

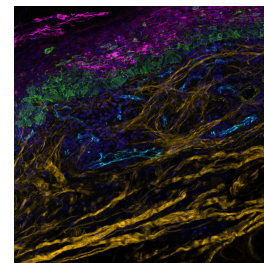
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3D Tissue Cyclic Immunofluorescence (3D CyCIF)

Forked from [Tissue Cyclic Immunofluorescence \(t-CyCIF\) version 3](#)

DOI

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We use this protocol and it's working.

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Abstract

Here, we describe a method for collecting 3-dimensional cyclic immunofluorescence (3D CyCIF) images of human tissue. The original cyclic immunofluorescence (CyCIF) method can generate images with >60 markers in 5-micron thick tissue samples. We extend this original method for confocal imaging of thick tissues (30-40 microns thick). 3D CyCIF can capture multiple layers of intact cells at high resolution, enabling precise analysis of cell phenotypes and complex three-dimensional cell-cell interactions.

This protocol builds off the original t-CyCIF method (see [dx.doi.org/10.17504/protocols.io.5qpvorbndv4o/v2](https://doi.org/10.17504/protocols.io.5qpvorbndv4o/v2)) and was developed as part of the following manuscript:

- Yapp C, Nirmal AJ, Zhou FY, Maliga Z, Tefft J, Montero Llopis P, Murphy GF, Lian C, Danuser G, Santagata S, Sorger PK. Multiplexed 3D Analysis of Immune States and Niches in Human Tissue. *bioRxiv*, 2023. DOI: 10.1101/2023.11.10.566670. PMCID: PMC10680601

Image Attribution

Image is from: <https://tissue-atlas.org/mel-3d-mis-2>



Materials

MATERIALS

☒ Hydrogen peroxide solution 50 wt. % in H₂O, stabilized **Merck MilliporeSigma (Sigma-Aldrich) Catalog #516813-500ML**

☒ 1N NaOH **Merck MilliporeSigma (Sigma-Aldrich) Catalog #1091371000**

☒ DPBS (10X), no calcium, no magnesium **Thermo Fisher Scientific Catalog #14200083**

☒ UltraPure Glycerol **Life Technologies Catalog #15514011**

☒ Hoechst 33342 **Cell Signaling Technology Catalog #4082**

☒ Coverslips thickness 1 1/2 24×50 mm **Corning Catalog #2980-245**

☒ Tissue-Tek® Vertical 24 Slide Rack **American Master Tech Scientific Catalog #LWS2124**

☒ Tissue-Tek Slide Staining Set (Dishes and Baths) **American Master Tech Scientific Catalog #LWS19**

☒ Light Therapy Lamp - UV Free Bright White Led Therapy Light with 15000 LUX **Amazon**

☒ Graduated Glass Cylinder 100 mL **American Master Tech Scientific Catalog #LWG0726**

☒ Centrifuge Tubes 15 mL **Corning Catalog #430790**

☒ Centrifuge tubes 50 mL **Catalog #430808**

☒ SuperBlock®; (PBS) Blocking Buffer **Thermo Fisher Catalog #37515**

Additional Materials:

- Zeiss ZEN Software (protocol based on version 3.9), with the LSM Plus and Stitching plugins
- Precision Cover Glasses, #1.5H Thickness, 24 × 50 mm (Thorlabs, CG15KH1)
- Antibodies (experiment-specific)
- Deionized water
- Ice box
- Pipette tips

Before start

Note that t-CyCIF is optimized for FFPE specimens, which must first undergo dewaxing and antigen retrieval to expose antigenic sites for antibody binding. An example of an automated version of this process can be found here: dx.doi.org/10.17504/protocols.io.4zpgx5n.

Pre-Staining and Blocking

- 1 Pre-staining and blocking take approximately 8 hours.

Place slides in the slide tray, cover all tissues with the secondary antibody solution used in the pre-staining procedure to and incubate in the dark at 4 °C overnight to block non-specific binding. The secondary antibody solution consists of fluorescent secondary antibodies diluted at 1:500 in **Superblock Blocking Buffer**. The species of the secondary antibodies should match any primary antibodies used in the first cycle (if applicable).

- **CRITICAL STEP** Place damp paper towels in the slide tray to maintain humidity and prevent evaporation of the antibody solution.
- **CRITICAL STEP** Do not use a hydrophobic barrier pen on the slides, as we have found that this adversely affects subsequent cycles.
- **CRITICAL STEP** Be careful not to scratch the tissue with pipette tip when applying the antibody solution.
- **CRITICAL STEP** Avoid Alexa Fluor 546-, Alexa Fluor 568-, and Alexa Fluor 594-conjugated antibodies because they are difficult to inactivate.

- 2 Wash slides 1 time with 1X PBS at Room temperature for 00:03:00 (max 5 min) per wash. Slides can be placed into a slide rack and lowered into a staining dish of PBS. Slide(s) should be lowered and raised slowly and gently in PBS .



- 3 Make fluorophore bleaching solution. Combine 25 mL 1X PBS, 4.5 mL 30% (wt/vol) H₂O₂, and 0.8 mL 1 Mass Percent NaOH in a 50-ml centrifuge tube. The final working concentration is 4.5% (wt/vol) H₂O₂ and 20 millimolar (mM) NaOH in PBS. 30 mL fluorophore bleaching solution is enough for four standard slides.

- **CRITICAL STEP** Fluorophore bleaching solution should be prepared immediately before use.

- 4 Place slides flat in a plastic transparent container with the tissue facing up, and then gently pour fluorophore bleaching solution into the container to completely cover tissue. Place the container between two **LED light panels** (one above and one below) at Room temperature for 01:00:00 .

1h

- **CRITICAL STEP** The pre-bleaching step is critical for reducing autofluorescence in the tissue and to inactivate the fluorophores of the secondary antibody from the pre-



staining step.

- **CRITICAL STEP** Light sources that produce excessive heat can damage tissues. LED light sources are therefore preferable, and large flat LED panels are now readily available at low cost (see **"Materials"** for our preferred light panel).
- **CRITICAL STEP** Completely immerse the tissue sections in fluorophore bleaching solution. The solution should be poured very gently over the slide to minimize tissue movement. During the subsequent bleaching process, bubbles will appear and gradually increase in size and number. This indicates that the oxidation reaction is proceeding as expected.

5 Bleach the fluorophores for  01:00:00 at  Room temperature as described.

1h

6 Wash the slides 1 time with 1X PBS at  Room temperature for  00:10:00 .

10m

- **CRITICAL STEP** Wash the slides to remove the fluorophore bleaching solution completely which may affect subsequent t-CyCIF steps.
- **CRITICAL STEP** Slide(s) should be lowered and raised slowly and gently in PBS .



First Round of t-CyCIF

7 Each round of t-CyCIF takes approximately 16-24 hours.

15h

Dilute up to four unconjugated primary antibodies from different species to the appropriate concentration in **Superblock Blocking Buffer** with 1ug/ml Hoechst 33342, cover all the tissue, and incubate in the dark at  4 °C overnight.

- **CRITICAL STEP** In the first round of t-CyCIF, unconjugated primary antibodies can be used. As in conventional immuno-fluorescence, these antibodies must be from different species (e.g. rabbit, mouse, and rat) to allow for detection with species-specific secondary antibodies. The optimal dilution for primary antibodies must be optimized empirically; we usually test across a range of dilutions starting from 1:100, guided by manufacturer's recommendations. The times listed for antibody incubation can be adjusted empirically; we use long incubations at  4 °C for convenience. (See Lin et al., 2018 and for information on increasing the throughput of t-CyCIF experiments.)

8 Wash slides 4 times with 1X PBS at  Room temperature for  00:03:00 (max 5 min) per wash.

15m



- 9 Cover the tissue with secondary antibodies and incubate in the dark at 4 °C for Overnight . 2h
- 10 Wash slides 4 times with 1X PBS at Room temperature for 00:03:00 (max 5 min) per wash. 15m
- 11 Mount coverslips onto slides with 150 µL of 70% glycerol in PBS to prevent dehydration during imaging. Carefully position coverslips over the center of each slide and lower slowly onto the slide to avoid producing bubbles between the coverslip and to prevent scratching tissues. Do not allow coverslip to overhang the edge of the slide. Dry excess liquid by gently pressing the long edges of the slide against a paper towel. 2m

Taking a preview image

- 12 Bring sample to a Zeiss LSM980 laser scanning confocal microscope. Carefully place slide(s) into a rigid slide holder that is compatible with the microscope stage. Acquire a preview image at a low magnification (such as 5x) of Hoechst in wide field imaging mode in the Tile window. 10m
- **CRITICAL STEP** - The slide holder should hold the slide firmly and in a reproducible manner using mechanisms such as springs. Rails are not recommended. Push the slide towards one corner of the holder. The same corner should be used every cycle.
 - **CRITICAL STEP** - Ensure that the stage and focus calibration has been performed after the last power cycle of the microscope. The stage and stage holder should also be level.
 - **CRITICAL STEP** - Save preview image for later. Use the preview image to select regions of interest (ROI) by drawing rectangle or the freehand tool.

First Round of t-CyCIF

- 13 Image at high resolution. Switch to 40x/1.3NA and add appropriate immersion oil when asked to do so. Set appropriate channel bandpasses, excitation lasers, laser power, gain settings, pinhole size (1 Airy unit), frame size, bit depth (16bit), bidirectional scanning, and Z-stack height and spacing. 16h
- **CRITICAL STEP** - Do not change the position of the slide within the slide holder. Use the saved ROI in the imaging software so that the exact same region of tissue is imaged for every cycle of t-CyCIF.
 - **CRITICAL STEP** - Select Autosave and choose a storage location with sufficient storage space.



- **CRITICAL STEP** - If using bidirectional scanning, run Auto correction to minimize aliasing of punctate structures.
- **CRITICAL STEP** - Place at least 4 focus support points in the ROI and choose tilted plane. When setting the focus height for each support point, it is helpful to set this Z-position at the 'far' side of the sample farthest from the objective lens. While focusing through the tissue with auto contrast off, this will appear when the image appears dark and noisy. Repeat for all support points. Set the focus compensation of the reference channel to be half of the Z-stack height but with a negative value. Start the scan and make small adjustments as needed so that the Z-stack starts and stops below and above the sample respectively.
- **TROUBLESHOOTING** - *Weak signal*. Possible reason: Low signal can result because of low level antigen expression. Direct immunofluorescence using conjugated antibodies does not provide the signal amplification that can be generated in indirect immunofluorescence. Solution: Widen the channel bandpass window but without significant crossover into the next channel; increase the detector gain up to ~800V; increase the laser power up to ~5%; increase the antibody concentration during staining step; use the corresponding unconjugated antibodies in the first round instead of the conjugated antibody to see if signal amplification from indirect immunofluorescence improves signal; if necessary, find an alternative antibody.
- **TROUBLESHOOTING** - *Saturating signal*. Possible reason: Abundant antigen in sample or excessive amount of antibody. Solution: Decrease the antibody concentration used during the staining steps; decrease the incubation time of the sample with antibody; decrease the laser power followed by detector gain.
- **TROUBLESHOOTING** - *Channel bleedthrough*. Possible reason: Hoechst has a long emission spectra that can bleed into other channels. Solution: Use multitrack imaging and arrange channels on different tracks. Put Hoechst, Alexafluor555/eFluor570, and Alexafluor 750 on track 1 and put Alexafluor 488 and Alexafluor 647/eFluor660 on track 2. Each track should only have the relevant lasers on. Choose Frame Fast or Line so that optical components do not need to move between tracks. This will decrease image acquisition time.
- **TROUBLESHOOTING** - *Aliasing artifacts in punctate channels*. Possible reason: galvo mirrors not set up for bi-directional scanning. Solution: Run Auto to calibrate.
- **TROUBLESHOOTING** - *Strong signal gradient across each tile*. Possible reason: The microscope stage, slide holder or sample is not level. Solution: Adjust levelling screws or contact a service engineer.
- **TROUBLESHOOTING** - *Strong background signal*. Possible reason: There is dissociation of one or more antibodies into the mounting media. Solution: Reduce antibody concentration during staining and/or use more wash steps for longer duration.

14 After image acquisition, remove coverslips by placing the slides in 1X PBS in a slide staining dish (which holds slides vertically). This can take an hour.

1h



- **CRITICAL STEP** - De-coverslipping is another procedure that requires practice. Always allow coverslips to fall away through gravity. Do not push the coverslips as this will scratch and damage tissues.

15 Perform fluorophore bleaching for ⌚ 01:00:00 at 🌡 Room temperature as described. 1h

16 Wash slides at least 3 times with 1X PBS at 🌡 Room temperature for ⌚ 00:10:00 per wash. 10m

- **CRITICAL STEP** - Wash slides thoroughly to remove fluorophore bleaching solution, since carry-over can adversely affect subsequent t-CyCIF cycles.
- **PAUSE POINT** - Slides may be stored in 1X PBS at 🌡 4 °C for several days. Make sure the entire tissue is covered in 1X PBS. Otherwise, the tissue may become dry and yield poor staining results.

Process Image

17 Process the image using **ZEISS ZEN software** (these instructions are based on ZEN version 3.9). 8h

- Use "LSM Plus" to denoise the image. Use 3D processing. This can take several hours.
- Then use "Stitching" to stitch the image using the nuclear channel as a reference channel. Select Fuse Tiles. This can take several hours. Save the result when done.
- **CRITICAL STEP** - In the ZEN software, use identical parameters for all imaging cycles.

Subsequent Cycles of t-CyCIF

1d 9h 20m

18 Subsequent cycles of t-CyCIF take approximately 16-24 hours each. The maximum number of cycles for 3D CyCIF depends on tissue type. Less dense tissues will degrade more quickly, which is evaluated by counting nuclei in the Hoechst channel. We are able to perform >10 cycles for most tissue types and >20 cycles for some resilient/dense tissues, such as tonsil. 8h

Dilute up to four conjugated antibodies conjugated with different fluorophores in **SuperBlock (PBS) Blocking Buffer** with 1ug/ml Hoechst 33342. Cover all tissue with antibody solution and incubate in the dark at 🌡 Room temperature for 6-8 hours .

- **CRITICAL STEP** - We typically use Alexa Fluor 488-, Alexa Fluor 555-, Alexa Fluor 647-, and Alexa Fluor 750- conjugated primary antibodies. Dilution is optimized empirically starting from 1:100 (vol:vol). If the microscope is equipped with NIR



detectors, alternative antibodies emitting up to 900nm are also suitable but will suffer from lower sensitivity. Consider putting antibodies targeting high expressing non-punctate proteins in this range.

- **CRITICAL STEP** - Avoid using Alexa Fluor 546-, Alexa Fluor 568-, and Alexa Fluor 594- conjugated secondary antibodies, as these fluorophores are difficult to bleach.

19 Wash the slides 4 times with 1X PBS at Room temperature for 00:03:00 (max 5 min) per wash. 20m

20 Mount coverslips onto slides with 150 μ L of 70% (vol:vol) glycerol in PBS and image the saved ROI with the Zeiss LSM980 laser scanning confocal as described. 16h

- **CRITICAL STEP** To reuse previous settings and load ROI, open a previous cycle and click Reuse. Ensure that the objective lens, pinhole size, Z-step size, ROI size, and frame size are consistent for the duration of the experiment.

21 Remove the coverslips as described.

22 Wash the slides 4 times with 1X PBS at Room temperature for 03:00:00 (max 5 min) per wash.

23 Bleach the fluorophores for 01:00:00 at Room temperature as described. 1h

24 Wash the slides 4 times with 1X PBS at Room temperature for 00:03:00 (max 5 min) per wash.

25 Process the image using **ZEISS ZEN software** (these instructions are based on ZEN version 3.9). 8h

- Use "LSM Plus" to denoise the image. Use 3D processing. This can take several hours.
- Save the result when done. Note that stitching is not needed after the first cycle.

- **CRITICAL STEP** - In the ZEN software, use identical parameters for all imaging cycles.

26 Start next t-CyCIF cycle: repeat steps 18-25 for each additional cycle.

- **TROUBLESHOOTING** Blurry images. (*See details in Step 13*)
- **TROUBLESHOOTING** Weak signal. (*See details in Step 13*)
- **TROUBLESHOOTING** Saturating signal. (*See details in Step 13*)
- **TROUBLESHOOTING** Cell loss. Possible reason: Difficult tissue type (very low cell density). Insufficient tissue fixation. Damage from t-CyCIF procedure (*e.g.* rough



handling of samples during washing). Solution: Check pre-analytical variables. Cautious handling of samples during application of antibodies and washing steps as well as during manipulation of coverslips.

- **TROUBLESHOOTING** Signal present after fluorophore bleaching step. Possible reason: Insufficient fluorophore inactivation. Solution: Avoid Alexa Fluor 546-, Alexa Fluor 568-, and Alexa Fluor 594- conjugated antibodies because they are difficult to inactivate; dilute conjugated antibodies further; extend fluorophore bleaching time; check that light is hitting the sample.