endogenous Biotin-Blocking Kit) to the cells or tissue and incubate for 15–30 minutes at room temperature in a humid environment.

5. Rinse samples with 1X PBS at room temperature.

6. Add one or two drops of the biotin reagent (Component B from the Endogenous Biotin-Blocking Kit) and incubate for 15–30 minutes at room temperature in a humid environment.

7. Rinse samples with 1X PBS at room temperature.

Optional

If using HRP-conjugated streptavidin secondary, block endogenous biotin in the sample. We recommend using the Invitrogen Endogenous Biotin-Blocking Kit (Cat. No. E21390).

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Multiplexed SuperBoost tissue staining with tyramides, cont.

Block samples for non-specific binding.

8. Add 2–3 drops (approximately 100–150 µL) of Blocking buffer (Component A from the Tyramide SuperBoost kit) to the sample.

9. Incubate for 60 minutes at room temperature in a humid environment.

Label with primary and poly-HRP secondary antibody

1. Label the tissue with primary antibody with mouse or rabbit as the host. Dilute the antibody in Blocking buffer (10% goat serum, Component A).

2. Incubate with the tissue for 30-60 minutes at room temperature or overnight at 2–8°C in a humid environment.

3. Wash the tissue for 2 minutes with 1X PBS at room temperature on a rocker. Repeat this step three times.

4. Add 2–3 drops (approximately 100–150 µL) of poly-HRP-conjugated secondary antibody for unlabeled primary antibodies or HRP-conjugated streptavidin for biotinylated primary antibodies (Component B) to the tissue.

5. Incubate for 10-60 minutes at room temperature or overnight at 2–8°C in a humid environment.

6. Wash the tissue for 2 minutes with 1X PBS at room temperature on a rocker in a humid environment. Repeat this step three times.

Note

If multiplexing using an automated slide stainer, the anti-mouse and anti-rabbit poly-HRP-conjugated secondary antibodies can be combined 1:1 into a single staining solution. This simplifies the procedure so that the same secondary antibody staining solution can be used when staining with either mouse or rabbit primary antibodies.

Note

We recommend optimizing the primary antibody staining concentration.

Note

If using a SuperBoost kit with Streptavidin, use a biotin-conjugated primary antibody.

Note

If you observe non-specific signal, you can shorten this incubation period.

This protocol is continued on page 93

Label with tyramide fluorophore(s)

- Prepare all reagents before starting these steps.
- Complete optimizing both the tyramide working solution and the reaction stop reagent incubation periods before proceeding.

Label with tyramide

1. Apply 100 µL of the tyramide working solution to the tissue.

2. Incubate according to the optimized time (2–10 minutes) at room temperature in a humid environment. The reaction can be stopped (1) using reaction stop reagent or (2) by performing HIER. We recommend using HIER when multiplexing with primary antibodies.

Using stop reagent; recommended for single TSA reaction

3. Apply 100 µL of reaction stop reagent working solution.

4. Rinse the tissue three times with 1X PBS at room temperature.

5. SuperBoost kits containing Biotin-XX tyramide only: If using a kit containing Biotin-XX tyramide, then use conjugated streptavidin. See “Streptavidin conjugates recommended for the detection of Biotin-XX tyramide” table.

Note

If using Biotin-XX tyramide with a fluorescent streptavidin, do not perform HIER after labeling with streptavidin, as that will also remove the fluorescent streptavidin.

This protocol is continued on page 94

Multiplexed SuperBoost tissue staining with tyramides, cont.

Performing antibody removal/stripping (HIER); recommended when multiplexing with primary antibodies from the same or different species.

For multiplexing on FFPE tissue samples, Tyramide SuperBoost kits are compatible with the citrate buffer/microwave method described by Tóth and Mezey (J Histochem Cytochem, 2007) to remove primary and secondary antibodies from the sample and eliminate peroxidase activity. This method does not significantly affect the fluorescence of the covalently reacted tyramide and allows the tissue to be reprobed with a primary antibody from either the same or a different species followed by subsequent Tyramide SuperBoost signal amplification.

1. Dilute Citrate Buffer (pH 6.0), Concentrate (Cat. No. 005000) 1:20 in distilled water.
2. After completing the “Label with tyramide” steps above, 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).
3. Reduce the power to 20% and keep microwaving for an additional 15 minutes.
4. Let the tissue sample cool to room temperature while keeping it in the citrate buffer.
5. Wash the sample twice with 1X PBS.
6. Repeat the “Block samples for non-specific binding”, “Label with primary and poly-HRP secondary antibody”, and “Label with tyramide” steps above with a primary antibody of the same or a different species.
7. Perform additional rounds of citrate buffer/microwaving followed by blocking, antibody staining, and Tyramide SuperBoost signal amplification (see “Label with primary and poly-HRP secondary antibody” and “Label with tyramide” steps) as needed for additional targets.

This protocol is continued on page 95

Counterstain and detect

1. Counterstain the tissue as needed using standard protocols. Some reagents recommended for nuclear counterstaining are listed below.

Counterstain type	Product	Ex/Em (nm)
Ready-to-use nuclear counterstains	NucBlue Fixed Cell ReadyProbes Reagent (Cat. No. R37606)	360/460
	NucGreen Dead 488 ReadyProbes Reagent (Cat. No. R37109)	504/523
	NucRed Dead 647 ReadyProbes Reagent (Cat. No. R37113)	642/661
Concentrated nuclear counterstains	DAPI (Cat. No. D1306)	358/461
	SYTOX Green (Cat. No. S7020)	504/523
	SYTOX Deep Red (Cat. No. S11381)	660/682

2. Mount the coverslips using a mountant with antifade properties:
   - ProLong Glass Antifade Mountant (Cat. No. P36984) is recommend for hard-set mounting and long-term archiving of tissue samples.
   - SlowFade Glass Soft-set Antifade Mountant (Cat. No. S36917) is recommended for soft-set mounting if coverslip removal is required for tissue samples. For optimal results, follow the instructions provided with the mountant.

3. Analyze the tissue using a compatible imaging instrument.

How to pick secondary antibodies

SuperBoost TSA recommends using secondary antibodies conjugated to poly-HRP and are provided in the full kits. Secondary antibodies conjugated to HRP may not provide the optimal signal.

Biotin-XX Tyramide requires the use of fluorescent streptavidins, as listed in the table.

Streptavidin conjugates recommended for the detection of Biotin-XX tyramide	Ex/Em (nm)
Alexa Fluor 350 Streptavidin (Cat. No. S11249)	346/442
Alexa Fluor 405 Streptavidin (Cat. No. S32351)	402/421
Alexa Fluor 488 Streptavidin (Cat. No. S11223)	495/519
Alexa Fluor 514 Streptavidin (Cat. No. S32353)	518/540
Alexa Fluor 555 Streptavidin (Cat. No. S21381)	555/565
Alexa Fluor 594 Streptavidin (Cat. No. S11227)	590/617
Alexa Fluor 647 Streptavidin (Cat. No. S21374)	650/668
Alexa Fluor 680 Streptavidin (Cat. No. S21378)	679/702
Alexa Fluor 700 Streptavidin (Cat. No. S21383)	702/723
Alexa Fluor 750 Streptavidin (Cat. No. S21384)	749/775

References

Lin JR, Chen YA, Campton D, Cooper J, Coy S, Yapp C, Tefft JB, McCarty E, Ligon KL, Rodig SJ, Reese S, George T, Santagata S, Sorger PK. High-plex immunofluorescence imaging and traditional histology of the same tissue section for discovering image-based biomarkers. Nat Cancer. 2023 Jul;4(7):1036-1052. doi: 10.1038/s43018-023-00576-1. PMID: 37349501

ViewRNA tissue fluorescence assay protocol

Introduction

Visualizing RNA transcripts in tissue samples can provide insight into cellular processes maintained in healthy states or impacted in diseased conditions. Invitrogen ViewRNA kits use branched DNA (bDNA) technology to enable single-molecule sensitivity in diverse sample types, such as FFPE and cryopreserved tissue. The fluorescent ViewRNA ISH Tissue Assay allows for multiplexing, enabling detection of multiple RNA transcripts at one time in a single sample.

Multiple variations of the kit are available: Cat. Nos. QVT0700 and QVT0800 contain 4 fluorescent probe types (488, 546/594, 647, 750) with variations in 546/594 in QVT0700/800 respectively. Other core kits and modules contain individual probes to be mixed and matched by the user. Please see Tables 1 and 2 in the Appendix for more information on fluorophores and kit options.

Materials

ViewRNA reagents and accessories

- ViewRNA Tissue Assay Fluorescence Kits (Suggested Cat. No. QVT0700 or QVT0800 see Table 2 in Appendix for all sizes and options)
- Probe set(s), see Table 1 in Appendix for probe set Type/fluorophore selection
- ReadyProbes Hydrophobic Barrier Pap Pen (Cat. No. R3777)
- UltraPure DNase/RNase-free Distilled Water (Cat. No. 10977023)
- Xylene (Cat. No. 396931000) and 100% Ethanol (Cat. No. T038181000) (FFPE only)
- Phosphate buffered saline (10X) pH 7.4, RNase-free (Cat. No. AM9624)
- Mounting medium
  - ProLong Glass Antifade Mountant with NucBlue Stain (Cat. No. P36981)
  - SlowFade Glass Soft-set Antifade Mountant (Cat. No. S36917)
- Tissue Tek Staining Dishes (3) (Cat. No. NC9507198)
- Tissue Tek Clearing Agent Dishes (2) (Cat. No. NC9012500)
- Hybridization system — Dry oven (Cat. No. 15-103-0503) or similar
- ViewRNA Temperature Validation Kit (Cat. No. QV0523)
- Image-iT Fixative Solution (Cat. No. R37814) or 4% Formaldehyde prepared in 1X PBS

This protocol is continued on page 97

General lab equipment

- Slide-staining moisture chamber:
  eBioscience StainTray (Cat. No. 44-0404-10) or similar
- Vertical slide rack
- 1 L glass beaker
- Hotplate
- Microtube centrifuge
- Vortex

Optional materials

- Microplate shaker
- ReadyProbes Tissue Autofluorescence Quenching Kit (Cat. No. R37630)

Important protocol notes

- Incubation times and temperatures should be strictly followed to ensure successful hybridization.
- All steps involving the use of RNase-free water should be performed using nuclease-free labware and disposable pipette tips to avoid RNase contamination.
- For the hybridization system, a humified tissue culture incubator is not recommended for this protocol.
- For all wash steps use 200 mL of indicated wash solution and use good washing technique. When performing washes, flick the slides to remove excess liquid carefully, do not completely dry the sample out.
- Prewarm Diluent Reagents to 40°C before beginning the protocol. Due to the viscous nature of Diluent Reagents, it is suggested to not make aliquots, warm entire bottle to 40°C and remove liquid as needed.
- There is an optional overnight storage step.

Positive and negative controls

- We recommend incorporating positive and negative controls in each assay to qualify and interpret your results.

Protocol tip

Negative control examples:

- Omit the target probe set
- Use a probe set designed to the sense strand of the target
- Use a probe set for a target that is not present in your tissue sample

Positive control examples:

- Housekeeping genes: ACTB, GAPDH, or UBC (please refer to User Guide, page 13 for more information on control probe selection)

This protocol is continued on page 98

ViewRNA tissue fluorescence assay protocol, cont.

ViewRNA ISH Fluorescence Assay Kit (Cat. Nos. QVT0700, QVT4700, QVT0800, QVT4800) contents and storage

Box 1 Store at –20°C*	Box 2 Store at 2–8°C*	Box 3 Store at 15–30°C*
QVC Pre-Amplifier Mix (25X)	100X Pretreatment Solution	100X Wash Buffer Component 1
Amplifier Mix, Types 1,4, 6, and 10 (25X)	Protease QF	400X Wash Buffer Component 2
Label Probes, Types 1, 4, 6 and 10 (25X each)	Probe Set Diluent QT	Detergent QC
100X DAPI	Amplifier Diluent QF	
	Label Probe Diluent QF	

* Reagents have a minimum shelf life of 6 months from date of receipt when stored as recommended. See kit labels for expiration dates and the package insert for component quantities.

Procedure

Buffer and equipment preparation

1. Prepare the solutions for target probe hybridization

1X PBS

Prepare 3 L of 1X PBS (RNase-free)

Reagent	Volume (3 L)
10X PBS	300 mL
ddH_2O	2.7 L

Wash Buffer

Prepare 1 L of Wash Buffer according to the table below. Mix well with 900 mL of ddH_2O and adjust total volume by adding an additional 87.5 mL ddH_2O.

Prepare 1 L of Wash Buffer

Reagent	Volume (4 L)
ddH_2O	900 mL + 87.5 mL
100X Wash Buffer Component 1	10 mL
400X Wash Buffer Component 2	2.5 mL

1X Pretreatment Solution

Prepare 500 mL of 1X Pretreatment Solution according to the table below in a 1 L glass beaker.

Prepare 500 mL of 1X Pretreatment Solution

Reagent	Volume (500 mL)
100X Pretreatment Solution	5 mL
ddH_2O	495 mL

This protocol is continued on page 99

Storage Buffer (optional for overnight storage)
Prepare 200 mL of Storage Buffer if using the optional stopping point according to the table below.

Prepare 200 mL of Storage Buffer

Reagent	Volume (200 mL)
Wash Buffer Component 2	60 mL
ddH_2O	140 mL

Note

The volume of storage buffer can be adjusted depending on experimental requirements.

2. Thaw probe set(s) on ice. Mix, briefly centrifuge and place on ice until use.

3. Pre-warm entire bottle of Probe Set Diluent to 40°C.

Note

If performing entire assay in one day, also prewarm Amplifier and Label Probe Diluent to 40°C.

Prepare the equipment

Calibrate the temperature of the dry oven to 40°C using the ViewRNA Temperature Validation Kit. When using the dry oven and humidifying chamber such as StainTray slide holder, ensure that the appropriate water level is attained. StainTray slide holder can be replaced by an enclosed plastic chamber supplied with a wetted tissue paper and elevated platform to create a hybridization chamber.

Note

FFPE and cryopreserved tissues can be used in this protocol. Tissue preparation steps differ depending on the sample. Proceed to the appropriate section to prepare your tissue.

Tissue preparation

FFPE tissue preparation

1. Bake the slides (optional)

Note

Baking tissue samples at 60°C for 1 hour can promote tissue adhesion to sample slides. If you are making your own FFPE sample, this step may be required. If purchasing FFPE samples, please check with the provider to see if baking has already been conducted.

Deparaffinize the slides

1. Deparaffinize with xylene and rehydrate with ethanol according to standard protocols.

This protocol is continued on page 100

ViewRNA tissue fluorescence assay protocol, cont.

Perform heat pretreatment

1. Cover the 1X Pretreatment Solution beaker tightly with aluminum foil and place on a hot plate. Heat to 90–95°C.
2. Place slides in a vertical slide rack and submerge into the heated 1X Pretreatment Solution using forceps. Cover the glass beaker with aluminum foil and incubate at 90–95°C for optimal time.
3. Remove slide rack with forceps and submerge slides into dd H_2O using a staining dish containing 200 mL of dd H_2O. Wash for 1 minute with frequent agitation.
4. Decant and repeat the wash with 200 mL of fresh dd H_2O.
5. Transfer the slide rack to a staining dish containing 1X PBS. Proceed to “Draw hydrophobic barrier” section.

Note

For guidelines, refer to user guide, page 29 “ Pretreatment optimization procedure”

Critical note

Do not let the tissue sections dry out from this point forward. After heat pretreatment, sections can be stored covered in 1X PBS at room temperature overnight.

Store slides (optional)

1. After heat pretreatment, slides can be stored overnight. Store slides in a clear staining dish containing 200 mL of Storage Buffer at room temperature for up to 24 hours. Cover the dish with a lid or sealing film to prevent evaporation.

Cryopreserved tissue preparation

Prepare compound-embedded tissue

1. Add tissue sections to pre-chilled 4% formaldehyde (Image-iT fixative solution) and fix overnight (16–19 hours) at 4°C.
2. Wash the slides with 200 mL 1X PBS for 1 minute. Proceed to “Draw hydrophobic barrier” section.

This protocol is continued on page 101

Store slides (optional)

1. After fixation, slides can be stored overnight. Store slides in a clear staining dish containing 200 mL of Storage Buffer at room temperature for up to 24 hours. Cover the dish with a lid or sealing film to prevent evaporation.

Draw hydrophobic barrier

1. Use a Pap Pen to draw a hydrophobic barrier around the tissue section. Lightly trace the barrier 2 to 4 times. Cover tissue with 100–200 µL 1X PBS, enough to cover the sample completely without touching the hydrophobic barrier to ensure the tissue does not dry out.

2. Allow the barrier to dry at room temperature for 15–20 minutes.

3. Proceed to “Protease digestion and fixation” section.

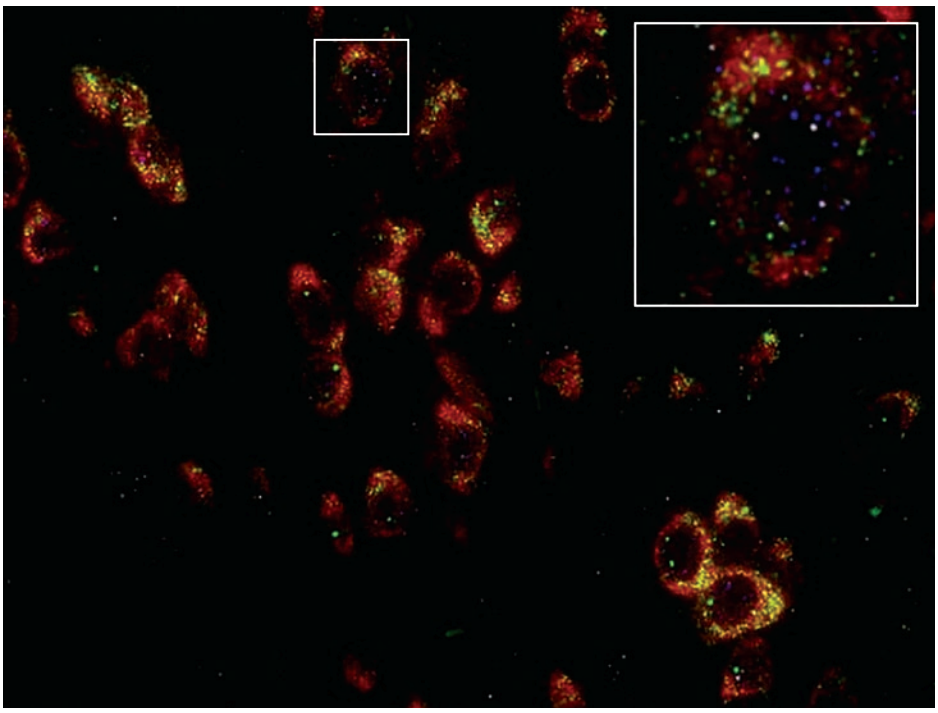


Figure 23. RNA-ISH in mouse brain tissue using ViewRNA ISH Tissue Assay. Mouse brain (FFPE) tissue was processed and Peptidyl-prolyl cis-trans isomerase B (PPIB), glutamate decarboxylase 2 (GAD2), RNA polymerase II subunit A (POLR2A), and GAPDH mRNA was labeled using ViewRNA probe sets. These probe sets were detected using ViewRNA ISH Tissue Assay while using Type 4 Alexa Fluor 488 for GAD2 (shown in green), Type 1 Alexa Fluor 594 for GAPDH (shown in red), Type 6 Alexa Fluor 647 for PPIB (shown in blue) and Type 10 Alexa Fluor 750 for POLR2A (shown in white). Fluorescent image was taken using EVOS M7000 imaging system and post-processed for visualization.

This protocol is continued on page 102

ViewRNA tissue fluorescence assay protocol, cont.

Protease digestion and fixation

1. Prepare the working protease solution by diluting the Protease Solution 1:100 in prewarmed 1X PBS and briefly vortex.

Note

Ensure that 1X PBS is pre-warmed to 40°C before starting this procedure.

2. Remove each slide from rack and flick to remove excess 1X PBS.

3. Place slides face up be face up in slide chamber (such as StainTray) and immediately add 400 µL of the working Protease Solution on the tissue section, making sure tissue section is covered.

4. Transfer slides to the dry oven and incubate at 40°C for optimal time. See user guide for tissue type and optimal incubation time guidance

5. Decant the working protease solution from the slides, wash the slides 200 mL 1X PBS staining dish and gently agitate for 1 min. Repeat the wash 1 more time with fresh 1X PBS.

6. Transfer the slide rack to a staining dish containing 200 mL of fixation solution and fix for 5 minutes at room temperature under fume hood.

7. Wash the slides with 200 mL of fresh 1X PBS for 1 minute with frequent agitation.

Target probe set hybridization

Note

Ensure Probe Set Diluent is prewarmed to 40°C before starting this procedure. Prepare sufficient volume to add 400 µL per sample.

1. Prepare the working probe set solution by diluting the ViewRNA Probe Sets 1:40 in prewarmed Probe Set Diluent. Dilute 10 µL of each target probe set in prewarmed Probe Set Diluent to a reach total volume of 400 µL per slide. Vortex briefly to mix. See Table 3 in the Appendix for suggested dilutions for 1–4 plex assays.

Note

For low-abundance targets, dilute target probe(s) 1:20 or 1:30. Remove each slide from rack and flick to remove excess 1X PBS.

2. Add up to 400 µL of the pre-warmed Probe Set Diluent to the negative control and up to 400 µL of working probe set solution to each test sample.

3. Transfer the slides to the dry oven and incubate at 40°C for 2 hours.

4. Decant the working probe set solution from the slides and submerge slides into a slide rack containing Wash Buffer.

This protocol is continued on page 103

5. Wash the slides 3 times with fresh 200 mL Wash Buffer at room temperature for 2 minutes with constant agitation.

6. Proceed to “Store slides” if you plan to perform the assay over two days, otherwise proceed to “Amplify and detect signal”.

Store slides (optional)

1. Store slides in a clear staining dish containing 200 mL of Storage Buffer at room temperature for up to 24 hours. Cover the dish with a lid or sealing film to prevent evaporation.

Amplify and detect signal

Note

Before beginning this step, make sure reagents for amplification and detection have been warmed to 40°C.

Wash stored slides (skip for one-day protocol)

1. Remove from Storage Buffer. Transfer the rack to a clear staining dish containing Wash Buffer, and wash for 2 minutes with frequent agitation.

2. Decant Wash Buffer and wash again with 200 mL of fresh Wash Buffer for 2 minutes with frequent agitation. Repeat the wash 1 more time with fresh Wash Buffer.

Perform signal amplification

Note

Ensure that Amplifier Diluent QF is pre-warmed to 40°C before starting this procedure.

1. Prepare working Pre-Amplifier Mix solution by mixing and then diluting 1:25 in pre-warmed Amplifier Diluent QF.

2. Remove each slide and flick to remove Wash Buffer. Place slides face up be face up in slide chamber (such as StainTray) and immediately add 400 µL of the working Pre-Amplifier Mix on the tissue section, making sure tissue section is covered.

3. Transfer the slides to the hybridization system and incubate at 40°C for 30 minutes.

4. Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.

This protocol is continued on page 104

ViewRNA tissue fluorescence assay protocol, cont.

5. Decant the Pre-Amplifier Mix from the slides and insert them into the slide rack in the Wash Buffer.

6. Wash the slides at room temperature for 2 minutes with constant agitation. Repeat this step with fresh Wash Buffer for a total of 3 washes.

7. Prepare the working Amplifier Mix solution by briefly swirling and diluting 1:25 in pre-warmed Amplifier Diluent QF.

8. Remove each slide and flick to remove Wash Buffer. Place slides face up be face up in slide chamber (such as StainTray) and immediately add up to 400 µL of the Amplifier Mix on the tissue section, making sure tissue section is covered.

9. Transfer the slides to the hybridization system and incubate at 40°C for 30 minutes.

10. Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.

11. Decant the Amplifier Mix from the slides and insert them into the slide rack in the Wash Buffer.

12. Wash the slides at room temperature for 2 minutes with constant agitation. Repeat this step with fresh Wash Buffer for a total of 3 washes.

This protocol is continued on page 105

Perform label probe hybridization

1. Prepare working Label Probe Mix solution by briefly swirling and diluting label probes 1:25 in prewarmed Label Probe Diluent QF. Dilute 16 µL of each label probe in prewarmed Label Probe Diluent to reach a total volume of 400 µL per slide. Vortex briefly to mix. See Table 4 in the Appendix for suggested dilutions for 1–4 plex assays.

2. Remove each slide and flick to remove Wash Buffer. Place slides face up be face up in slide chamber (such as StainTray) and immediately add 400 µL of the pre-warmed working Label Probe Mix on the tissue section, making sure tissue section is covered.

3. Transfer slides to the hybridization system and incubate at 40°C for 30 minutes.

4. Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.

5. Decant the Label Probe Mix from the slides and insert them into the slide rack in the Wash Buffer.

6. Wash the slides at room temperature for 2 minutes with constant agitation. Repeat this step with fresh Wash Buffer.

7. Perform a final wash with Wash Buffer for 10 minutes with frequent agitation. Do not wash longer than 30 minutes.

8. Proceed to “Counterstain and mount for detection”.

Note

Ensure that Label Probe Diluent QF is pre-warmed to 40°C before starting this procedure.

This protocol is continued on page 106

ViewRNA tissue fluorescence assay protocol, cont.

(Optional): Reduce tissue autofluorescence

1. Remove each slide from the Wash Buffer, then flick to remove excess Wash Buffer.
2. Wash the slides three times with fresh 1X PBS for 3 minutes at room temperature with frequent agitation.
3. Prepare the ReadyProbes Tissue Autofluorescence Quenching Kit by combining equal volumes of Components A, B, and C.
4. Add 400 µL of the prepared ReadyProbes solution to each sample, then incubate for 5 minutes at room temperature.
5. Wash the slides three times with fresh 1X PBS for 3 minutes at room temperature with frequent agitation.
6. Proceed to “Counterstain and mount for detection”

Counterstain and mount for detection

1. Counterstain the tissue with DAPI using standard protocols.
2. Mount the coverslips using a mountant with antifade properties. ProLong Glass reagent is recommended for hard-set mounting and long-term archiving of tissue samples. SlowFade Glass reagent is recommended for soft-set mounting if coverslip removal is required.
3. Analyze the tissue using a compatible imaging system such as inverted fluorescent microscope or HCS platform.

Note

For the ViewRNA Tissue Fluorescence Assay, mounting media with antifade are highly recommended.

Note

For optimal results, follow the instructions provided with the mounting reagent.

This protocol is continued on page 107

Appendix for ViewRNA Tissue Fluorescence Assay Protocol

Table 1. Definition of Fluorescence Probe set types. Spectral properties of these fluorophores can be found in our online fluorophore selection guides.

Probe type	Detection label	Fluorescent readout
1	Alexa Fluor 594	Texas Red
1	Alexa Fluor 546	TRITC/RFP
4	Alexa Fluor 488	FITC/GFP
6	Alexa Fluor 647	Deep Red/Cy5
10	Alexa Fluor 750	Near-infrared/Cy7

Table 2. ViewRNA Tissue fluorescence kit and module options.

ViewRNA Tissue Fluorescence Kit options	Assay size
ViewRNA Tissue Fluorescence 4-Plex Assay Core Kit (green, orange, deep red, near IR)	QVT0700 (24 assays) QVT4700 (96 assays)
ViewRNA Tissue Fluorescence 1-Plex Assay Core Kit	
ViewRNA Tissue Alexa Fluor 488 (Type 4) Module	
ViewRNA Tissue Alexa Fluor 546 (Type 1) Module	
ViewRNA Tissue Alexa Fluor 750 (Type 10) Module	
ViewRNA Tissue Fluorescence 4-Plex Assay Core Kit (green, red, deep red, near IR)	QVT0800 (24 assays) QVT4800 (96 assays)
ViewRNA Tissue Fluorescence 1-Plex Assay Core Kit	
ViewRNA Tissue Alexa Fluor 488 (Type 4) Module	
ViewRNA Tissue Alexa Fluor 594 (Type 1) Module	
ViewRNA Tissue Alexa Fluor 750 (Type 10) Module	
ViewRNA Tissue Fluorescence 1-Plex Assay Core Kit with Alexa Fluor 647 (Type 6)	QVT0600C (24 assays) QVT4600C (96 assays)
ViewRNA Tissue Fluorescence Module options†	**Assay size**
ViewRNA Tissue Alexa Fluor 488 (Type 4) Module	QVT0688B (24 assays) QVT4688B (96 assays)
ViewRNA Tissue Alexa Fluor 546 (Type 1) Module	QVT0646B (24 assays) QVT4646B (96 assays)
ViewRNA Tissue Alexa Fluor 594 (Type 1) Module	QVT0694B (24 assays) QVT4694B (96 assays)
ViewRNA Tissue Alexa Fluor 750 (Type 10) Module	QVT0640B (24 assays)

† Modules can be added to the ViewRNA Tissue Fluorescence 1-Plex Assay Core Kit with Alexa Fluor 647 (Type 6) for 2- to 4-plex detection.

This protocol is continued on page 108

ViewRNA tissue fluorescence assay protocol, cont.

Appendix for ViewRNA Tissue Fluorescence Assay Protocol, cont.

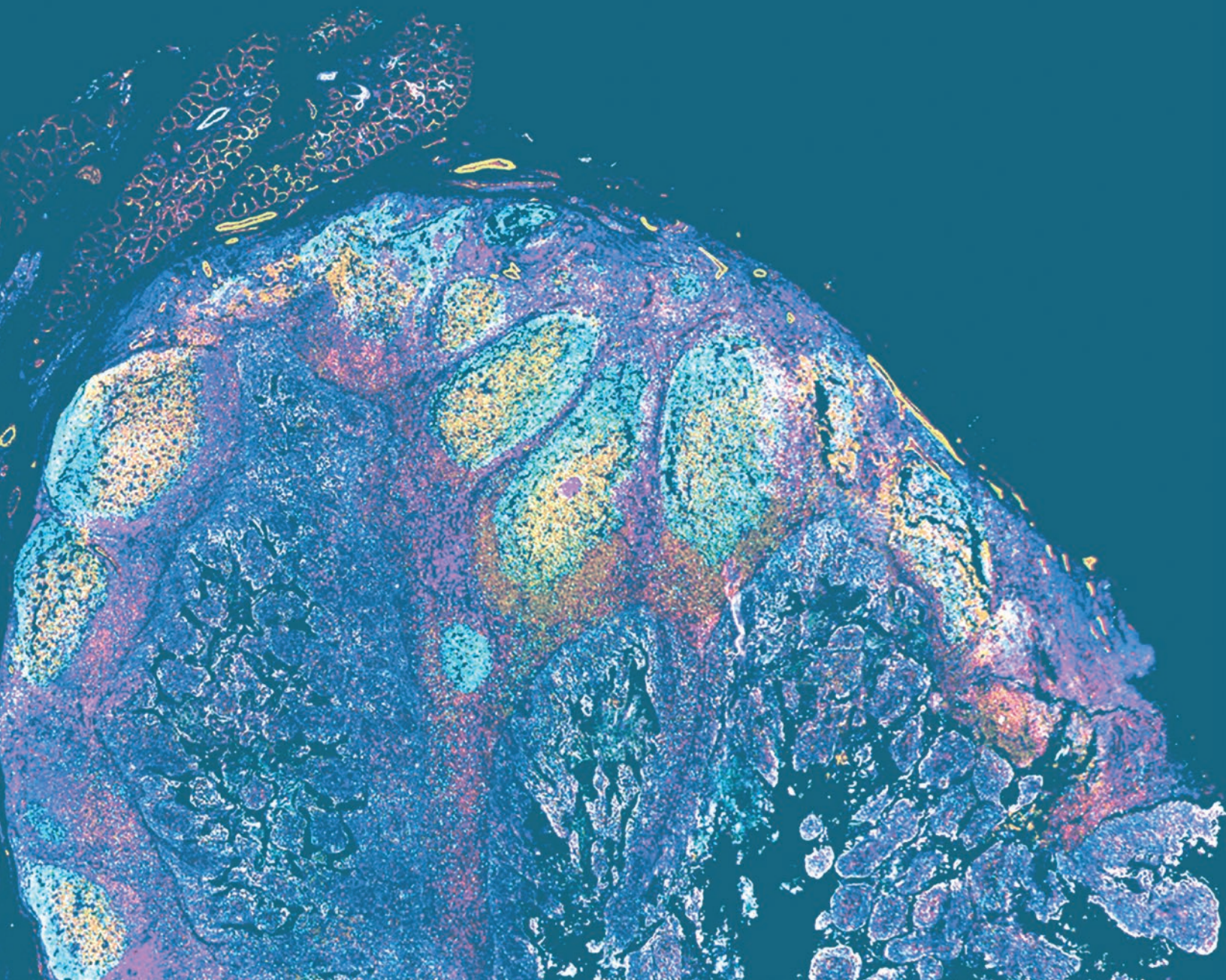
Table 3. Examples of Target Probe set dilutions.

Reagent	1-plex (400 µL/slide total volume)	2-plex (400 µL/slide total volume)	3-plex (400 µL/slide total volume)	4-plex (400 µL/slide total volume)
Probe Set Diluent (prewarmed to 40°C)	390 µL	380 µL	370 µL	360 µL
Target Probe 1	10 µL	10 µL	10 µL	10 µL
Target Probe 2	—	10 µL	10 µL	10 µL
Target Probe 3	—	—	10 µL	10 µL
Target Probe 4	—	—	—	10 µL

Table 4. Suggested Label Probe dilutions.

Reagent	1-plex (400 µL/slide total volume)	2-plex (400 µL/slide total volume)	3-plex (400 µL/slide total volume)	4-plex (400 µL/slide total volume)
Label Probe Diluent QF (prewarmed to 40°C)	384 µL	368 µL	352 µL	336 µL
Label Probe Mix (25X) (Type 6)	16 µL	16 µL	16 µL	16 µL
Label Probe 2	—	16 µL	16 µL	16 µL
Label Probe 3	—	—	16 µL	16 µL
Label Probe 4	—	—	—	16 µL

Cell painting



Cell painting

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 24). Collectively, this panel requires an image acquisition configuration of at least five fluorophores and fluorescence channels to capture the eight markers.

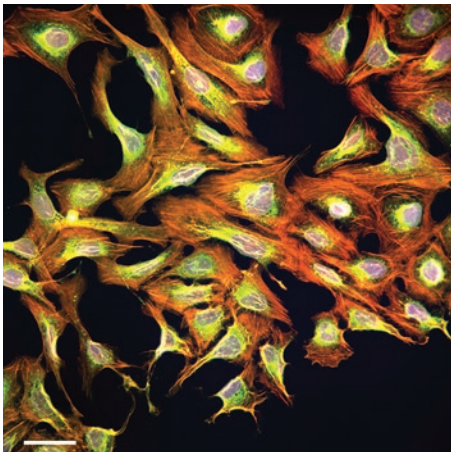


Figure 24. Cell painting screening of U2OS cells. The cells were labeled using the Image-iT Cell Painting Kit, and analyzed on the CellInsight CX7 LZR Pro HCS Platform with a 20X 0.7 NA objective to screen for nuclei (channel 1), endoplasmic reticulum and cytoplasmic RNA and nucleoli (channel 2), Golgi 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 110

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 ddH_2O 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 ddH_2O 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).

Note

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

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% CO_2, and ~95% relative humidity to allow overnight recovery and growth of the plated cells.

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 111

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% CO_2, and ~95% relative humidity. To avoid DMSO vehicle effects, be sure to keep the final DMSO concentration below 0.5%.

Critical note

Do not remove the medium.

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% CO_2, 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.

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

11. Incubate for 30 minutes at room temperature.

This protocol is continued on page 112

Cell painting, cont.

12. Discard solution and wash twice with 1X HBSS.

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

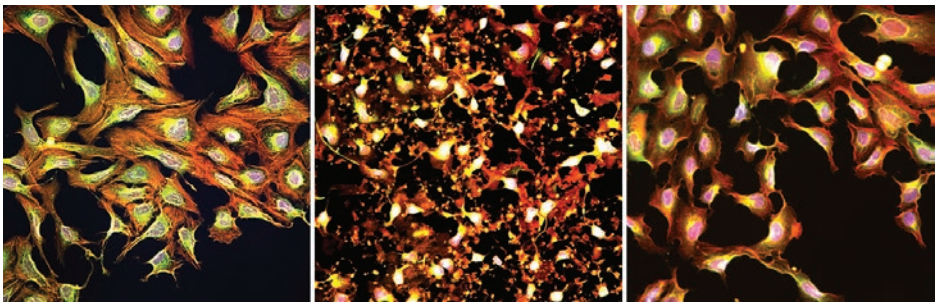
14. Tightly seal the plates with adhesive seals. Plates are now ready for screening.

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 25.



Untreated | 10 µM Staurosporine | 1 µM Taxol

Figure 25. Phenotypic comparisons of untreated versus pharmacological control exposure in U2OS cells. Cells were treated with compounds of interest at 1–100 µM final concentrations for 48 hours in 96-well imaging plates. After compound treatment, cells were immediately labeled using the using the Image-iT Cell Painting Kit and analyzed using the CellInsight CX7 LZR Pro HCS Platform.

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