Chapter 2

General Staining and Segmentation Procedures for High Content Imaging and Analysis

Kevin M. Chambers, Bhaskar S. Mandavilli, Nick J. Dolman, and Michael S. Janes

Abstract

Automated quantitative fluorescence microscopy, also known as high content imaging (HCI), is a rapidly growing analytical approach in cell biology. Because automated image analysis relies heavily on robust demarcation of cells and subcellular regions, reliable methods for labeling cells is a critical component of the HCI workflow. Labeling of cells for image segmentation is typically performed with fluorescent probes that bind DNA for nuclear-based cell demarcation or with those which react with proteins for image analysis based on whole cell staining. These reagents, along with instrument and software settings, play an important role in the successful segmentation of cells in a population for automated and quantitative image analysis. In this chapter, we describe standard procedures for labeling and image segmentation in both live and fixed cell samples. The chapter will also provide troubleshooting guidelines for some of the common problems associated with these aspects of HCI.

Key words High content screening, High content imaging, CellMask, Segmentation, Nuclear segmentation, CellTracker

1 Introduction

HCI combines the spatial resolution provided by fluorescence microscopy with algorithm-based quantitation of a variety of parameters, providing multiplex data relating to many aspects of cellular function, structure, and toxicity across populations of cells. Rapid developments in instrumentation, software, and reagents over the last decade have enabled HCI to emerge as a powerful platform for cell-based interrogation of biological processes with higher throughput and increased statistical significance [1–3]. In order to generate reliable data by HCI, accurate identification and segmentation of each cell in a population is required. Segmentation by HCI typically employs the use of fluorescent probes which bind nucleic acids for nuclear staining, protein-reactive dyes for whole

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Table 1
Whole cell and nuclear stains for segmentation

Segmentation tool	Excitation/emission approximate maxima (nm)	Target
HCS NuclearMask™ Blue stain	350/461	Nuclear
HCS NuclearMask™ Red stain	622/645	Nuclear
HCS NuclearMask™ Deep Red stain	638/686	Nuclear
Hoechst 33,342 stain	350/461	Nuclear
HCS CellMask™ Blue stain	346/442	Whole cell
HCS CellMask™ Green stain	493/516	Whole cell
HCS CellMask™ Orange stain	556/572	Whole cell
HCS CellMask™ Red stain	588/612	Whole cell
HCS CellMask™ Deep Red stain	650/655	Whole cell
CellTracker™ Blue CMAC stain	353/466	Whole cell
CellTracker™ Blue CMF_2HC stain	371/464	Whole cell
CellTracker™ Blue CMHC stain	372/470	Whole cell
CellTracker™ Violet BMQC stain	415/516	Whole cell
CellTracker™ Green CMFDA stain	492/517	Whole cell
CellTracker™ Green BODIPY stain	522/529	Whole cell
CellTracker™ Orange CMTMR stain	541/565	Whole cell
CellTracker™ Orange CMRA stain	548/576	Whole cell
CellTracker™ Red CMTPX stain	577/602	Whole cell
CellTracker™ Deep Red stain	630/660	Whole cell
CellMask™ Orange Plasma membrane stain	556/572	Plasma membrane
CellMask™ Deep Red Plasma membrane stain	650/655	Plasma membrane

cell staining, and to some extent, plasma membrane probes to delineate the cellular border (*see* Table 1 for a list of segmentation stains and dyes and their excitation and emission wavelengths). In addition to the fluorescence characteristics of segmentation tools and determination of compatible hardware configurations, selection criteria should account for the ability of such probes to label live cell or fixed cell samples and whether or not targets of interest are localized to the nuclear region [4]. In this chapter, we provide detailed protocols for labeling live or fixed cells for segmentation from whole cells or nuclei along with representative images of

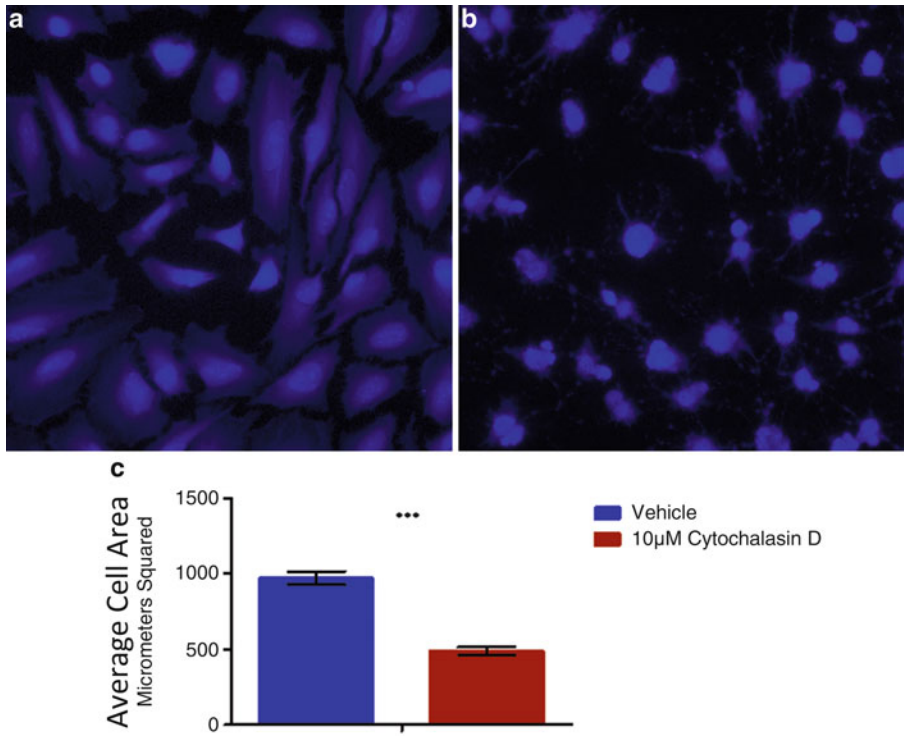

Fig. 1 HeLa cells labeled with HCS CellMask™ Blue stain and treated with vehicle (**a**) or 10 μM cytochalasin D for 3 h (**b**). Automated imaging and analysis of these two populations showed that the mean area of the cells decreased by half with 10 μM cytochalasin D treatment (**c**). $P \leq 0.0001$

proper segmentation and the quantitative data of drug-dependent change in morphology that can be measured with these segmentation tools (Figs. 1 and 2).

2 Segmentation Tools for Automated High Content Imaging and Analysis

Image segmentation is a critical step within automated image analysis, and reagents designed to facilitate this process represent integral tools in the development of HCI-based assays [1]. Image segmentation is a process that divides an image into one or more regions that represent defined objects of interest as cells or within cells and allows further determination and quantitation of their features in a spatial context. In order to obtain quantitative data from cell populations, the spatial distribution of the object and regions of interest within the object must be defined. Subsequently, the objects defined by segmentation tools and well-established image analysis algorithms may be utilized to measure changes in the number, fluorescence emission intensities, shape, texture, or motion of these objects within or across defined regions [4, 5]. Fluorescent dyes that stain nuclei, the cytoplasm or the plasma

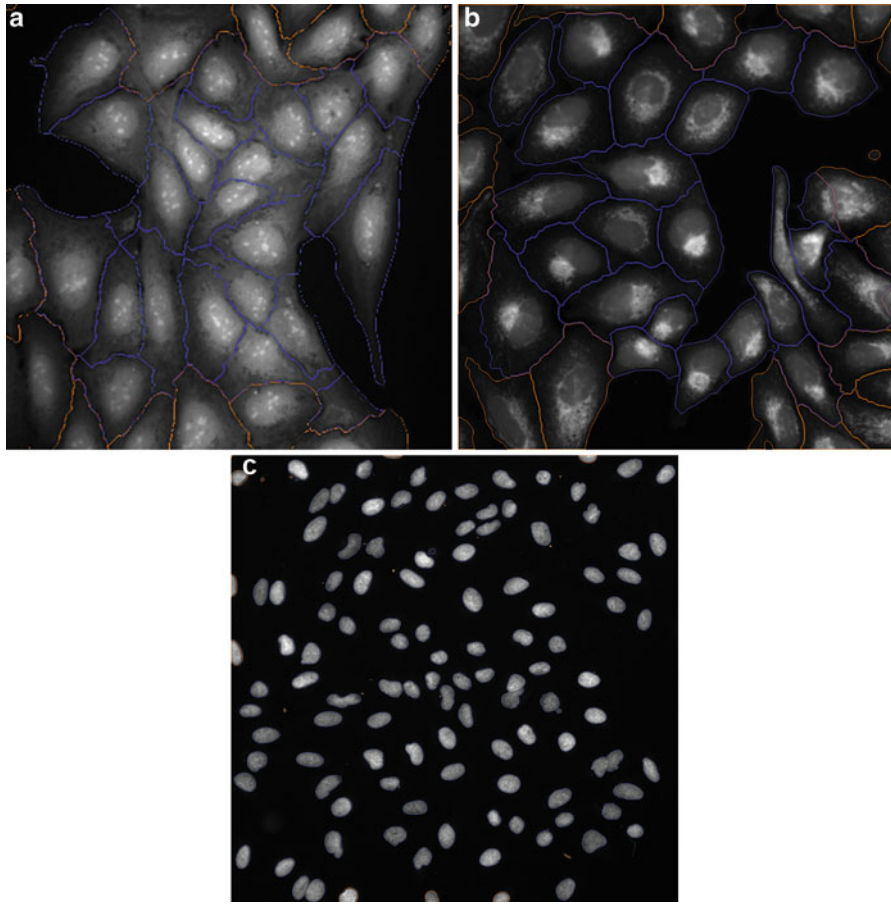

Fig. 2 Image segmentation of U-2 OS cells based on whole cell labeling with HCS CellMask™ Blue stain (**a**), CellTracker™ Deep Red stain (**b**), or based on nuclear labeling with Hoechst 33342 stain (**c**). Accepted objects are outlined in *blue* and rejected objects that cannot be completely quantified are in *orange*

membrane are very useful labeling tools that, in combination with segmentation algorithms, enable robust HCI-based assays (Table 1). In addition to demarcation of objects and regions of interest, stains used for image segmentation may also provide information about cell health including cellular and nuclear morphology. Here we describe example protocols that utilize HCS CellMask™ stains [2] and CellTracker™ probes to label the nuclei and cytoplasm of fixed and live cells, respectively. The whole cell staining that results from these probes enable robust segmentation for automated analysis of changes in cellular size and morphology across populations (Fig. 1). In addition, we provide an example protocol for cell segmentation based upon labeling of nuclei in live or fixed cells with the common, blue-fluorescent nucleic acid stain, Hoechst 33342 [2].

3 Fixed Cell Staining with HCS CellMask™ Stains

3.1 Materials

1. HCS CellMask™ Stain (cytoplasmic/nuclear stain; 2 Component Kit: Component A = HCS CellMask Stain, Component B = DMSO).
2. HeLa (Fig. 1) or U-2 OS (Fig. 2) cells.
3. Fetal bovine serum (FBS).
4. MEM (HeLa cells) or McCoy's 5A (U-2 OS cells) media.
5. 96-well tissue culture microplate (*see* **Note 1**).
6. 16% formaldehyde (*see* **Note 2**).
7. 100% Triton® X-100.
8. Laminar flow hood.
9. Cell culture incubator.
10. Phosphate buffered saline (PBS).
11. High content imaging instrument with appropriate objectives and filter sets for the dye being used (Table 2) (*see* **Note 3**).

3.2 Reagent Preparation

1. Prepare complete growth medium by supplementing MEM (HeLa cells) or McCoy's 5A (U-2 OS cells) with 10% FBS.
2. Prepare a 10 mg/mL HCS CellMask™ stock solution by dissolving the entire contents of the HCS CellMask™ vial (Component A) in 25 µL of DMSO (Component B) (*see* **Note 4**).
3. Prepare the fixative by diluting 16% aqueous formaldehyde solution in PBS to obtain a 4% formaldehyde solution.
4. Prepare the permeabilization solution by adding 10 µL of Triton® X-100 to 10 mL of PBS.

3.3 Methods

1. Seed U-2 OS or HeLa cells into a 96-well plate at a density of 3000 cells/well in 100 µL of McCoy's 5A (U-2 OS cells) or MEM (HeLa cells) supplemented with 10% FBS and incubate overnight under normal cell culture conditions (*see* **Note 1**).
2. Remove the medium and add 4% formaldehyde solution (prepared in Subheading 3.2, **step 3**) to each well and incubate for 15 min at room temperature (*see* **Note 5**).
3. Remove fixative and wash the fixed cells 2–3 times with PBS.
4. Add the 0.1% Triton® X-100 solution (prepared in Subheading 3.2, **step 4**) to each well and incubate for 15 min at room temperature (*see* **Note 6**).
5. Remove permeabilization solution and wash wells 2–3 times with PBS.

Table 2
HCS studio configuration for image segmentation using thermo fisher HCI platforms (see Note 7)

Parameter	HCS CellMask™ Blue	CellTracker™ Deep Red	Hoechst 33342
Bioapplication	Cytoskeletal rearrangement	Cytoskeletal rearrangement	General intensity measurement tool
Objective	40×	40×	20×
Exposure time	Fixed	Fixed	Fixed
Acquisition type	Widefield	Widefield	Widefield
Filter	386-23_BGRFRN_BGRFRN	650-13_BGRFRN_BGRFRN	BGRFR_386_23
Acquisition mode	1104 × 1104 (2 × 2 binning)	1104 × 1104 (2 × 2 binning)	1104 × 1104 (2 × 2 binning)
Autoexposure setting	25% peak target	25% peak target	25% peak target
Image preprocessing			
Channel	1	1	1
Object type	Bright	Bright	Bright
Background removal	Off	Off	Off
Primary object identification			
Smoothing	On	On	Off
Method	Uniform	Uniform	
Value	3	3	
Thresholding	On	On	On
Method	Fixed	Fixed	Fixed
Value	400	200	900
Segmentation	On	On	Off
Method	Intensity	Intensity	
Value	520	520	
Object cleanup	Off	On	On

6. Prepare a 1× HCS CellMask™ staining solution by adding 2 μL of the HCS CellMask™ stock solution (prepared in Subheading 3.2, **step 2**) to 10 mL PBS.
7. Add 100 μL of HCS CellMask™ staining solution (prepared in Subheading 3.3, **step 6**) to each well and incubate for 30 min at room temperature.

8. Wash each well 2–3 times in PBS to remove excess stain before plate sealing and imaging.
9. Optimize the exposure conditions to equal less than saturation limits of detector or camera for positive control, and then scan samples on any high content imaging instrument. The protocol used here was developed on a Thermo Scientific CellInsight CX7 platform with the Cytoskeletal Rearrangement Assay BioApplication and are available in Table 2 (Fig. 2a) (*see* **Note 7**).

4 Live Cell Labeling with CellTracker™ Deep Red Dye

4.1 Materials

1. CellTracker™ Deep Red Dye (Cell permeant dye that becomes impermeant inside the cell; 20 × 15 μg Kit, 1 component).
2. U-2 OS cells.
3. Fetal bovine serum (FBS).
4. McCoy's 5A Medium.
5. 96-well tissue culture plate (*see* **Note 1**).
6. Dimethylsulfoxide (DMSO).
7. Cell culture incubator.
8. Phosphate buffered saline (PBS).
9. High content imaging instrument with objectives and filter set appropriate for detecting CellTracker™ Deep Red stain (Table 2) (*see* **Note 3**).

4.2 Reagent Preparation

1. Prepare complete growth medium by supplementing McCoy's 5A (U-2 OS cells) with 10% FBS.
2. Prepare a 1 mM CellTracker™ Deep Red stock solution (1000×) by dissolving the entire contents of one vial of CellTracker™ Deep Red dye (Component A) in 20 μL of DMSO (*see* **Note 11**).

4.3 Methods

1. Seed U-2 OS cells into a 96-well plate at a density of 2500 cells/well in 100 μL of complete growth medium and incubate overnight under normal cell culture conditions (*see* **Note 1**).
2. Prepare a 1× CellTracker™ Deep Red working solution by adding 10 μL of the CellTracker™ Deep Red stock solution (prepared in Subheading 4.2, **step 2**) to 10 mL unsupplemented MEM.
3. Add 100 μL of CellTracker™ Deep Red working solution (prepared in Subheading 5.3, **step 2**) to each well and incubate for 30 min at room temperature.
4. Remove CellTracker™ Deep Red working solution from wells.
5. Add 100 μL of complete growth media to each well of the 96-well plate.

6. Optimize the exposure conditions and scan samples on any high content imaging instrument. The protocol used here was developed on a Thermo Scientific CellInsight CX7 platform with the Cytoskeletal Rearrangement Assay BioApplication (Fig. 2b) (Table 2) (*see* **Note 7**).

5 Live or Fixed Cell Labeling of the Nuclei with Hoechst 33342

5.1 Materials

1. Hoechst 33342 stain, trihydrochloride, trihydrate—10 mg/mL aqueous solution (nucleic acid dye).
2. U-2 OS cells.
3. Fetal bovine serum (FBS).
4. McCoy's 5A Medium.
5. 96-well tissue culture microplate (*see* **Note 1**).
6. 16% formaldehyde (*see* **Note 12**).
7. 100% Triton® X-100.
8. Laminar flow hood.
9. Cell culture incubator.
10. Phosphate buffered saline (PBS).
11. High content imaging instrument with objectives and filter sets appropriate for Hoechst 33342 (Table 2) (*see* **Note 3**).

5.2 Reagent Preparation

1. Prepare complete growth medium by supplementing McCoy's 5A (U-2 OS cells) with 10% FBS.
2. Prepare the fixative by diluting 16% aqueous formaldehyde solution in PBS to obtain a 4% formaldehyde solution (fixed cell labeling only).
3. Prepare the permeabilization solution by adding 10 μL of Triton® X-100 to 10 mL of PBS (fixed cell labeling only).

5.3 Methods

1. Seed U-2 OS cells into a 96-well plate at a density of 2500 cells/well in 100 μL of complete growth medium and incubate overnight under normal cell culture conditions (*see* **Note 4**).
2. *For Fixed Cell Experiments Only*: Remove the medium and add 4% formaldehyde solution (prepared in Subheading 5.2, **step 2**) to each well and incubate for 15 min at room temperature (*see* **Note 12**).
3. *For Fixed Cell Experiments Only*: Remove fixative and gently wash the fixed cells 2–3 times with PBS.
4. *For Fixed Cell Experiments Only*: Add the 0.1% Triton® X-100 solution (prepared in Subheading 5.2, **step 3**) to each well and incubate for 15 min at room temperature.

5. *For Fixed Cell Experiments Only*: Remove permeabilization solution and gently wash wells 2–3 times with PBS.
6. Prepare a 1× Hoechst 33342 staining solution by adding 5 μL of the 10 mg/mL stock solution to 10 mL PBS for fixed cells or complete media for live cells (See **Note 13**).
7. Add 100 μL of Hoechst 33342 staining solution (prepared in Subheading 5.3, **step 6**) to each well and incubate for 15 min at room temperature.
8. Optimize the exposure conditions and scan samples on any high content imaging instrument. The protocol used here was developed on a Thermo Scientific ArrayScan™ V^{TI} platform with the General Intensity Measurement Tool BioApplication (Fig. 2c) (Table 2) (*see* **Note 7**).

6 Notes

1. Plates should be selected for an available form factor and thickness for the HCI platform being used and cells should be plated uniformly at lower densities for good separation of cells and accurate segmentation of objects (Subheadings 3.1, **item 5**, 3.3, **step 1**, 4.1, **item 5**, 4.3, **step 1**, 5.1, **item 5**, and 5.3, **step 1**).
2. Other fixation methods such as methanol have not been validated for use with HCS CellMask™ stains (Subheading 3.1, **item 6**). Literature searches may be helpful to determine if other fixation methods have been tested for these dyes.
3. While this study used a 40× objective, procedures outlined here are applicable to 10× and 20× magnifications (Subheading 3.1, **item 11**, Subheading 4.1, **item 9**, and 5.1, **item 10**).
4. Store any unused HCS CellMask™ stain at −20 °C, protected from light. For optimal results, use frozen aliquots within 6 months of preparation. Avoid freeze/thaw cycles (Subheadings 3.2, **step 2** and 3.3, **step 9**).
5. For assays where there may be apoptotic or dead cells, fixative can be added to the existing media in a 2× solution (Subheading 3.3, **step 2** and 5.3, **step 2**).
6. Other incubation times and Triton-X concentrations may be used. The researcher should use the optimal permeablization conditions for the cell type and fluorophore being used (Subheading 3.3, **step 4**).
7. Optimize the labeling conditions and software parameters for segmentation so at least an average of 95% of cells per field are segmented correctly (Table 2) (Subheadings 3.3, **step 9**, 4.3, **step 6**, and 5.3, **step 8**) (Fig. 3).

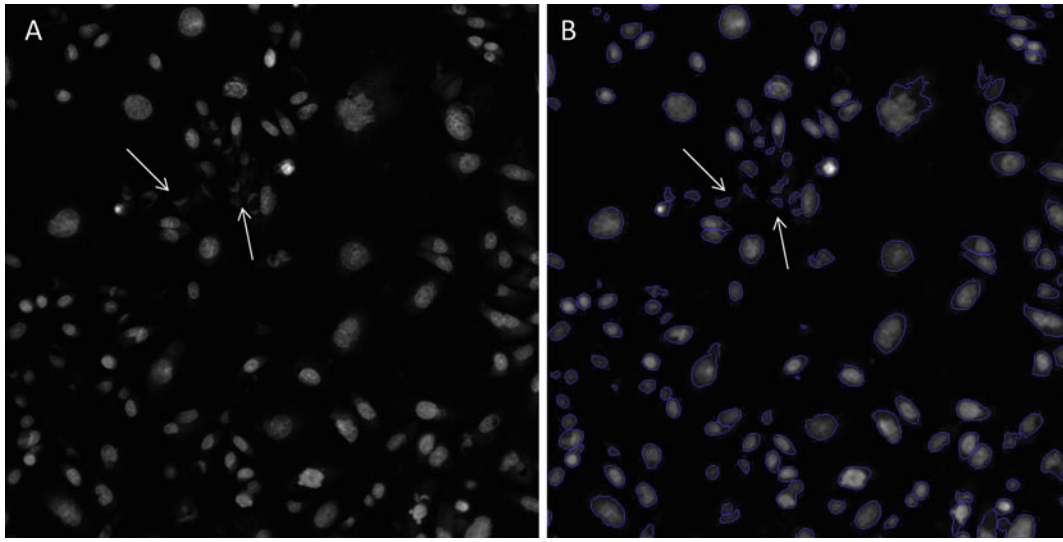

Fig. 3 Improper labeling conditions can affect segmentation of cells. Improper labeling conditions for Hoechst 33342 results in only half of some nuclei being labeled (*arrows* in **a**) which prevents proper segmentation (*arrows* in **b**) (see **Note 7**)

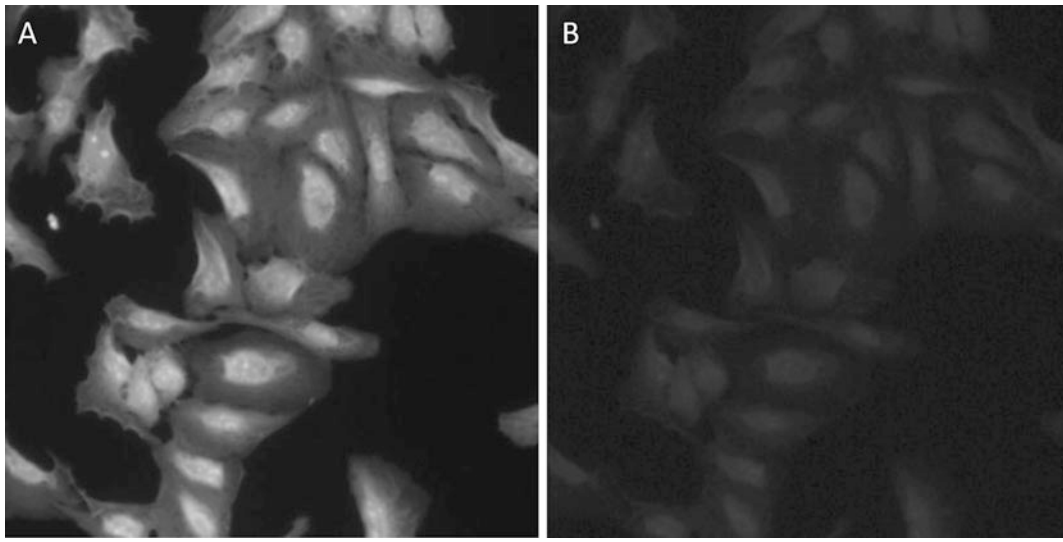

Fig. 4 Dyes "bleeding through" into other channels can affect assay and data integrity. Images from 60 ms exposures in the Texas Red (**a**) and TRITC (**b**) channels showed HCS CellMask Red "bleeding through" into the shorter wavelength TRITC channel obscuring dim targets (See Notes 8, 9, and 10)

8. When multiplexing HCS CellMask™ stains, CellTracker™ dyes, or Hoechst 33342 with other fluorophores, first perform small-scale optimization labeling conditions to ascertain fluorescence compatibility prior to large-scale screening. In a

96-well plate format, serial dilutions can be used effectively to optimize staining and labeling conditions (Fig. 4).

9. Given the brightness of these stains, "bleed through" into neighboring fluorescence channels can easily be mitigated by reducing the final concentration of the HCS CellMask™ reagent down to as low as 1 μg/mL in the staining solution. HCS CellMask™ reagents bleed through more prominently into channels with emission wavelengths shorter than the ideal emission wavelength of the dye, so it is advisable to assign less-abundant targets to longer wavelength emission channels when designing experiments (Fig. 4).
10. HCS CellMask™ stains are compatible only with fixed and permeabilized cells. When combining with antibody-based probes, labeling with HCS CellMask™ should be performed as the last step to avoid competitive blocking of epitopes intended for binding by primary antibodies (Fig. 4).
11. Any unused CellTracker™ Deep Red working solution should be discarded (Subheading 4.2, **step 2**).
12. Other fixation methods such as methanol may be used instead of formaldehyde (Subheadings 5.1, **item 6** and 5.3, **step 2**).
13. Store any unused Hoechst 33342 staining solution at 4 °C, protected from light (subheading 5.3, **step 6**).

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