# tCyCIF protcol (Leica BOND) version 0.1

**Jia-Ren Lin,Benjamin Izar,Shaolin Mei,Shu Wang,Peter Sorger**

**Abstract**

**The architecture of normal and diseased tissues strongly influences the development and progression of disease as well as responsiveness and resistance to therapy. We describe a tissue-based cyclic immunofluorescence (t-CyCIF) method for highly multiplexed immuno-fluorescence imaging of formalin-fixed, paraffin-embedded (FFPE) specimens mounted on glass slides, the most widely used specimens for histopathological diagnosis of cancer and other diseases. t-CyCIF generates up to 60-plex images using an iterative process (a cycle) in which conventional low-plex fluorescence images are repeatedly collected from the same sample and then assembled into a high dimensional representation. t-CyCIF requires no specialized instruments or reagents and is compatible with super-resolution imaging; we demonstrate its application to quantifying signal transduction cascades, tumor antigens and immune markers in diverse tissues and tumors. The simplicity and adaptability of t-CyCIF makes it an effective method for pre-clinical and clinical research and a natural complement to single-cell genomics.**

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## Guidelines

Key resources, reagents and software used in this study are listed on-line at the HMS LINCS Center Publication Page http://lincs.hms.harvard.edu/lin-elife-2018/ (RRID:SCR_016370). This page provides links to an OMERO image database from which individual images can be obtained; stitched and registered image panels can be obtained at www.cycif.org.(RRID:SCR_016267) and a video illustrating the t-CyCIF method can be found at https://vimeo.com/269885646.

## Materials

Hydrogen peroxide solution contains inhibitor, 30 wt. % in H2O, ACS reagent
216763-500ML by Sigma Aldrich

1N NaOH by Contributed by users

Phosphate Buffered Saline: 5 L of 20X
sc-362299 by Santa Cruz Biotechnology

Odyssey® Blocking Buffer (PBS) 927-40000 by Licor

Hoechst 33342, Trihydrochloride, Trihydrate - 10 mg/mL Solution in Water H3570 by Invitrogen - Thermo Fisher

## Protocol

### Pre-processing and pre-staining tissues for t-CyCIF (Leica BOND RX)

***Step 1.***

Pre-processing of FFPE tissue and tumor slices mounted on slides was performed on a Leica BOND RX automated stained using the following protocol:

| Step | Reagent | Supplier | Incubation (min) | Temp. (°C) |
|---|---|---|---|---|
| 1 | *No Reagent | N/D | 30 | 60 |
| 2 | BOND Dewax Solution | Leica | 0 | 60 |
| 3 | BOND Dewax Solution | Leica | 0 | R.T. |
| 4 | BOND Dewax Solution | Leica | 0 | R.T. |
| 5 | 200 proof ethanol | User* | 0 | R.T. |
| 6 | 200 proof ethanol | User* | 0 | R.T. |
| 7 | 200 proof ethanol | User* | 0 | R.T. |
| 8 | Bond Wash Solution | Leica | 0 | R.T. |
| 9 | Bond Wash Solution | Leica | 0 | R.T. |
| 10 | Bond Wash Solution | Leica | 0 | R.T. |
| 11 | Bond ER1 solution | Leica | 0 | 99 |
| 12 | Bond ER1 solution | Leica | 0 | 99 |
| 13 | Bond ER1 solution | Leica | 20 | 99 |
| 14 | Bond ER1 solution | Leica | 0 | R.T. |
| 15 | Bond Wash Solution | Leica | 0 | R.T. |
| 16 | Bond Wash Solution | Leica | 0 | R.T. |
| 17 | Bond Wash Solution | Leica | 0 | R.T. |
| 18 | Bond Wash Solution | Leica | 0 | R.T. |
| 19 | Bond Wash Solution | Leica | 0 | R.T. |
| 20 | IF Block | User* | 30 | R.T. |
| 21 | Antibody Mix | User* | 60 | R.T. |
| 22 | Bond Wash Solution | Leica | 0 | R.T. |
| 23 | Bond Wash Solution | Leica | 0 | R.T. |
| 24 | Bond Wash Solution | Leica | 0 | R.T. |

| | | | | |
|---|---|---|---|---|
| 25 | Hoechst Solution | User* | 30 | R.T. |
| 26 | Bond Wash Solution | Leica | 0 | R.T. |
| 27 | Bond Wash Solution | Leica | 0 | R.T. |
| 28 | Bond Wash Solution | Leica | 0 | R.T. |

Steps 2-10: Dewaxing & Rehydration with Leica Bond Dewax Solution Cat. AR9222.
Steps 11-14: Antigen retrieval with BOND Epitope Retrieval solution 1 (ER1; Cat. AR9961)
Steps 15-19: Washing with Leica Bond Wash Solution (Cat. AR9590).
Steps 20-28 Pre-staining procedures as shown in Figure 1A:
Step 20: IF Block - Immunofluorescence blocking in Odyssey blocking buffer (LI-COR, Cat. 927401)
Step 21: Antibody Mix - Incubation with secondary antibodies diluted in Odyssey blocking buffer
Step 25: Staining with Hoechst 33342 at 2 µg/ml (w/v) in in Odyssey blocking buffer

EQUIPMENT
Equipment brand:
Leica BOND RX
SKU:
3342171
Specifications:
https://www.leicabiosystems.com/ihc-ish-fish/ihc-ish-instruments/products/leica-bond-rx/

leica_bond_rx.jpg

## Prestaining (manual)

***Step 2.***

Dewaxed specimens were blocked by incubation with Odyssey blocking buffer for 30 mins by applying the buffer to slides as a 250-500 µl droplet at room temperature; evaporation was minimized by using a slide moisture chamber (Scientific Device Laboratory, 197-BL). Slides were then pre-stained by incubation with diluted secondary antibodies (listed above) for 60 minutes, followed by washing 3 times with 1xPBS. Finally, slides were incubated with Hoechst 33342 (2 µg/ml) in 250-500 µl Odyssey blocking buffer for 30 min in a moisture chamber and washed 3 times with 1xPBS in vertical staining jars. After imaging, cells were subjected to a round of fluorophore inactivation (see below). Following fluorophore inactivation, slides were washed 4 times with 1x PBS by dipping them in a series of vertical staining jars to remove residual inactivation solution.

## Performing cyclic immunofluorescence

***Step 3.***

All primary antibodies (fluorophore-conjugated and unconjugated) were diluted in Odyssey blocking buffer. Slides carrying tissues that had been subjected to pre-staining, or to a previous t-CyCIF stain and bleach cycle, were incubated at 4°C for 12 hr with diluted primary or fluorophore-conjugated antibody (250-500 µl per slide) in a moisture chamber. Long incubation times were a matter of convenience and many antibodies only require short incubation with sample. Slides were then washed four times in 1x PBS by dipping in a series of vertical staining jars.
For indirect immunofluorescence, slides were incubated in diluted secondary antibodies in a moisture chamber for 1 hr at room temperature followed by four washes with 1xPBS. Slides were incubated in Hoechst 33342 at 2 µg/ml in Odyssey blocking buffer for 15 min at room temperature, followed by four washes in 1xPBS. Stained slides were mounted prior to image acquisition (see the Mounting section below).

## Mounting & de-coverslipping

***Step 4.***

Immediately prior to imaging, slides were mounted with 1xPBS or, if imaging was expected to take longer than 30 minutes, for example, in the case of samples larger than 2-4 $cm^2$ (corresponding to about 200 fields of view with a 10X objective) PBS was supplement with 10% Glycerol. Slides were covered using 24 x 60mm No. 1 coverslips (VWR 48393-106) to prevent evaporation while facilitating subsequent de-coverslipping via gravity. Following image acquisition, slides were placed in a vertical staining jar containing 1xPBS for at least 15 min. Coverslips were released from slides (and the tissue sample) via gravity as the slides were slowly drawn out of the staining jar

## Fluorophore inactivation (bleaching)

***Step 5.***

After imaging, fluorophores were inactivated by placing slides horizontally in 4.5% $H_2O_2$ and 24 mM NaOH made up in PBS for 1 hour at RT in the presence of white light. Following fluorophore inactivation, slides were washed 4 times with 1x PBS by dipping them in a series of vertical staining jars to remove residual inactivation solution.

## Image acquisition (RareCyte CyteFinder)

***Step 6.***

Stained slides from each round of CyCIF were imaged with a CyteFinder slide scanning fluorescence microscope (RareCyte Inc. Seattle WA) using either a 10X (NA=0.3) or 40X long-working distance objective (NA = 0.6). Imager5 software (RareCyte Inc.) was used to sequentially scan the region of interest in 4 fluorescence channels. These channels are referred to by the manufacturer as a: (i) “DAPI channel” with an excitation filter having a peak of 390 nm and half-width of 18nm and an emission filter with a peak of 435nm and half-width of 48nm; (ii) “FITC channel” having a 475nm/28nm excitation filter and 525nm/48nm emission filter (iii); “Cy3 channel” having a 542nm/27nm excitation filter and 597nm/45nm emission filter and (iv); “Cy5 channel” having a 632nm/22nm excitation filter and 679nm/34nm emission filter. Imaging was performed with 2x2 binning to increase sensitivity, reduce exposure time and reduce photo bleaching.

## Image processing (Software requirments)

***Step 7.***

Code and scripts used in this study are listed in Table 3 and also on-line at the HMS LINCS Center publication page (http://lincs.hms.harvard.edu/lin-elife-2018/). ImageJ is available at https://imagej.nih.gov/ij/

BaSic is available at https://www.helmholtz-muenchen.de/icb/research/groups/quantitative-single-cell-dynamics/software/basic/index.html. Matlab scripts used in this paper and ASHLAR registration/stitching algorithm is available at our GitHub repositories (https://github.com/sorgerlab/cycif and https://github.com/sorgerlab/ashlar.

SOFTWARE PACKAGE (WINDOWS - )

**ImageJ/Fiji, 1.8.0**

National Institutes of Health
http://wsr.imagej.net/distros/win/ij152-win-java8.zi

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## Image processing (Background subtraction and image registration)

***Step 8.***

Background subtraction was performed using the previously established rolling ball algorithm (with a 50-pixel radius) in ImageJ. Adjacent background-subtracted images from the same sample were then registered to each using an ImageJ script as described previously[20]. All images with 2x2 binning in acquisition were partially de-convoluted with unsharp masking. DAPI images from each cycle were used to generate reference coordinates by Rigid-body transformation. To generate virtual hyper-stacked images, the transformed coordinates were applied to images from four channel imaging of each t-CyCIF cycle.

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http://wsr.imagej.net/distros/win/ij152-win-java8.zip

## Image processing (Image Stitching, Shading and flat-field correlation)

***Step 9.***

The BaSiC algorithm[23] was used for shade and flat-field correction in the create of the multi-panel montage images shown in Figures 2B, 6B, 9A and 11A. Additional information can be found on the BaSiC website (https://www.helmholtz-muenchen.de/icb/research/groups/quantitative-single-cell-dynamics/software/basic/index.html). An example of the performance of BaSiC is shown in Figure 2-figure supplement 1. The ImageJ plugin of BaSiC was applied for whole image stacks using the default options. After processing with BaSiC, images stack were stitched with ImageJ/Fiji “Grid stitch” plugin with default options. ASHLAR was used to stich, register and scale images available at http://www.cycif.org/.

## Image processing (Single-cell segmentation & quantification)

***Step 10.***

To obtain intensity values for single cells, images were segmented using a previously described[20] Watershed algorithm based on nuclear staining by Hoechst 33342. Images were initially thresholded using the OTSU algorithm and binarized in the Hoechst channel, which was then used to generate a nuclear mask image. The mask images were then subjected to the Watershed algorithm in ImageJ to obtain single-cell regions of interest (ROIs). From the nuclei, the cytoplasm was captured by centripetal expansion of either of 3 pixels in images obtained with a 10X objective or of 6 pixels in images obtained with a 40X objective, until cell reaching the cell boundaries (cell membrane). The cytoplasm was then defined as the region between the cell membrane and the nucleus. Following cell segmentation, these cell boundaries were used to compute mean and integrated intensity values from all channels. Because ROIs are (initially) defined only by the nuclear signal, this approach is likely to over- or under- segment cells with irregular shapes, which can lead to nuclear, cytosolic or cell membrane “signal contamination” between neighboring and/or stacked cells. Further experimental (e.g. including membrane markers to guide whole-cell rather than nuclear-only segmentation) and analytical algorithms to more accurately segment individual cells (e.g. using deep learning methods to register and apply additional features) would help to improve segmentation. All imageJ scripts used in this manuscript can be found in our Github

repository (https://github.com/sorgerlab/cycif)

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**ImageJ/Fiji, 1.8.0**

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http://wsr.imagej.net/distros/win/ij152-win-java8.zip