

A OneStep Solution to Fix and Stain Cells for Correlative Live and Fixed Microscopy

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Correlating the location of subcellular structures with dynamic cellular behaviors is difficult when working with organisms that lack the molecular genetic tools needed for expressing fluorescent protein fusions. Here, we describe a protocol for fixing, permeabilizing, and staining cells in a single step while imaging on a microscope. In contrast to traditional, multi-step fixing and staining protocols that take hours, the protocol outlined here achieves satisfactory staining within minutes. This approach takes advantage of well-characterized small molecules that stain specific subcellular structures, including nuclei, mitochondria, and actin networks. Direct visualization of the entire process allows for rapid optimization of cell fixation and staining, as well as straightforward identification of fixation artifacts. Moreover, live imaging prior to fixation reveals the dynamic history of cellular features, making it particularly useful for model systems without the capacity for expressing fluorescent protein fusions. © 2021 The Authors. Current Protocols published by Wiley Periodicals LLC.

Basic Protocol: Fixing, permeabilizing, and staining mammalian cells in one step on the microscope

Keywords: cell biology • correlative microscopy • cytoskeleton • live imaging • microscopy • staining

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INTRODUCTION

Cell staining is a widely used technique in cell biology that allows visualization of cellular structures, often in fixed cells. Protocols for fixing and staining generally require multiple steps that take hours to complete, and preclude the association of cell dynamics with staining results. Here, we present a protocol for imaging cells while fixing and staining by adding a single “OneStep” solution containing a fixative, a detergent, and fluorescent dyes. This combined fixation and staining protocol has three main advantages compared to traditional staining protocols: (1) it saves time—we can achieve satisfactory staining in minutes instead of hours; (2) it allows direct comparison of the same cell before and after staining—this means that any artifacts due to buffers, detergents,

or fixatives are immediately apparent, allowing for quick optimization; and (3) cellular structures and behaviors observed prior to fixation are easily correlated with subcellular localization information obtained by staining. This ability to correlate subcellular localization with cellular function makes this protocol especially advantageous for studies of non-traditional model organisms that lack the capacity for expressing fluorescent protein fusions.

Here, we provide a detailed protocol for performing a OneStep fixation and staining of mammalian cells while filming. We outline steps for optimizing this protocol for use with other cell types, and describe the modifications we made for its use with *Naegleria gruberi* amoebae and the chytrid fungus *Batrachochytrium dendrobatidis*.

CAUTION: This protocol involves the use of paraformaldehyde (PFA)–containing solutions. Please consult with your institution’s environmental health and safety department, as recommendations for working with PFA may vary. If appropriate, this hazard can be mitigated by using a microscope that fits inside a chemical fume hood (see Figure S1 in Supporting Information).

BASIC PROTOCOL

FIXING, PERMEABILIZING, AND STAINING MAMMALIAN CELLS IN A SINGLE STEP ON THE MICROSCOPE

Materials

Cells

The type of cells can vary. In the example shown in Figure 1, we used an LLC-Pk1 pig kidney epithelial cell line.

0.25% trypsin-EDTA (ThermoFisher; 25200-056) if needed to detach adherent cells

Cell culture medium

The type of medium can vary. In the example shown in Figure 1, we grew the cells in a 1:1 mixture of Opti-MEM and F-10 medium supplemented with 7.5% fetal bovine serum and 1% antibiotic/antimycotic.

OneStep Solution (optimized for LLC-Pk1 pig kidney epithelial cells; see recipe)

Type B immersion oil for microscopy (Cargille; 16484) or similar immersion oil

Imaging plate: 96-well glass-bottom plate (Brooks; MGB096-1-2-LG-L), or similar glass bottom dish

1.5-ml microcentrifuge tubes (Capp; 5101505, or similar)

Micropipettes and micropipette tips (P-2.5, P-20, P-200, P-1000)

Fluorescence microscope with a camera capable of time-lapse imaging.

For Figures 1-3, we used a Nikon Ti2 eclipse equipped with a Plan Apo λ 100 $\times$ oil objective (1.45 NA), a Crest spinning disk (50 μ m), a Prime 95B CMOS camera, and a Spectra III/Celesta light source (with excitation wavelengths of 405, 477, 546, and 638 nm). This microscope was controlled through NIS Elements software.

Chemical hood for preparing paraformaldehyde solutions

Tissue culture hood for seeding mammalian cells (any Class II Type A2 Biological Safety Cabinet)

A humidified incubator set to 37°C + 5% CO₂

1. Seed cells into imaging plates or dishes.

Follow your preferred protocol for seeding cells, choosing a seeding time that ensures that the cells are displaying the desired behaviors during imaging. In our example, the day before the experiment, we lifted cells from a subconfluent 25-cm² culture flask using 200 μ l of 0.25% trypsin-EDTA. Once detached, cells were suspended in 1000 μ l of medium, and 20 μ l of this suspension were added to wells of a 96-well plate containing 150 μ l/well of medium. We then allowed the cells to settle for ~24 hr in an incubator at 37°C,

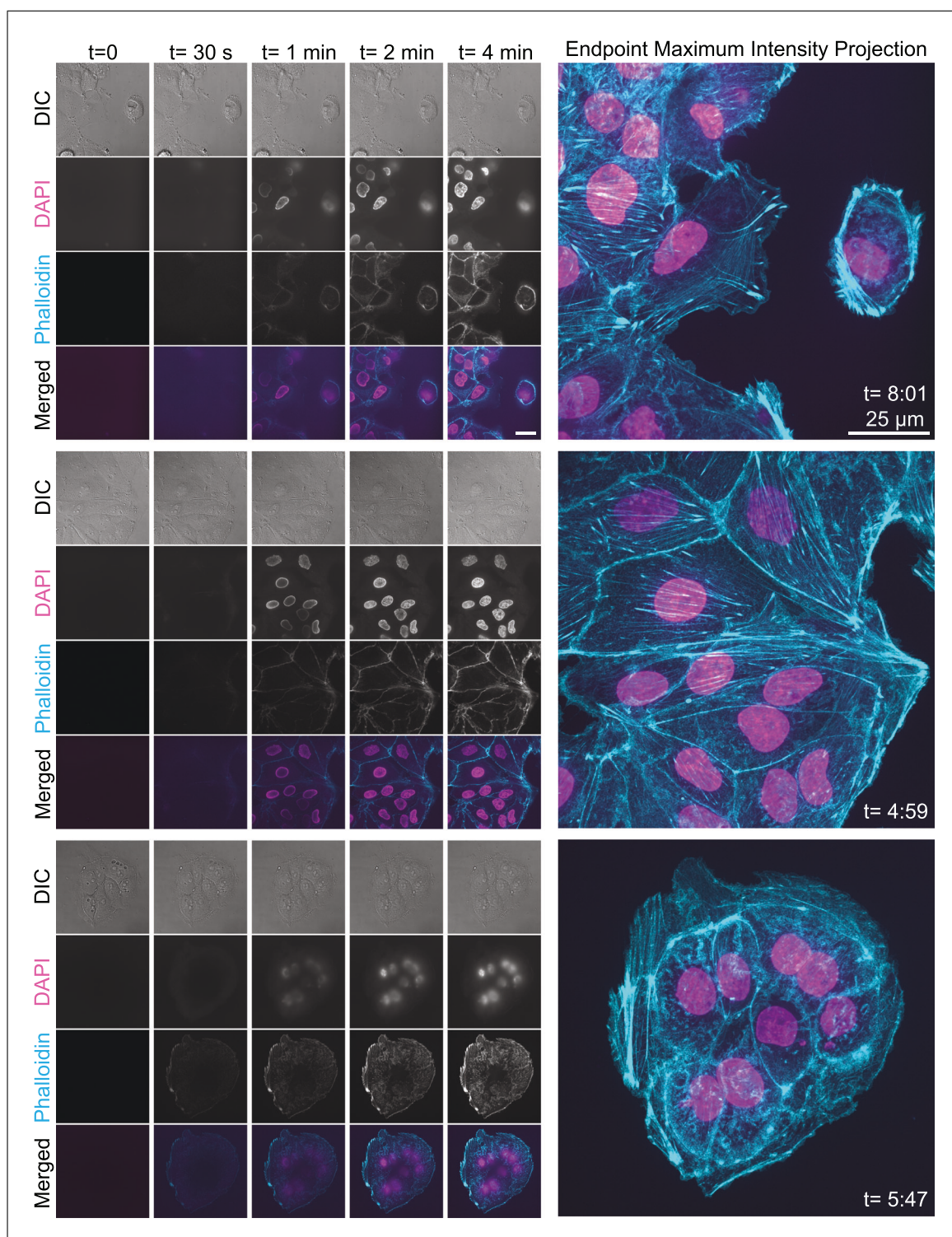


Figure 1 Robust staining of DNA and polymerized actin occurs within minutes of adding OneStep Solution. Mammalian cells were imaged prior to (before and including $t = 0$) and during a time course with $2\times$ OneStep solution added to an equal volume of cells in $1\times$ PBS. Cells were imaged until the phalloidin fluorescence signal plateaued, at which time the field of view was imaged as a z-stack to record multiple focal planes within the sample. Maximum intensity projections from these z-stacks are shown on the right, with endpoint times given in min, and scale bars showing $25\text{ }\mu\text{m}$. Representative timepoints are shown from three replicates. DAPI staining of DNA is shown in magenta, and phalloidin staining of actin polymer is shown in cyan.

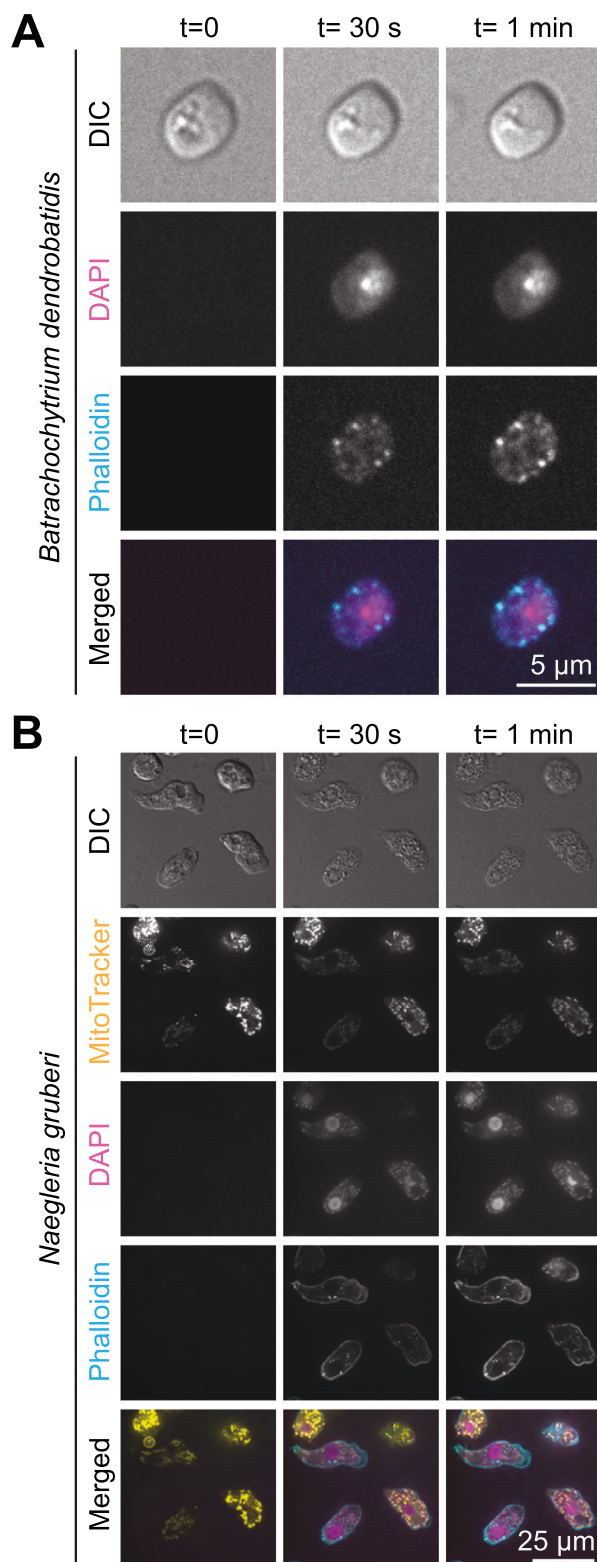


Figure 2 OneStep fixing and staining is adaptable to different cell types. **(A)** *Batrachochytrium dendrobatidis* and **(B)** *Naegleria gruberi* cells were imaged prior to treatment (before and including $t = 0$), and throughout the fixation/staining process, during which the $2\times$ solution was added to an equal volume of cells in water. Representative cells are shown 30 and 60 s after the addition of the OneStep solution. DAPI staining of DNA is shown in magenta, and phalloidin staining of actin polymer is shown in cyan. *Naegleria gruberi* cells were treated with MitoTrackerTM Red (displayed in yellow) prior to imaging.

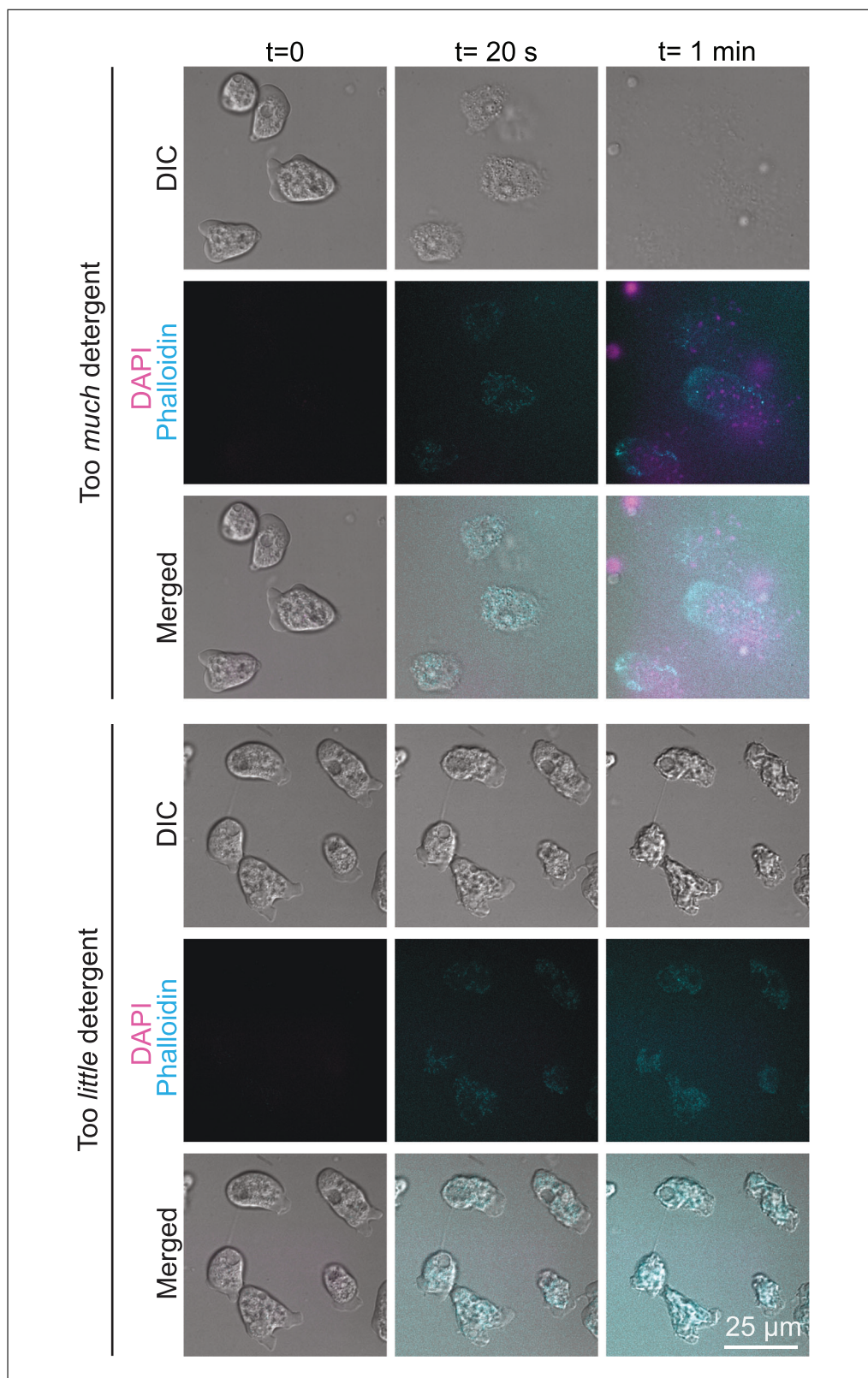


Figure 3 *Naegleria gruberi* cells were treated with OneStep solutions containing too much detergent (top, 0.2% Triton X-100) or too little detergent (bottom, 0.02% Triton X-100). Note that nuclei float away to unknown destinations when too much detergent is added, while cells shrivel like raisins when treated with too little detergent.

with 5% CO₂. We recommend seeding at least 10 wells during initial optimization of new cell lines to allow for troubleshooting. Cells can also be seeded into other types of dishes or plates, provided that they are suitable for imaging. For optimal optics, we recommend glass coverslip-bottom dishes.

2. Prepare a “2×” OneStep solution on the day of the experiment.

If the protocol has not yet been optimized for your desired cell type, make up a few different formulations of OneStep solution, varying the concentration of paraformaldehyde (PFA) and detergent (Triton X-100 or NP-40 Alternative). As a starting point, we recommend testing the concentrations you typically use for fixing and staining, keeping in mind that the fixatives/detergents/dyes will need to be at twice their final concentrations (“2×”) because the OneStep mixture will be added to an equal volume of buffer and cells in the well. For example, if you typically use a fixation buffer of PBS + 3.7% PFA, a permeabilization buffer of PBS + 0.1% Triton X-100, and a staining solution of 1 μg/ml DAPI + 66 nM labeled phalloidin in PBS, make up a solution of: 1× PBS + 7.4% PFA + 0.2% Triton X-100, 2 μg/ml DAPI, and 132 nM labeled phalloidin. Also make up formulations with higher and/or lower concentrations of PFA and Triton X-100 (we have found that lower levels of detergent are usually more effective—here, 0.05% Triton X-100 was optimal).

3. Gently aspirate the medium out of the wells.

Pipette or aspirate the medium out of the corner of the well while tilting the plate slightly to remove as much medium as possible without dislodging the cells.

4. Gently add 150 μl of PBS to each well.

Do this immediately following step 3, so as not to let the cells dry out (this is critical). If your cell type does not tolerate PBS, test your preferred buffer. For long-term imaging of mammalian cells, we recommend waiting until step 5, then gently removing medium and replacing it with PBS on the microscope before step 6, or testing a buffer (other than the growth medium) that is preferable for longer incubations (e.g., HBSS). If you seeded cells into a different type of dish, add an amount of buffer up to half of the volume you typically use for that dish/well type.

5. Begin imaging the cells using fluorescence and DIC or phase/contrast microscopy as a time series.

Be sure to use the appropriate filters for the dyes you are using. In our example, we used DAPI and TRITC fluorescence channels. After imaging the first well, optimize the exposure settings before moving to the next well. We recommend a time window of 5-20 s between frames.

6. After a few minutes (or as long as you would like to observe the live cells), add 150 μl of the appropriate OneStep solution to the imaging well. Continue imaging until you are satisfied with the staining intensity. We typically image until the fluorescence signal plateaus.

When the time series is complete, it can be helpful to image the cells as a z-stack to observe structures outside of the initial focal plane. See Figure 1 for examples of representative time points and maximum intensity projections of z-stacks, and Figure 2 for additional cell types.

REAGENTS AND SOLUTIONS

Phosphate-buffered saline (PBS) 10×

80 g/L NaCl (Fisher; BP358-1)
2 g/L KCl (Fisher; P217-500)
14.4 g/L Na₂HPO₄ (Sigma Aldrich; S9763-1KG)
2.4 g/L KH₂PO₄ (Sigma Aldrich; P0662-1KG),
Adjust pH to 7.4
Bring up volume to 1 L

Autoclave
Store at room temperature

OneStep Solution (to make 1 ml, optimized for LLC-Pk1 pig kidney epithelial cells)

532.5 μ l of 1 $\times$ PBS (make 1 $\times$ from 10 $\times$ stock; see recipe)
5 μ l of 10% Triton X-100 (Promega; H5142) or NP-40 Alternative (Millipore 492016-500 ml); make a 10% solution in PBS, vortex to mix (final concentration: 0.05%)
462.5 μ l of 16% paraformaldehyde (PFA; Electron Microscopy Sciences; final concentration, 7.4%)
2 μ l of 1 mg/ml DAPI (4',6-diamidino-2-phenylindole; Life Technologies; D1306; final concentration 2 μ g/ml)
4 μ l of $\sim$ 66 μ M Alexa FluorTM 568 Phalloidin (Invitrogen; A12380) in DMSO (final concentration 132 nM); or substitute another labeled phalloidin
Protect from light, use the same day

CAUTION: Paraformaldehyde should be added in a fume hood to avoid inhalation. Please consult with your institution's environmental health and safety division, as recommendations for best practices for working with PFA vary.

COMMENTARY

Background Information

Distinct types of microscopy have unique advantages for visualizing cell structures and/or behaviors. For example, light microscopy can be used to observe the behavior of virtually any cell type, while electron microscopy can reveal subcellular structures at much higher resolution in fixed cells. To take advantage of more than one type of microscopy at a time, cell biologists have developed several techniques to correlate data from multiple imaging modalities (Fernandes, Saavedra-Villanueva, Margeat, Milhiet, & Costa, 2020; Gómez-Varela et al., 2020; Navikas et al., 2021; Phillips et al., 2020). For example, Correlative Light and Electron Microscopy (CLEM) allows direct association of light microscopy data (e.g., fluorescent protein localization) with higher-resolution electron microscopy (reviewed in de Boer, Hoogenboom, & Giepmans, 2015). Additionally, protocols for conducting *in situ* immunofluorescence on a microscope allow biologists to correlate live cell imaging data with that from super-resolution microscopy (Bálint, Verdeny Vilanova, Sandoval Álvarez, & Lakadamyali, 2013; Tam et al., 2014).

Cells are densely packed with organelles, proteins, and a plethora of small molecules. To allow detection of specific cellular structures within this teeming mass, cell biologists have also developed a variety of dyes and staining procedures. These staining procedures typically include a series of distinct steps, which take hours. Although this approach is appropriate for many applications, some cell

dyes do not require such complex protocols, and can label subcellular structures in a single step (Taylor, 1980).

We developed this OneStep protocol to combine the simplicity of single-step staining with correlative microscopy. This approach allows rapid association of live cell behavior with the visualization of underlying cellular structures. We recently employed this protocol to determine that actively growing, filopodia-like structures in *Naegleria gruberi* were filled with polymerized actin (Velle & Fritz-Laylin, 2020). While particularly advantageous for this and other emerging model systems that lack robust genetic tools, we also use this OneStep staining protocol to rapidly correlate behaviors and structures in genetically tractable systems.

Critical Parameters

To adapt this protocol to a new cell type, titrating detergent and fixative concentrations is key. Too *much* detergent dissolves membranes rapidly, causing cytoplasm and entire organelles to leak out and drift away before they can be crosslinked in place (Fig. 3, top). Too *little* detergent leaves cells impermeable to dyes, and can cause fixation artifacts such as shrivelling (Fig. 3, bottom). Because fixative concentration can also influence these outcomes, we advise setting up a parameter matrix to empirically determine the appropriate concentration of both detergent and fixative for each cell type. We suggest starting with at least three detergent concentrations between $\sim$ 0.005% and $\sim$ 0.1%, and three

Table 1 Troubleshooting Guide for Live Fixation/Staining

Problem	Possible cause	Solution(s)
Cytosol spills out of cells (Fig. 3, top)	Too much detergent or too little fixative	Try using detergent at half the strength first. If the problem persists, and further adjustments of detergent concentration do not resolve the issue, also try a higher concentration of PFA.
Cells shrivel	Not enough detergent, or the osmotic strength of the buffer is too high	Try using a higher concentration of detergent and less PFA, and/or adjust the buffer composition to lower the osmolality
Cells shrivel and do not stain (Fig. 3, bottom)	Not enough detergent	Try using higher concentrations of detergent
Cells show signs of phototoxicity before fixation	Illumination used to detect fluorescent signal is damaging cells	Set the software to only image every other frame with fluorescence, and/or adjust the exposure settings to allow the use of less light intensity

Table 2 Successful OneStep Solution Parameters (given as 2×) for Selected Cell Types

Cell type	Buffer system	Fixative	Detergent	Staining
Mammalian cells (LLC-Pk1 pig kidney epithelial cells, Fig. 1)	PBS	7.4% PFA	0.05% Triton X-100	2 µg/ml DAPI + 132 nM labeled phalloidin
Amoeboid cells (<i>Naegleria gruberi</i> , NEG-M, Fig. 2B)	130 mM sucrose + 50 mM sodium phosphate buffer, pH 7.2	3.6% PFA	0.025% NP-40 Alternative	
Chytrid fungi (<i>Batrachochytrium dendrobatidis</i> , Fig. 2A)	65 mM sucrose + 25 mM sodium phosphate buffer, pH 7.2	4% PFA	0.1% NP-40 Alternative	

dilutions of PFA ranging from 1.8% to 8%. See Table 1 for additional troubleshooting information, and Table 2 for example parameters that were successful in our experiments with three distinct cell types.

Another critical aspect of this protocol is even mixing of the OneStep solution with the sample. We use a “2×” concentration of OneStep solution so that we can add an equal volume to the sample, which provides adequate mixing. Adding a smaller volume of a higher concentration may result in fixative and detergent concentrations that are locally concentrated or diluted. This variability will make optimization difficult.

In addition to the concentration and mixing of OneStep solution, consideration should

also be given to the suitability of the sample for this type of protocol. Samples that are thick (e.g., embryos, tissue slices) may require additional time for the solution to penetrate, and may demand further optimization. Cells that are weakly adherent or non-adherent may also detach or drift when the solution is added. We have avoided this by using tissue culture–treated glass for *Naegleria* cells, and concanavalin A–coated glass for *B. dendrobatidis*.

Troubleshooting

Table 1 lists problems that may arise with the protocol along with their possible causes and solutions.

Understanding Results

We have used this protocol to visualize actin polymer networks and DNA in three cell types: mammalian (pig) epithelial cells (Fig. 1), a chytrid fungus (*Batrachomyces dendrobatidis*; Fig. 2A), and the amoeba *Naegleria gruberi* (Fig. 2B). We have included specific OneStep formulations for each in Table 2. We have also successfully applied this protocol to *Naegleria gruberi* cells pre-incubated with fixable MitoTracker (MitoTracker™ Red CM-H2Xros; Invitrogen M7513) to visualize mitochondria in living and fixed cells (Fig. 2B).

Time Considerations

After optimization, OneStep fixing and staining of pre-seeded cells can be fully executed in well under an hour. When optimizing for a new cell line, it may be more efficient to prepare several formulations of “OneStep solution” with varying concentrations of fixative and detergent for testing in parallel.

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Author Contributions

Katrina B. Velle: conceptualization, formal analysis, funding acquisition, investigation, methodology, visualization, writing original draft, writing review and editing; **Carline Fermino do Rosario:** investigation, resources, validation, writing review and editing; **Patricia Wadsworth:** resources, writing review and editing; **Lillian K. Fritz-Laylin:** conceptualization, funding acquisition, methodology, project administration, resources, supervision, writing original draft, writing review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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