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Cyclic immunofluorescence (CycIF), a highly multiplexed method for single-cell imaging

Jia-Ren Lin[1*], Mohammad Fallahi-Sichani[2], Jia-Yun Chen[2] and Peter K. Sorger[1,2*]

[1] HMS LINCS Center

Laboratory of Systems Pharmacology

[2]Department of Systems Biology

Harvard Medical School

200 Longwood Avenue

Boston, Massachusetts, 02115 USA

*Correspondence to Peter K Sorger (peter_sorger@hms.harvard.edu) and Jia-Ren Lin (jia-ren_lin@hms.harvard.edu)

Jia-Ren Lin telephone: 617-432-6925

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Immunofluorescence has been widely used in different fields of biological and medical research for decades. The ability to obtain *in situ* and single-cell information makes this technique particularly important in post-genomic era. However, biochemical and optical constraints limit the number of signals that could be captured simultaneously within the same sample. Here we introduce CycIF (Cyclic Immunofluorescence), an easy and low-cost method to increase the multiplexity of conventional immunofluorescence. The CycIF method has been applied in pre-clinical drug discovery, cancer and stem cell biology, and it has the potential to significantly improve our understanding of cell biology in health and disease.

ABSTRACT

Probing the phenotypic heterogeneity within biological samples is essential for understanding the contributions of genetic and epigenetic variations in cancers and other diseases. Fluorescence microscopy with high-content imaging capability is particularly useful in unraveling the protein and signaling variability in drug response and resistance. However, the main obstacle for fluorescent imaging is the number of proteins/signals that can be simultaneously measured. Thus, we have developed a Cyclic Immunofluorescence (CycIF) method to overcome this technical barrier. By applying a mild chemical inactivation to the Alexa Fluor© conjugated antibodies, CycIF allows multiple rounds of immunostaining on the same fixed samples. This method,

unlike other antibody-stripping methods, is specifically optimized for fragile, monolayer cell cultures. In addition, this protocol has deployed a chemical method to inactivate genetically encoded fluorescent proteins. Thus, CycIF is an easy, robust and versatile protocol for collecting highly multiplexed single cell data and can be used for high-throughput, high-content screening in drug discovery and basic research.

Keywords: CycIF, immunofluorescence, high-content imaging, multiplexing, systems pharmacology.

INTRODUCTION

One of the major challenges in biomedical research is to understand how heterogeneity contributes to diverse cellular responses within different single cells. It is especially crucial in cancer research, given the drastic genetic and epigenetic alternations within tumors that lead to differential cellular growths, transformations and drug responses (Brooks, Burness, & Wicha, 2015; Gerdes et al., 2014). These cellular variabilities are usually masked by conventional, bulk measurements like ELISA or western blotting. Thus, single-cell approaches are required to uncover the cellular heterogeneity and allow the identification of interesting sub-populations (eg. stem-like or drug-resistant) (Irish & Doxie, 2014). Among these approaches, immunofluorescence-based high-content microscopy is unique in its ability to integrate high-throughput screening pipelines (eg. compounds or siRNAs) with multiplexed measurements of diverse cellular phenotypes both quantitatively and qualitatively (Zanella, Lorens, & Link, 2010)). Moreover, the rapid development of live-cell reporters expands the measurement repertoire to time space, making it possible to study the spatiotemporal dynamics of signaling networks at single cells. Nevertheless, the number of cellular features that can be obtained

within the same single cell is usually limited by the available fluorescence channels (Gerdes et al., 2013). Mass cytometry and several other recently developed technologies have overcome this limitation and demonstrated its powerfulness in highly-multiplexed and high-dimensional measurements at single cells (Bendall et al., 2011). However, these multiplexing approaches are mainly focused on *in situ* profiling of tissue sections, and often employ harsh antigen-retrieval methods to enable serial immunostainings and stripping cycles. These methods cannot apply to adherent cell cultures, one of the most commonly used tools in drug screen, because adherent cells are not as durable as tissue sections. To fulfill this unsatisfied need, a Cyclic ImmunoFluorescence (CycIF) method that is optimized for adherent cells has been developed. It is an easy and robust assay for highly multiplexed and high-content imaging experiments (Lin, Fallahi-Sichani, & Sorger, 2015). CycIF applies a unique fluorophore inactivation method followed by sequential stainings and microscopy imaging. By simply bleaching the fluorescent dyes but leaving the antibody intact, this method is gentle enough to be used in various adherent cell lines and even in suspension cells. Furthermore, an alternative method to bleach genetic encoded fluorescent proteins has also been deployed with the CycIF protocol. It is particularly useful for experiments combining live-cell imaging and multiplexed high-content screening. These methods will be discussed in more details in the following sections.

STRATEGIC PLANNING

Choosing dye-antibody conjugates.

In CycIF, direct immunofluorescence with dye-conjugated antibodies is preferred for several reasons. First, the multiplexity of indirect immunofluorescence is constrained by the number of available dyes and the isotypes of secondary antibodies. Second, co-staining of polyclonal

antibodies with the same species requires tedious procedures to avoid cross-reactivity. Third, to employ a maximum number of channels in each cycle of staining (herein three dyes plus one DNA marker), the use of conjugated antibodies are more favorable than use of primary-secondary antibody combinations. Though dye-conjugated antibodies are excellent for multiplexing, there are some potential issues. Without amplification of secondary antibodies, the signal to noise ratio can be low for conjugated antibodies. In addition, the chemical process of crosslinking dyes to antibodies might affect the affinity or the specificity of directly conjugated antibodies. Therefore, the commercially available Alexa fluor© conjugated antibodies should be used if available. The Alexa 488/555/647 dyes provide brighter and more stable fluorescence than FITC/Cy3/Cy5 dyes, and this feature is essential to overcome weak signals in direct immunofluorescence (Panchuk-Voloshina et al., 1999). Furthermore, many vendors provide Alexa Fluor conjugated antibodies which have been extensively tested for immunofluorescence or immunohistochemistry. By using the commercial sources, the CycIF protocol should be consistent and widely applicable across different laboratories. The antibodies validated for CycIF applications are listed in **Table 1**. Meanwhile, a website hosted by HMS-LINCS will keep updating the availability of CycIF-compatible antibodies (http://lincs.hms.harvard.edu/lin-NatCommun-2015/).

Planning the sequence of antibody combinations in different cycles.

In order to reach maximal multiplexity with CycIF, we stain with at least three different antibodies (with Alexa 488/555/647) in each cycle. However, each Alexa dye has a different brightness, and the combination might be adjusted based on instrument-specific emission/excitation filters and the light sources. In general, the Alexa Fluor© 488 is the brightest dye (quantum yield: 0.92) although there is a higher background fluorescence in the

same channel. The Alexa Fluor© 647 is the second brightest dye (quantum yield: 0.33) with the least background fluorescence from the far-red region. The Alexa Fluor© 555 is the weakest dye here (quantum yield: 0.1) but it outperforms the commonly used Cy3 dye. In addition, though it is minimal, there is still potential spectrum crosstalk amongst the three dyes when combined. Therefore, we suggest selecting an Alexa 488 conjugate for the lowest affinity antibody and when staining low-abundance proteins, while leaving an Alexa 555 conjugate for the highest affinity antibody and when staining abundant proteins.

In general, only minimal antigen loss is observed during the CycIF procedure, and that fluorophore inactivation is nearly complete for Alexa 488/555/647 dyes (Lin et al., 2015). However, in order to acquire quantitative results from cyclic stainings without residual signal interferences from the previous cycles, the general principle is to stain first with low-signal antibodies (eg. antibodies against phospho-proteins) and then apply high-signal antibodies (eg. antibodies against cytoskeleton or abundant proteins) in the later cycles. An example order of antibodies used in the six-round CycIF experiment is listed in **Table 2**.

How far can you go? (Tips for preventing cell loss in CycIF)

The main constraint for the CycIF procedure is cell loss over staining cycles due to inadvertent mechanical disruption during washing steps. To prevent this, prolonged fixation and gentle washes are necessary. We combined paraformaldehyde (PFA) with methanol to achieve optimal fixation for adherent cells. It is also helpful to pre-coat plates with poly-lysine or collagen. In addition, you should try to minimize the overall length of the experiment from the first to the last

cycle. Even stored at 4^{o}C in PBS solution, the fixed cells will gradually detach over time. An automated plate washer, such as BioTek EL406, is highly recommended for gentle washing and dispensing. Listed below are the detailed washing steps we set up for the BioTek EL406 machine for a CycIF assay run in 96-well microplates.

<Protocol begin>

Step Details: W-Wash

Pre-dispense before washing: No

Bottom Wash: No

Number of Wash Cycles: 4 Aspirate per cycle

Vacuum Filtration: False

Travel Rate: 3 CW 7.3 mm/sec

Delay: 0 msec

Z Offset: 35 steps (4.45 mm above carrier)

X Offset: -40 steps (1.83 mm left of center)

Y Offset: 0 steps (center of well)

Secondary Aspirate: No

Dispense per cycle

Buffer: A Volume: 250 µL/well

Flow Rate: 2

Z Offset: 110 steps (13.97 mm above carrier)

X Offset: -25 steps (1.14 mm left of center)

Y Offset: 0 steps (center of well)

Pre-dispense: not available

Delay start of Vacuum until

Volume dispensed: 300 µL/well

Shake/Soak after dispense:

No Pre-dispense between cycles: No

Final Aspirate: No

<Protocol end>

Ways to multiplexing via CycIF.

Though a protease-mediated antibody stripping method is described in Lin et al 2015, the protocol here will focus on chemical fluorophore inactivation. The reasons are: (1) Protease digestion will deteriorate bio-samples and degrade antigens, particularly with cultures grown as mono-layers. (2) Different antibodies (isotypes or host species) will be digested at different rates by proteases. (3) Depending on the antibodies and samples used, protease-stripping method needs to be optimized individually.

With the chemical fluorophore inactivation method, the main constraint will be the availability of dye conjugated antibodies suitable for immunofluorescence. In addition, signal amplification by secondary antibodies might be required to detect certain low-abundance proteins. To overcome these constraints, indirect immunofluorescence using labeled secondary antibodies in the first round of CycIF can be followed by cycles of direct immunofluorescence. The Alexa dyes conjugated to secondary antibodies in the first round will also be chemically inactivated. Since only dye conjugated primary antibodies will be used in the following cycles, there is no concern of cross-reactivity from secondary antibodies (see **Alternative Protocol 1**, **Figure 1**).

Non-antibody labeling steps (eg. using Phalloidin, Annexin V and Mitotracker) could be incorporated in the CycIF procedure to increase its multiplexing potential. Many Alexa Fluor© based subcellular labeling products are available from ThermoFisher/Invitrogen, and they are fully compatible with the chemical inactivation method. Furthermore, non-Alexa dyes might be used in the last staining cycle and the chemical inactivation process omitted. However, the integrity of subcellular structures and organelles might be compromised by peroxidation and

washing steps. For example, the Mitotracker CMXRos (Invitrogen, Cat: M-7512) is sensitive to oxidation and should be applied before fixation. Thus, further investigation and optimization steps might be required for use of different fluorescent labelling kits.

The rapid growth of fluorescent protein-based live-cell sensors and the advancement of time-lapse imaging system have opened up new avenues for single-cell research. The spatiotemporal resolution at the single cell level allows us to dissect the causal relationship of cellular heterogeneity in depth (Hoppe, Coutu, & Schroeder, 2014). The genetically encoded fluorescent reporters offer researchers great ways to probe the dynamics of signaling networks, but remnants of their fluorescence after fixation hinder options for further immunostaining cycles for these cells. Thus, in **Alternative Protocol 2**, a chemical method is introduced for inactivation of fluorescent proteins making the samples compatible with the subsequent CycIF pipeline **(Figure 1)**. By combining fluorescent reporters with CycIF, researchers for the first time have the ability to obtain time-resolving and highly multiplexed information together at a single cell level.

PROTOCOLS:

The protocols below have been optimized for adherent cells grown in 96-well plates. Cell lines tested are: MCF7, MCF10A, COLO858, WM115, WM1552C, LOXIMVI , RPE1, human primary fibroblasts, human iPSCs. Cultured cells are seeded at 20-50% confluency and then treated with drugs or growth factors if necessary. Typically, cells are fixed and stained 2~3 days after seeding, which leads to 70-90% final confluency in general. Over- or under- confluent cells might result in detachment during CycIF procedures; further details are discussed in the Troubleshooting section.

Basic Protocol (Type I CycIF): Cyclic immunofluorescence with only Alexa fluorophore-conjugated antibodies

Materials

Corning 96 well Plates, Clear Bottom, black (Sigma-Aldrich, Cat: CLS3603)

16 % Paraformaldehyde/PFA (Electron Microscopy Sciences, Cat:15710): For making 4% PFA, diluted 1:4 in PBS.

20x Phosphate-buffered Saline/PBS (Santa Cruz Biotech, Cat:SC-362299): Dilute 1:20 in milli-Q water to make 1x solution.

Methanol 99.9% (Fisher Scientific, Cat: AC61009), store in -20^{o}C

Odyssey blocking buffer (LICOR Odyssey, Cat:927-40125)

Hoechst 33342 10 mg/mL (Invitrogen, Cat: H3570)

Hydrogen peroxide/H_2O_2 solution 30 wt. % in H_2O (Sigma-Aldrich, Cat: 216763)

Sodium hydroxide/NaOH 2M solution, (Sigma-Aldrich, S5881)

Biotek EL406 Washer/dispenser

GE Cytell Cell Imaging System

Step 1. Fixation: Fix cells in 96-well plates with 140 ul 4% PFA at room temperature for 30 minutes. Then wash 4 times with 250 µl PBS per wash.

**Extensive fixation is required. For loosely-adherent cells the fixation time may be extended up to 1 hr.*

Step 2. Permeabilization: Add 140 μl ice-cold methanol, and allow the plates to sit at room temperature for 10 minutes. Then wash 4 times with 250 μl PBS per wash.

Step 3. Blocking: Incubate the cells with 50 μl Odyssey blocking buffer at room temperature for 1 hr.

Step 4. Staining: Dilute antibodies in 50 ul Odyssey buffer, and add to the cells. Incubate at $4^{o}C$ overnight, and then wash 4 times with 250 μl PBS per wash.

**All washes should be as gentle as possible. A plate washer (e.g. Bio-Tek EL406) is recommended.*

*** The optimal dilutions of antibodies tested for CycIF are provided in Table 1. The detailed guideline for antibody dilution in immunofluorescence can be found in these articles (Donaldson, 2002; Hoffman, Le, & Sita, 2008).*

Step 5. Hoechst staining: Dilute Hoechst 33342 (1mg/ml stock) 1:5000 in 140 μl PBS and incubate with the cells for 15 minutes at room temperature. Then wash 4 times with 250 μl PBS per wash. Leave 10-20 μl of PBS in each well after the last wash step.

Step 6. Imaging: Use a Cytell (GE Healthcare Life Sciences) or Operetta (Perkin Elmer) imager or the equivalent. For the Cytell, the fixed filter setting is required for 4-channel immunofluorescence (Hoechst, 488 nm, 555 nm, and 647 nm) to minimize crosstalk. In addition, a binning option or high-sensitivity camera may be required for weak signals, such as those from directly-conjugated primary antibodies.

Step 7. Fluorophore inactivation: After imaging, remove the PBS and add 140 µl fluor-inactivation solution (3% H_2O_2 in PBS plus 20 mM NaOH). Incubate at room temperature for 1 hr with light (using a tabletop lamp or equivalent). Then wash 4 times with 250 µl PBS per wash, leaving 10-20 µl of PBS in each well after the last wash step. (After washing, the cells can be stored at 4oC before proceeding, but it is best to proceed with the next round of imaging as soon as possible.)

** Light treatment is optional, but it may increase the efficiency of fluorophore inactivation by 10-20%, depending on the light source. A fluorescent or LED-source of white lamps have been used in the CycIF protocol. A detailed set-up of light-treated device is described in the* ***Reagents, Supplies and Instruments section.***

Step 8. Start CycIF: After fluor inactivation, you may begin a second round of labeling starting from step 3 (blocking) or step 4 (staining). However, imaging again after dye inactivation and before the next round of labeling is recommended to ensure a complete removal of dye signal.

** Re-blocking (from step 3) will reduce the background from non-specific antibody binding, but this extra step might increase the chance of cell loss. Therefore, re-blocking is normally omitted unless the antibody background is high.*

Alternative Protocol 1 (Type II CycIF): Cyclic immunofluorescence with non-conjugated antibodies mixed with fluorophore-conjugated antibodies

Materials

Corning 96 well Plates, Clear Bottom, black (Sigma-Aldrich, Cat: CLS3603)

16 % Paraformaldehyde/PFA (Electron Microscopy Sciences, Cat:15710): For making 4% PFA, diluted 1:4 in PBS.

20x Phosphate-buffered Saline/PBS (Santa Cruz Biotech, Cat:SC-362299): Dilute 1:20 in milli-Q water to make 1x solution.

Methanol 99.9% (Fisher Scientific, Cat: AC61009), store in -20^{o}C

Odyssey blocking buffer (LICOR Odyssey, Cat:927-40125)

Hoechst 33342 10 mg/mL (Invitrogen, Cat: H3570)

Hydrogen peroxide/H_2O_2 solution 30 wt. % in H_2O (Sigma-Aldrich, Cat: 216763)

Sodium hydroxide/NaOH 2M solution, (Sigma-Aldrich, S5881)

Biotek EL406 Washer/dispenser

GE Cytell Cell Imaging System

Step 1-3. *Same as basic protocol.*

**Note: the Alternative protocol 1 should be applied only in the first CycIF cycle, and followed by the Basic Protocol (Step 4-8)*

Step 4a. Primary antibody staining: Dilute antibodies in 50 µl Odyssey buffer, and add to the cells. Incubated at 4oC overnight, and then wash 4 times with 250 µl PBS per wash.

*** The optimal dilutions of antibodies tested for CycIF are provided in Table 1. The detailed guideline for antibody dilution in immunofluorescence can be found in these articles* (Donaldson, 2002; Hoffman et al., 2008).

Step 4b. Secondary antibody staining: Dilute Alexa conjugated secondary antibodies (1:1000~1:2000) in 50 μl Odyssey buffer, add to the cells. Incubated at room temperature for 1 hr, and then wash 4 times with 250 μl PBS per wash.

Step 5. & 6. (Hoechst staining and Imaging) *Same as basic protocol.*

Step 7. Fluorophore inactivation: After imaging, remove the PBS and add 140 μl fluor-inactivation solution (4.5% H_2O_2 in PBS plus 25 mM NaOH). Incubate at room temperature for 1-1.5 hr, then wash 4 times with 250 μl PBS per wash, leaving 10-20 μl of PBS in each well after the last wash step.

**The secondary antibodies with Alexa dyes usually have stronger signals and take longer to inactivate. Before proceeding to the next step, make sure the inactivation is complete by imaging the residual signals.*

Step 8. Start CycIF: After fluor inactivation, you may begin a second round of labeling starting from step 3 (blocking) or step 4 (staining). However, only fluorophore-conjugated antibodies should be used from this point to avoid cross-reactivity from secondary antibodies.

** Re-blocking (from step 3) will reduce the background from non-specific antibody binding, but this extra step might increase the chance of cell loss. Therefore, re-blocking is normally omitted unless the antibody background is high.*

Alternative Protocol 2 (Type III CycIF): Cyclic immunofluorescence for cells expressed fluorescent proteins (FPs) followed by antibody staining

Materials

Corning 96 well Plates, Clear Bottom, black (Sigma-Aldrich, Cat: CLS3603)

16 % Paraformaldehyde/PFA (Electron Microscopy Sciences, Cat:15710): For making 4% PFA, diluted 1:4 in PBS.

20x Phosphate-buffered Saline/PBS (Santa Cruz Biotech, Cat:SC-362299): Dilute 1:20 in milli-Q water to make 1x solution.

Methanol 99.9% (Fisher Scientific, Cat: AC61009), store in -20^{o}C

Odyssey blocking buffer (LICOR Odyssey, Cat:927-40125)

Hoechst 33342 10 mg/mL (Invitrogen, Cat: H3570)

Hydrogen peroxide/H_2O_2 solution 30 wt. % in H_2O (Sigma-Aldrich, Cat: 216763)

Sodium hydroxide/NaOH 2M solution, (Sigma-Aldrich, S5881)

Hydrogen Chloride/HCl 1M solution, (dilute from 12 M solution; Sigma-Aldrich, cat. #258148)

Biotek EL406 Washer/dispenser

GE Cytell Cell Imaging System

Step 1 to 6. *Same as basic protocol.*

Step 7a. Fluorophore inactivation for fluorescent proteins: After imaging, remove the PBS and add 140 µl acidic fluor-inactivation solution (3% H_2O_2 in PBS plus 20 mM HCl). Incubate at

room temperature for 1-1.5 hr, then wash 4 times with 250 µl PBS per wash, leaving 10-20 µl of PBS in each well after the last wash step.

**For Turquoise/ECFP fusion proteins, increase the inactivation time to 2 hours (or bleach the second time with fresh inactivation solution).*

**Note: this step (Step 7a) will be applied only in the first cycle.*

Step 7b. Fluorophore inactivation for Alexa Dyes: Remove the PBS and add 140 µl fluor-inactivation solution (3% H_2O_2 in PBS plus 20 mM NaOH). Incubate at room temperature for 1-1.5 hr, then wash 4 times with 250 µl PBS per wash, leaving 10-20 µl of PBS in each well after the last wash step.

Step 8. Start CycIF: After fluor inactivation, you may begin a second round of labeling starting from step 3 (blocking) or step 4 (staining). However, imaging after inactivation and before the next round of labeling is recommended to ensure complete removal of the signals from dyes & fluorescent proteins.

** Re-blocking (from step 3) will reduce the background from non-specific antibody binding, but this extra step might increase the chance of cell loss. Therefore, re-blocking is normally omitted unless the antibody background is high.*

REAGENTS, SUPPLIES AND INSTRUMENTS

- Corning 96 well Plates, Clear Bottom, black (Sigma-Aldrich, Cat: CLS3603)
- 4% paraformaldehyde/PFA (diluted from the 16% stock from Electron Microscopy Services, cat. #15710)

- Methanol 99.9% (Fisher Scientific, Cat: AC61009)
- Hoechst 33342 (Invitrogen, cat. #H3570)
- Odyssey blocking buffer (LI-COR Biosciences, cat. #927-40001)
- 20x Phosphate-buffered Saline/PBS (Santa Cruz Biotech, cat. #SC-362299):
- 30% hydrogen peroxide (Sigma-Aldrich, cat. #H1009)
- 1 M NaOH (dissolve from pellets; Sigma-Aldrich, cat. #S5881)
- 2 M HCl (dilute from 12 M solution; Sigma-Aldrich, cat. #258148)
- Fluor-inactivation solution for Alexa dyes: 3% H_2O_2 (1/10 dilution from 30% stock), 20 mM NaOH (1/50 dilution from 1 M stock) in PBS
- Fluor-inactivation solution for indirect IF, use up to 4.5% H_2O_2 and 25 mM NaOH.
- Fluor-inactivation solution for GFP/YFP/mCherry fusion proteins: 3% H_2O_2 (1/10 dilution from 30% stock), 20 mM HCl (1/100 dilution from 2M stock) in PBS
- Biotek EL406 Washer/dispenser
- GE Cytell Cell Imaging System or Perkin Elmer Operetta High Content Imaging System
- Light-treatment device, equipped with Lithona 211E71 LED light (UCLD 24 WH, Color temperature: 3000k with CRI:83 warm-white light). The peak wave-length of the light source is at 630 nm with second peak at 540 nm and third peak at 450 nm. (**Figure 2**).

IMAGE/DATA ANALYSIS

Imaging processing (registration and quantification)

Image registration (alignment) will be required for merging signals from different rounds of CycIF, and an imager with a robust stage/position control is necessary. Since only a portion of each image will be retained during image registration, stitching images from overlapping sites/fields prior to registration allows for a larger fraction of cells to be retained in the final registered image for downstream image analysis.

Image registration is done by ImageJ scripts and plugins (StackReg: http://bigwww.epfl.ch/thevenaz/stackreg/; MultiStackReg: http://bradbusse.net/downloads.html). In brief, Hoechst images from different cycles will be used as reference to generate registration information using the rigid body transformation algorithm. Then the same registration information will be applied to other channels and a hyperstack image containing all channels will be generated. Image segmentation and analysis will be performed using ImageJ with the scripts provided on the public website (http://lincs.hms.harvard.edu/lin-NatCommun-2015/). Hoechst images from the last round of CycIF will be converted to nuclear masks and regions of interest (ROIs). These ROIs will be applied to images for all data channels (488/555/647/Hoechst) and the mean fluorescent intensities will be gathered. The nuclear masks can also be converted into ring-shape ROIs outside the nuclei for quantifying the cytoplasmic intensity of other channels. The raw intensity data generated by ImageJ can be saved in comma-separated-values (CSV) format and processed by Matlab scripts for further data analysis. All ImageJ and Matlab scripts are available on the website (http://lincs.hms.harvard.edu/lin-NatCommun-2015/)

High-dimensional data analysis

The high-dimensional single-cell data generated by CycIF post challenges in both visualization and data analysis. To date, several computational tools developed for CyTOF data analysis,

including viSNE and Wanderlust algorithms, could be applied for CycIF data (Amir et al., 2013; Bendall et al., 2014; Lin et al., 2015). However, there are still increasing need for developing better computation tools for high-dimensional single-cell data, especially for resolving the complexity of non-intensity image features captured by CycIF. To accelerate this, sample datasets are available in the HMS-LINCS website and a data-oriented paper will be published soon (Honarnejad et. al., in preparation).

A few examples/tools for CycIF data are demonstrated in **Figure 4**. The Custom-developed Matlab scripts for high-dimensional data analysis can be obtained from HMS-LINCS website (http://lincs.hms.harvard.edu/lin-NatCommun-2015/) and the CYT package is available from the Pe'er lab webpage (http://www.c2b2.columbia.edu/danapeerlab/html/software.html). Principle component analysis and cross-correlation analysis can be performed with Matlab built-in functions via importing numerical data. viSNE and Wanderlust algorithms from the CYT package are useful to processing CycIF data. The raw intensity data generated from CycIF will be saved in CSV format and then be imported into CYT application. Please refer to Pe'er lab webpage for more detail descriptions on viSNE and Wanderlust algorithms. For Wanderlust, the raw intensity data files need to be normalized using the Wanderlust script with default parameters (L number = 30; K number = 5; Number of landmarks = 20; Number of graphs = 25; Distance Metric = Cosine).

COMMENTARY

Background Information

Antibody stripping with the purpose of increasing the multiplicity of tissue staining is not a new concept in immunohistochemistry. The heating and microwave steps used in antigen retrieval protocols are also frequently used to remove antibodies from formalin-fixed paraffin-embedded (FFPE) tissue samples (Robertson, Savage, Reis-Filho, & Isacke, 2008). However, this method is not applicable to monolayer cell cultures in a research setting because heating or microwaving would destroy these samples easily. Different technologies have been developed by others for multiplexed measurement in cells (Angelo et al., 2014; Schweller et al., 2012; Zrazhevskiy & Gao, 2013), but most of the techniques require special reagents (e.g. quantum dots, oligonucleotides or isotope conjugates), or special instrumentation (e.g. hyperspectral microscopes, microfluidic devices, mass cytometry) which are not widely available and have their own limitations. In contrast, immunofluorescence microscopy is an affordable and widely used technique with an additional advantage of obtaining information on cell morphology and microenvironment, but the multiplexity of conventional immunofluorescence is limited. Here, we present a simple and robust CycIF protocol using commercially available chemicals and antibodies to perform highly multiplexed single-cell immunofluorescence. We present different variants of CycIF involving direct immunofluorescence (with fluorophore-conjugated antibodies), indirect immunofluorescence, fluorescent proteins and other fluorescent compounds to reach maximal multiplexity.

The reaction behind chemical inactivation of Alexa dyes in our protocol is base-catalyzed oxidation. The dye molecules are irreversibly modified after oxidation, leaving the non-fluorescent antibodies within specimens. Recently, the Riordan group at Stanford University and the GE Global Research Center have reported the use of similar chemistry for multiplexed immunofluorescence in tissue samples, though their approaches are performed on less stable cyanine dyes (Cy3 and Cy5) (Gerdes et al., 2013; Riordan, Varma, West, & Brown, 2015).

Given the similar chemical principle behind our method and the two other methods, this suggests not only the possible application of our method in FFPE, but also inactivation of other fluorescent molecules (FITC, TRITC, Cy3, Cy5 etc.) with the same chemical process. We have tested our protocol using TRIC or FITC-conjugated antibodies and obtained effective inactivation with base-catalyzed oxidation. We are continually updating and optimizing our protocol (e.g. applying CycIF to FFPE tissue samples and frozen sections); and plan to post updated protocols on the public website (http://lincs.hms.harvard.edu/lin-NatCommun-2015/).

Critical Parameters

To achieve consistent staining using CycIF protocol, several key factors must be considered. First, samples/cells must be fixed extensively to prevent dissociation following multiple steps of washing. We suggest using a combination of paraformaldehyde and methanol fixation to achieve maximum fixation. However, combinations of other fixatives can be used depending on the type of antigen to be detected. For example, acetone/methanol fixation leads to better results when cytoskeleton or other structural proteins are of interest. Second, imaging devices used in CycIF require proper stage control capability as well as high-efficiency sensors. This is because multiplexed single-cell measurements in CycIF rely on revisiting the same position across different staining cycles. Post-processing with image re-registration is used to merge images from different cycles, although signals from cells on the edge might be lost during this process. Using low-magnification objectives (4x or 10x) and wide-field charge coupled device (CCD) or complementary metal oxide semiconductor (CMOS) image sensors will help to overcome this problem. Another key factor for a successful CycIF experiment is to obtain Alexa dye-conjugated antibodies suitable for immunofluorescence. We have tested antibodies from

different vendors, including Cell Signaling Technology, Abcam, and BioLegend and found that vendor-provided usage information (dilution/buffer/applications) for most antibodies is not optimal. Therefore, re-validation and optimization of these conditions for different cell types and/or sample preparation procedures seems to be essential (Hoffman et al., 2008). Here, we provide a list of antibodies we have tested in several different cell lines, and future updates will be available on our website (http://lincs.hms.harvard.edu/lin-NatCommun-2015/).

Troubleshooting

Insufficient inactivation

In general, a one-hour incubation in base-hydrogen peroxide mixture is sufficient for Alexa Fluor© 488/555/647 dyes to bleach near completely and to reach pre-staining fluorescence levels. However, if necessary, longer incubation can be used to eliminate residual fluorescence. In the case of strong fluorescent signals resulting, for example, from indirect immunofluorescence (primary/secondary antibody complex), increasing the H_2O_2 concentration from 3% to 4.5% is helpful. Photo-bleaching using white light improves the efficiency and the speed of fluorophore inactivation (**Figure 2**).

Uneven staining

It is essential to avoid uneven immunostaining that can result from different sources such as incomplete fixation or permeabilization, insufficient antibody incubation, or poor washing. Ample amounts of PFA and methanol should be used to ensure complete fixation and permeabilization. For antibody incubation, it is important to increase the volume of antibody solution in proportion to the area of the sample. Here, we suggest at least 50 µl of antibody solution per well in a 96-well plate. Applying sufficient volume of solution is essential to

achieve uniform staining and to increase signal to noise ratio. Finally, washing steps should be performed with plenty of PBS (250 μl per well per wash) using an automatic washer/dispenser if possible. Manual wash with gentle aspiration/dispense is also workable.

Cell loss

We discussed in previous sections that several steps are critical to reduce cell loss during CycIF, including sufficient fixation and gentle wash. Cell density is another key factor that influences cell-to-cell and cell-to-plate adhesion. Very low or high cell density both lead to dissociation of cells during CycIF. Collagen or poly-lysine coating might strengthen cell adhesion and reduce cell loss in loosely adherent cells. In addition, the cell detachment and the antigen degradation increase over time once cells are permeabilized, so you should try to finish the CycIF cycles as soon as possible.

Low signal

Since conjugated antibodies are used in the CycIF protocol, unamplified signals from direct immunofluorescence are difficult to detect in the case of low abundance proteins. Using bright dyes like Alexa Fluor© with sensitive CCD/CMOS cameras should help increase signal to noise ratio. Adding more antibodies enhances the signal but can also lead to higher background noise. When the signal from direct immunofluorescence is too weak for an antibody, an indirect immunofluorescence staining in the first cycle of CycIF is suggested.

Anticipated Results

For most of the adherent cells we have tested, five rounds of CycIF can be performed without significant cell loss. Fifteen different antibody signals can be collected from the whole procedure. **Figure 5** illustrates staining from 15 different antibodies in the same fields in RPE cell line. For

strongly adherent cells like MCF10A, >5 rounds of CycIF is feasible and yields highly multiplexed single-cell measurements. We have so far successfully performed 10 rounds of CycIF on these cells, yielding a dataset of ~15,000,000 data-points from ~400,000 cells in a single plate (Honarnejad et. al., in preparation). Such high dimensional single-cell data demand development of advanced image and data analysis methods in the near future.

Time Considerations

Since a typical round of CycIF can be performed within one day (covering overnight incubation of antibody plus imaging and inactivation time), a five-round CycIF experiment can be completed within one week. However, to maintain consistent signals and view fields for each round and the requirement of image registration, all imaging must be performed on the same instrument. Thus, the bottleneck limiting the CycIF throughput is usually imaging time because of the sequential nature of this procedure. In addition, high-dimensional single-cell image analysis is itself time-consuming and therefore requires high-end computers or CPU clusters for accelerating the process.

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LITERATURE CITED

Amir, E. D., Davis, K. L., Tadmor, M. D., Simonds, E. F., Levine, J. H., Bendall, S. C., … Pe'er, D. (2013). viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. *Nature Biotechnology*, *31*(6), 545–52. doi:10.1038/nbt.2594

Angelo, M., Bendall, S. C., Finck, R., Hale, M. B., Hitzman, C., Borowsky, A. D., … Nolan, G. P. (2014). Multiplexed ion beam imaging of human breast tumors. *Nature Medicine*, *20*(4), 436–442. doi:10.1038/nm.3488

Bendall, S. C., Davis, K. L., Amir, E.-A. D., Tadmor, M. D., Simonds, E. F., Chen, T. J., … Pe'er, D. (2014). Single-cell trajectory detection uncovers progression and regulatory coordination in human B cell development. *Cell*, *157*(3), 714–25. doi:10.1016/j.cell.2014.04.005

Bendall, S. C., Simonds, E. F., Qiu, P., Amir, E. D., Krutzik, P. O., Finck, R., … Nolan, G. P. (2011). Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science (New York, N.Y.)*, *332*(6030), 687–96. doi:10.1126/science.1198704

Brooks, M. D., Burness, M. L., & Wicha, M. S. (2015). Therapeutic Implications of Cellular Heterogeneity and Plasticity in Breast Cancer. *Cell Stem Cell*, *17*(3), 260–271. doi:10.1016/j.stem.2015.08.014

Donaldson, J. G. (2002). Immunofluorescence staining. *Current Protocols in Immunology / Edited by John E. Coligan ... [et Al.]*, *Chapter 21*, Unit 21.3. doi:10.1002/0471142735.im2103s48

Gerdes, M. J., Sevinsky, C. J., Sood, A., Adak, S., Bello, M. O., Bordwell, A., … Ginty, F. (2013). Highly multiplexed single-cell analysis of formalin-fixed, paraffin-embedded cancer tissue. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(29), 11982–7. doi:10.1073/pnas.1300136110

Gerdes, M. J., Sood, A., Sevinsky, C., Pris, A. D., Zavodszky, M. I., & Ginty, F. (2014). Emerging understanding of multiscale tumor heterogeneity. *Frontiers in Oncology*, *4*, 366. doi:10.3389/fonc.2014.00366

Hoffman, G. E., Le, W. W., & Sita, L. V. (2008). The importance of titrating antibodies for immunocytochemical methods. *Current Protocols in Neuroscience / Editorial Board, Jacqueline N. Crawley ... [et Al.]*, *Chapter 2*, Unit 2.12. doi:10.1002/0471142301.ns0212s45

Honarnejad, S, Lin, J.R., Bandara, S., Hafner, M. & Sorger, P.K. Measuring population averaged and single-cell drug response using multiplexed, low-cost imaging assays (in preparation)

Hoppe, P. S., Coutu, D. L., & Schroeder, T. (2014). Single-cell technologies sharpen up mammalian stem cell research. *Nature Cell Biology*, *16*(10), 919–27. doi:10.1038/ncb3042

Irish, J. M., & Doxie, D. B. (2014). High-dimensional single-cell cancer biology. *Current Topics in Microbiology and Immunology*, *377*, 1–21. doi:10.1007/82_2014_367

Lin, J.-R., Fallahi-Sichani, M., & Sorger, P. K. (2015). Highly multiplexed imaging of single cells using a high-throughput cyclic immunofluorescence method. *Nature Communications*, *6*, 8390. doi:10.1038/ncomms9390

Panchuk-Voloshina, N., Haugland, R. P., Bishop-Stewart, J., Bhalgat, M. K., Millard, P. J., Mao, F., & Leung, W. Y. (1999). Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. *The Journal of Histochemistry and Cytochemistry : Official Journal of the Histochemistry Society*, *47*(9), 1179–88. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10449539

Riordan, D. P., Varma, S., West, R. B., & Brown, P. O. (2015). Automated Analysis and Classification of Histological Tissue Features by Multi-Dimensional Microscopic Molecular Profiling. *PloS One*, *10*(7), e0128975. doi:10.1371/journal.pone.0128975

Robertson, D., Savage, K., Reis-Filho, J. S., & Isacke, C. M. (2008). Multiple immunofluorescence labelling of formalin-fixed paraffin-embedded (FFPE) tissue. *BMC Cell Biology*, *9*, 13. doi:10.1186/1471-2121-9-13

Schweller, R. M., Zimak, J., Duose, D. Y., Qutub, A. A., Hittelman, W. N., & Diehl, M. R. (2012). Multiplexed in situ immunofluorescence using dynamic DNA complexes. *Angewandte Chemie (International Ed. in English)*, *51*(37), 9292–6. doi:10.1002/anie.201204304

Zanella, F., Lorens, J. B., & Link, W. (2010). High content screening: seeing is believing. *Trends in Biotechnology*, *28*(5), 237–45. doi:10.1016/j.tibtech.2010.02.005

Zrazhevskiy, P., & Gao, X. (2013). Quantum dot imaging platform for single-cell molecular profiling. *Nature Communications*, *4*, 1619. doi:10.1038/ncomms2635

FIGURE LEGENDS

Figure 1: Three ways to achieve highly multiplexed read-outs in single cells. Please refer to the text for details.

Figure 2: The set-up of light device for fluorophore inactivation.

Figure 3. Experimental workflow with time estimations. Illustrated here is a typical CycIF cycle. Start from the step of antibody incubation, and follow by washing, imaging and preliminary data analysis. The fluorophore inactivation will be performed after the confirmation of image/data quality. Once full cycle can be completed in 24 hours.

Figure 4. High-dimensional analysis illustrations for CycIF data. Shown here are four methods for high-dimensional data analyses for CycIF results. The Principle Component Analysis (PCA) and viSNE algorithm are generally used for dimension reduction and visualization of CycIF data (Amir et al., 2013; Lin et al., 2015). The cross-correlation method is for probing the relationships between different signals measured by CycIF. The Wanderlust algorithm is a graph-based trajectory detection method to convert CycIF single-cell data to one-dimensional trajectory (Bendall et al., 2014; Lin et al., 2015).

Figure 5. Example images for a five-round CycIF experiment. Retinal pigment epithelium (RPE) cells were cultured and fixed in 96 wells. 5 rounds of CycIF staining were performed and representative images are shown with antibodies as indicated.

Table 1. List of antibodies tested for CycIF protocol

Alexa-488/FITC conjugated	Alexa-555/TRITC conjugated	Alexa-647 conjugated
***p-ERK1/2 T202/Y204 (CST #4344, Lot #12) : 1:200	***p-Rb S807/S811 (CST #8957, Lot #1) : 1:400	***p21 Waf1/Cip1 (CST #8587, Lot #3) : 1:200
***EGFR (CST #5616, Lot #4) : 1:400	***p-Histone H3 S10 (CST #3475, Lot #2) : 1:800	***p-S6 S235/S236 (CST #4851, Lot #22) : 1:400
***Lamin A/C (CST #8617, Lot #2) : 1:400	***β-Actin (CST #8046, Lot #1) : 1:200	***beta-Tubulin (CST #3624, Lot #4) : 1:200
***p-S6 S240/S244 (CST #5018, Lot #4) : 1:800	***VEGFR2 (CST #12872, Lot #1) : 1:400	***beta-Catenin (CST #4627, Lot #5) : 1:400
***PCNA (CST #8580, Lot #1) : 1:400	***Vimentin (CST #9855, Lot #1) : 1:200	***mTOR (CST #5048, Lot #2) : 1:300
***Ki-67 (CST #11882, Lot #4) : 1:400	***p-S6 S235/S236 (CST #3985, Lot #4) : 1:300	***pan-Akt (CST #5186, Lot #3) : 1:400
***Cyclin D1 (AB #AB190194, Lot #GR199456-1) : 1:400	***p-AuroraABC (CST #13464, Lot #1) : 1:200	***p65 NFkB (AB #AB190589, Lot #GR199457-1) : 1:800
***Bax (BIO #633603, Lot #B169774) : 1:400	***S6 (CST #6989, Lot #2) : 1:200	***p27 (AB #AB194234, Lot #GR200274-1) : 1:400
**EpCAM (CST #5198, Lot #9) : 1:100	**pan-Keratin (CST #3478, Lot #4) : 1:200	p75 NGF Receptor (AB #AB195180, Lot #GR203573-1) : 1:400
**c-JUN (AB #AB193780, Lot #GR203494-1) : 1:400	**p-Histone H2A.X S139 (CST #8228, Lot #3) : 1:200	***p-H2.AX S139 (CST #9270, Lot #15) : 1:400
**E-Cadherin (CST #3199, Lot #11) : 1:200	**LC3A/B (CST #13173, Lot #1) : 1:200	***Vimentin (CST #9856, Lot #7) : 1:800
*p-c-JUN (CST #12714, Lot #6)	**ActinRed 555 (Invitrogen #R371112, Lot #1646656)	***CD45 (BIO #304020, Lot #B1810139) : 1:400
*p-CREB (CST #9187, Lot #6)	**cPARP (CST #6894, Lot #1) : 1:200	***p-H2.AX S139 (BIO #613407, Lot #B199199) : 1:400
*p-HSP27 (CST #12172, Lot #1)	**p21 Waf1/Cip1 (CST #8493, Lot: #2) : 1:200	**p-Tyrosine (CST #9415, Lot #8) : 1:100
*Cyclin B1 (SC #SC-752, Lot #K1008)	*Sox2 (CST #5179, Lot #4)	**Her2 (BIO #324412, Lot #B179768) : 1:200
*cdc2/CDK1 (SC #SC-54, Lot #G0606)	*Oct-4A (CST #4439, Lot #1)	*FOXO3a (AB #AB196539, Lot #GR202407-1)
	*cMyc (SC #SC-40, Lot #B2813)	*Bcl-2 (BIO #658705, Lot #B180139)

*Has not worked in any cell line or condition tested to date	**Works in specific cells/conditions,	***Works across all cell lines and conditions tested to date

CST = Cell Signaling Technology AB = Abcam BIO = BioLegend SC = Santa Cruz Biotechnology

Table 2. An example of 6-round CycIF experiment using both indirect and direct IFs

	Round 1	Round 2	Round 3	Round 4	Round 5	Round 6
Alexa 488	Foxo3a(R)[1]	p-ERK	CycD1	p-S6(240)	Bax	PCNA
Alexa 555	Actin-555[2]	p-RB	p-Aurora	p-H3	pan-S6	Keratin
Alexa 647	p53(m)[3]	p21	p27	p-S6(235)	γH2ax	AKT

1. Rabbit Foxo3a antibody plusAlexa-488 goat anti-rabbit IgG
2. ActinRed™ 555 ReadyProbes® Reagent from ThermoFisher/Invitrogen
3. Mouse p53 antibody plus Alexa-647 goat anti-rabbit IgG

Figure 1

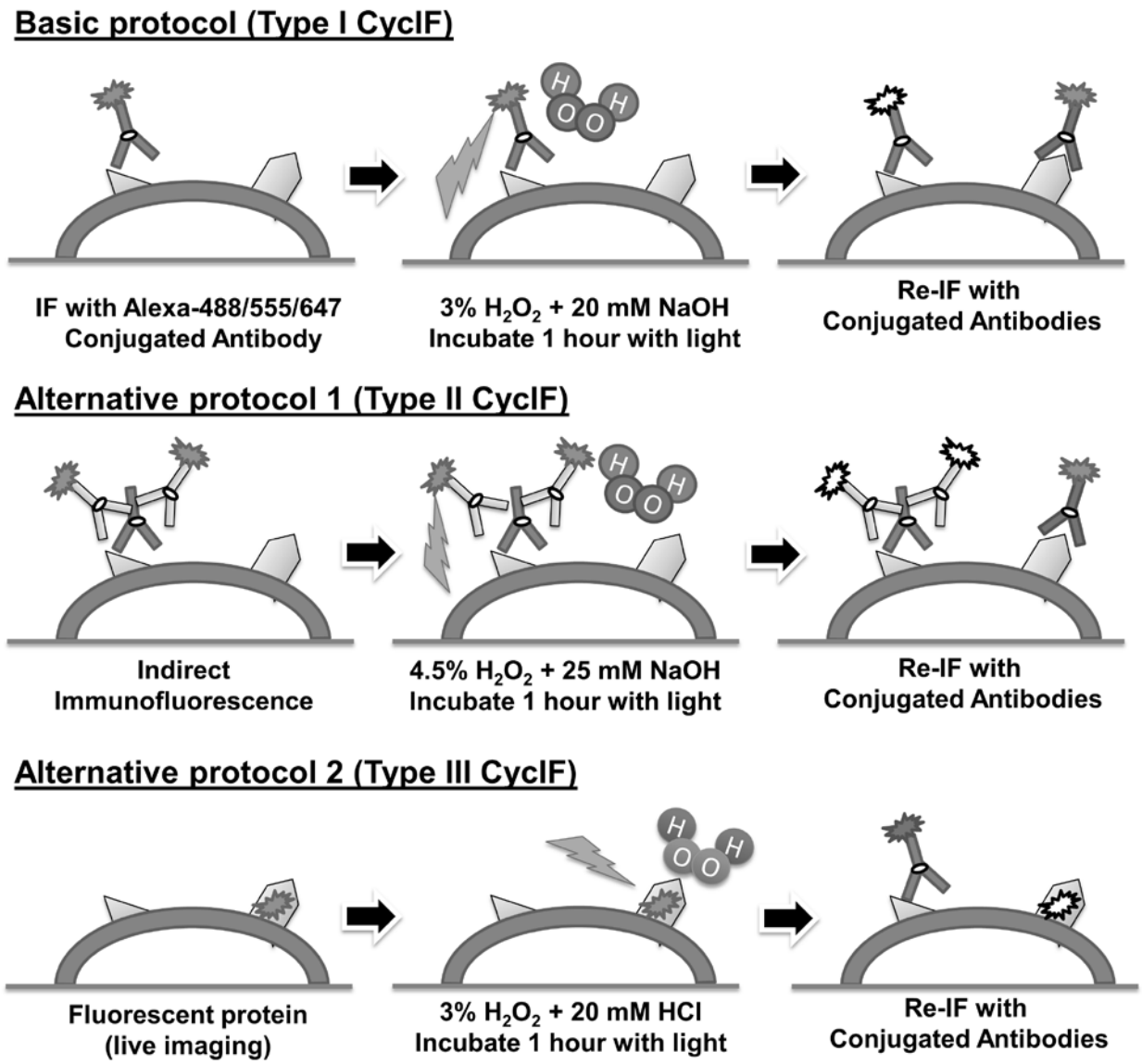

Basic protocol (Type I CycIF)
IF with Alexa-488/555/647 Conjugated Antibody
3% H_2O_2 + 20 mM NaOH Incubate 1 hour with light
Re-IF with Conjugated Antibodies
Alternative protocol 1 (Type II CycIF)
Indirect Immunofluorescence
4.5% H_2O_2 + 25 mM NaOH Incubate 1 hour with light
Re-IF with Conjugated Antibodies
Alternative protocol 2 (Type III CycIF)
Fluorescent protein (live imaging)
3% H_2O_2 + 20 mM HCl Incubate 1 hour with light
Re-IF with Conjugated Antibodies

Figure 2

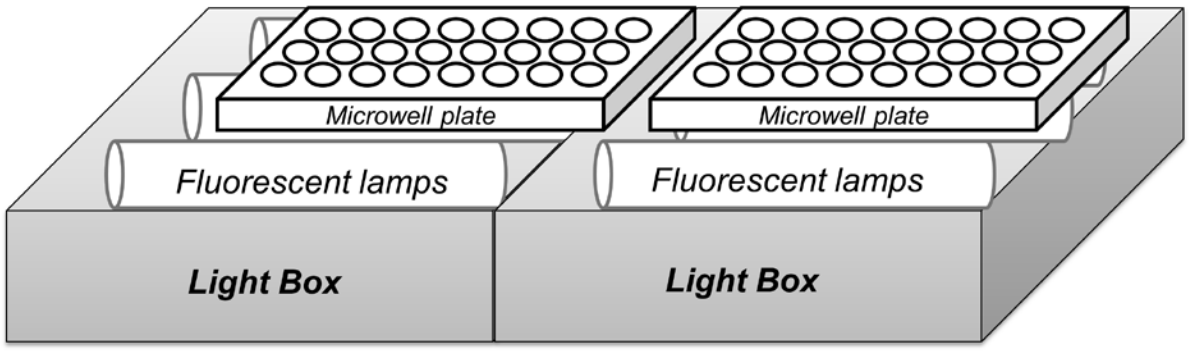

Microwell plate
Microwell plate
Fluorescent lamps
Fluorescent lamps
Light Box
Light Box

Figure 3

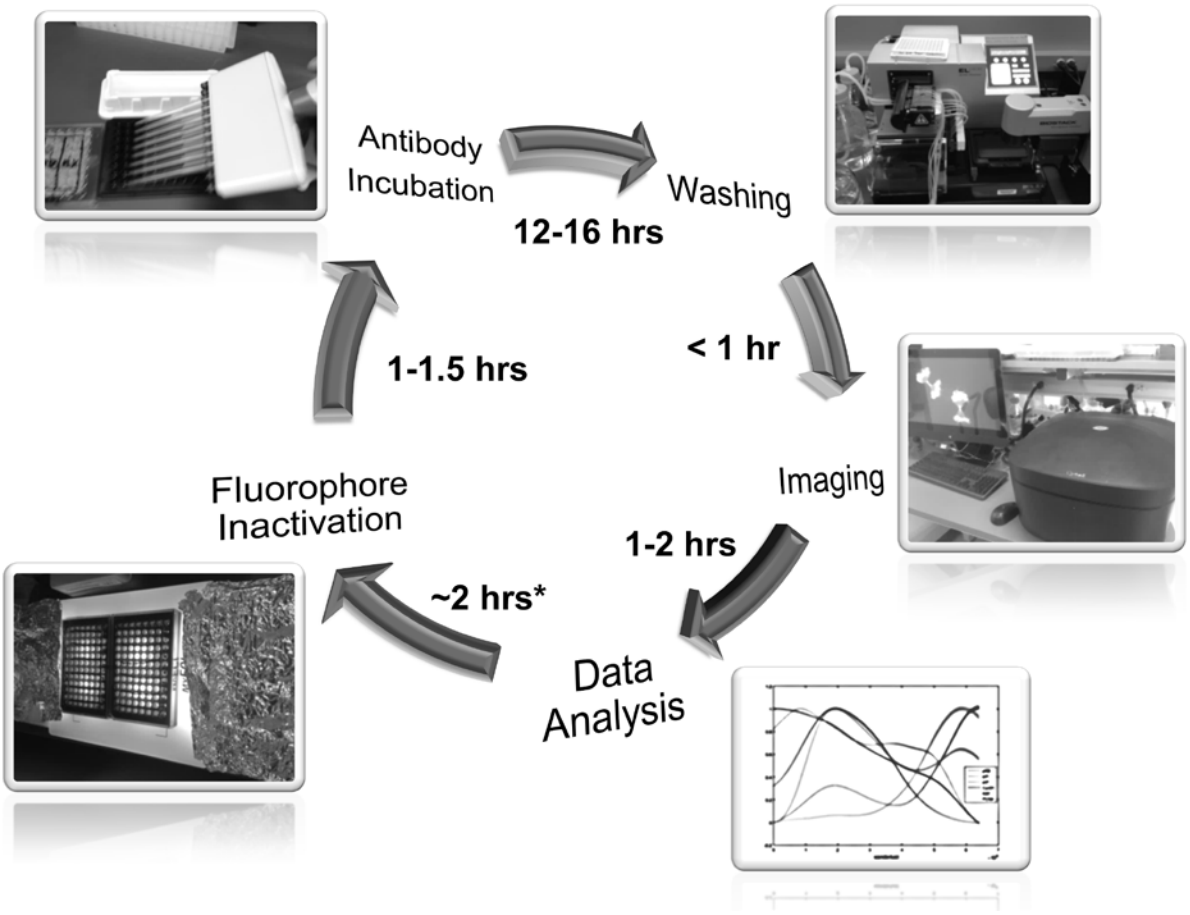

Antibody Incubation
12-16 hrs
Washing
< 1 hr
Imaging
1-2 hrs
Data Analysis
~2 hrs*
Fluorophore Inactivation
1-1.5 hrs

Figure 4

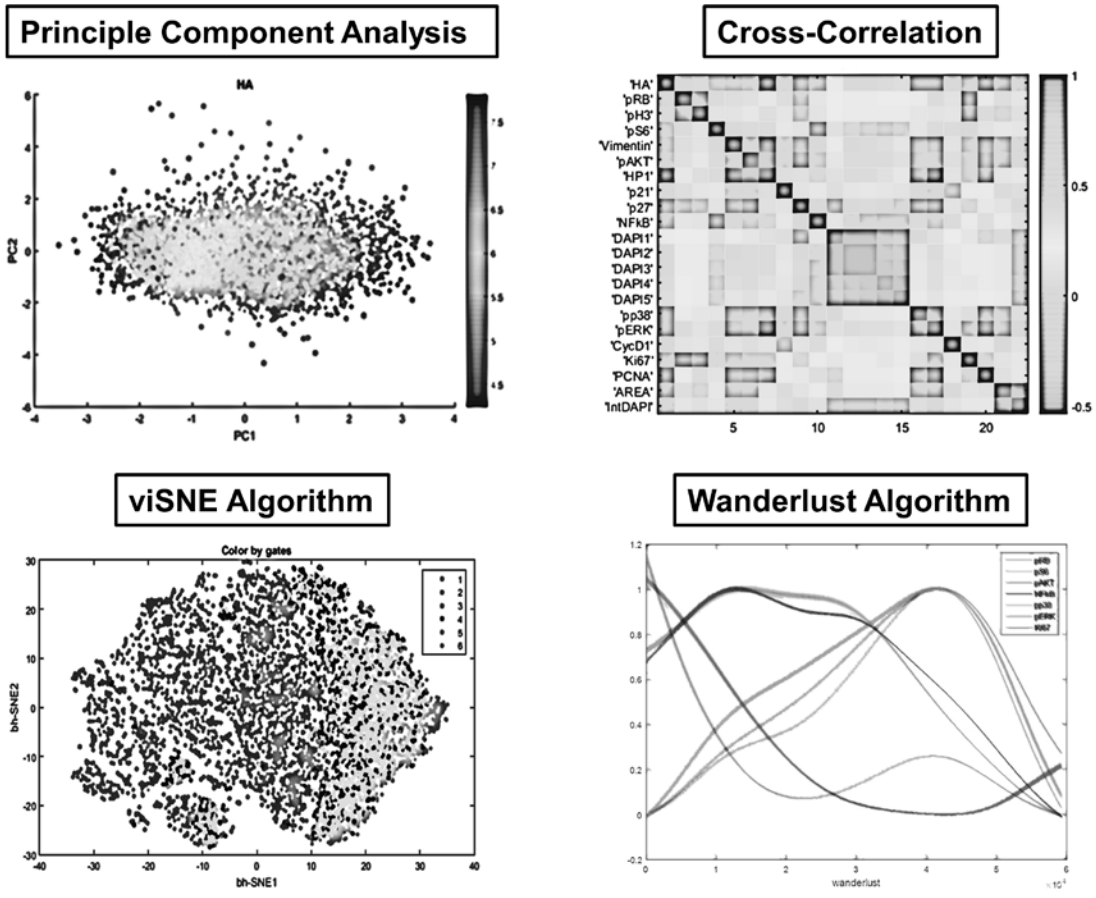
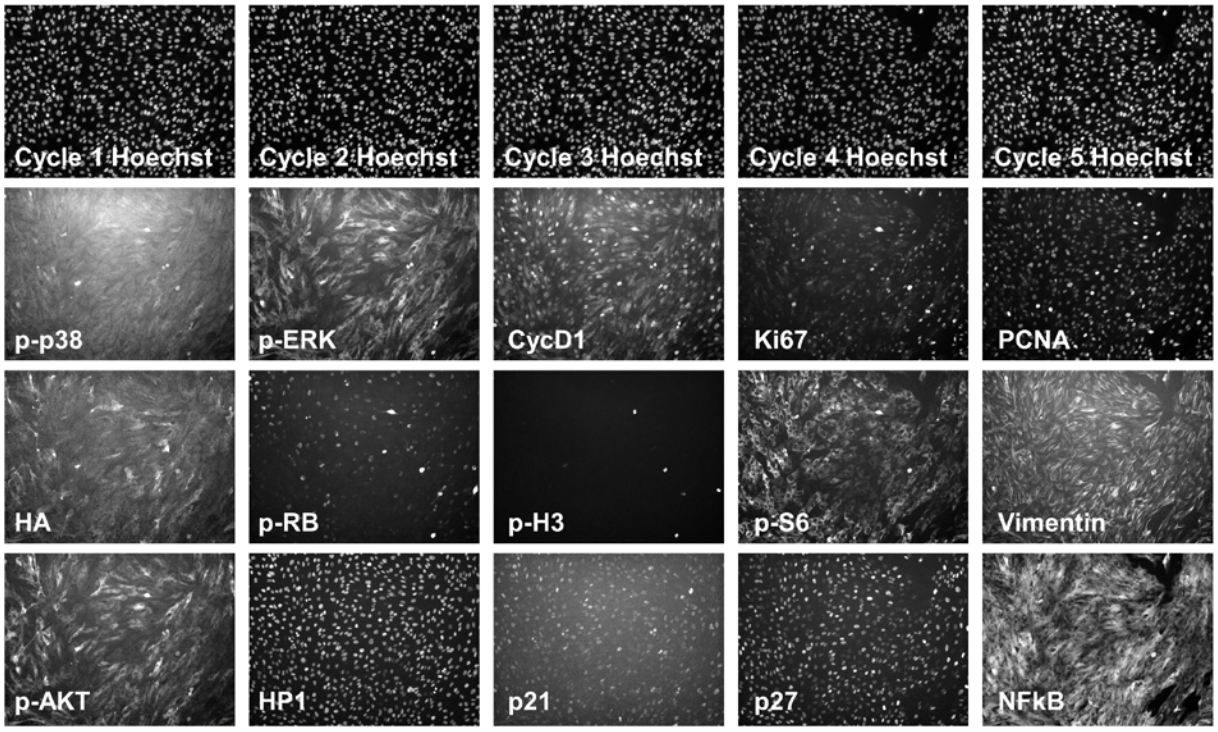

Principle Component Analysis
HA
PC1
PC2
Cross-Correlation
'HA'
'pRB'
'pH3'
'pS6'
'Vimentin'
'pAKT'
'HP1'
'p21'
'p27'
'NFkB'
'DAPI1'
'DAPI2'
'DAPI3'
'DAPI4'
'DAPI5'
'pp38'
'pERK'
'CycD1'
'Ki67'
'PCNA'
'AREA'
'IntDAPI'
viSNE Algorithm
Color by gates
bh-SNE1
bh-SNE2
Wanderlust Algorithm
wanderlust

Figure 5


Cycle 1 Hoechst
Cycle 2 Hoechst
Cycle 3 Hoechst
Cycle 4 Hoechst
Cycle 5 Hoechst
p-p38
p-ERK
CycD1
Ki67
PCNA
HA
p-RB
p-H3
p-S6
Vimentin
p-AKT
HP1
p21
p27
NFkB