# Sample preparation and imaging for large scale 3D spectral confocal imaging of tissues

Tarek Ashkar[1], Michael Ferkowicz[1]

[1]Indiana University School of Medicine, Indianapolis, Indiana

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KPMP
Tech. support email: info@kpmp.org

Stephanie Garceau
University of Washington

ABSTRACT

Frozen participant samples are first sized and preserved for high-resolution 3D imaging. Preserved biopsy sections are imaged, without staining, to understand the sample's structure and condition.Next, the biopsy sections are stained with antibodies and fluorescent dyes to identify specific structures and cells with pathological significance.This stained sample is imaged in its entirety giving a 3D image.Finally, the 3D image is analyzed by experts to assess known and novel pathologies.

This protocol summarizes both label-free imaging and labeling for fluorescence imaging including the labeling, mounting and imaging approaches.The fluorescent labels used herein include conjugated or fluorescent small molecules and conjugated antibodies.Protocol 2 uses both directly conjugated primary antibodies for identifying specific antigens and indirect labeling with a fluorophore conjugated secondary antibody that recognizes the primary antibody.

Multiple rounds of imaging are enabled by the use of a non-hardening mounting media, low charge slides and removable rubber cement as a sealant.Details are described below.

Two imaging approaches are outlined: protocol 1 and 2.Protocol 1 involves label free imaging of auto-fluorescent species and second harmonic generation (SHG) with multiphoton excitation.These modalities are followed by protocol 2, which involves staining with fluorescent small molecules and immuno-fluorescence and subsequent large scale 3D confocal fluorescence imaging.There are three different staining strategies used in protocols, summarized in Table 1.

These protocols start with fixed 50 µm tissue sections prepared as described in the Cryopreservation protocol.

**Table 1. Staining Strategy**
**Version 1**

| Structure/cell type | Target | Species | Vendor (cat #) | Dilution [stock] | Direct labeling | Indirect labeling |
|---|---|---|---|---|---|---|
| Nuclei | DAPI | - | ThermoFisher (D1306) | 1:200 [1 mg/mL] | - | - |
| vasculature | Phalloidin | - | ThermoFisher O7466 | 1:200 [2 U/mL] | Oregon Green | - |
| glomeruli | " | - | " | " | " | - |
| brush borders | " | - | " | " | " | - |
| TAL | THP | Sheep | R&D Systems (AF5144) | 1:200 [0.2 mg/mL] | Alexa 546 | - |
| Proximal tubules | AQP1 | Goat | Santa Cruz (sc-9878) | 1:50 [0.2 mg/mL] | - | donkey anti-goat Alexa 568 (1:200) |
| Neutrophils | MPO | Rabbit | Abcam (ab9535) | 1:50 | - | donkey anti-rabbit DyLight 594 (1:200) |
| macrophages | CD68 | Mouse | Dako (M0876) | 1:50 [0.3 mg/mL] | - | donkey anti-mouse Alexa 633 (1:200) |
| T-cells | CD3 | Mouse | BD Pharmingen (557706) | 1:50 | Alexa 647 | - |
| B-cells | CD45R | Rat | Abcam (ab64100) | 1:50 [0.2 mg/mL] | - | donkey anti-rat Alexa 660 (1:200) |

**Version 2**

| Structure/cell type | Target | Species | Vendor (cat #) | Dilution [stock] | Direct labeling | Indirect labeling |
|---|---|---|---|---|---|---|
| nuclei | DAPI | - | ThermoFisher (D1306) | 1:200 [1 mg/mL] | - | - |
| vasculature | Phalloidin | - | ThermoFisher O7466 | 1:200 [2 U/mL] | Oregon Green | - |
| glomeruli | " | - | " | " | " | - |
| brush borders | " | - | " | " | " | - |
| TAL | THP | Sheep | R&D Systems (AF5144) | 1:200 [0.2 mg/mL] | Alexa 546 | - |
| proximal tubules | AQP1 | Goat | Santa Cruz (sc-9878) | 1:50 [0.2 mg/mL] | - | donkey anti-goat Alexa 568 (1:200) |
| neutrophils | MPO | Rabbit | Abcam (ab9535) | 1:50 | - | donkey anti-rabbit DyLight 594 (1:200) |
| macrophages | CD68 | Mouse | Dako (M0876) | 1:50 [0.3 mg/mL] | - | donkey anti-mouse Alexa 633 (1:200) |
| T-cells | CD3 | Mouse | BD Pharmingen (557706) | 1:50 | Alexa 647 | - |
| eosinophils | SIGLEC8 | Mouse | Biolegend (347102) | 1:50 [0.5 mg/mL] | Alexa 660 | - |

**Version 2.1**

| Structure/cell type | Target | Species | Vendor (cat #) | Dilution [stock] | Direct labeling | Indirect labeling |
|---|---|---|---|---|---|---|
| Nuclei | DAPI | - | ThermoFisher (D1306) | 1:100 [2 mg/mL] | - | - |
| vasculature | Phalloidin | - | ThermoFisher O7466 | 1:200 [2 U/mL] | Oregon Green | - |
| glomeruli | " | - | " | " | " | - |
| brush borders | " | - | " | " | " | - |
| TAL | THP | Sheep | R&D Systems (AF5144) | 1:1000 [0.2 mg/mL] | Alexa 546 | - |
| Proximal tubules | AQP1 | Goat | Santa Cruz (sc-9878) | 1:50 [0.2 mg/mL] | - | donkey anti-goat Alexa 568 (1:200) |
| Neutrophils | MPO | Rabbit | Abcam (ab9535) | 1:50 | - | donkey anti-rabbit DyLight 594 (1:200) |
| macrophages | CD68 | Mouse | Dako (M0876) | 1:50 [0.3 mg/mL] | - | donkey anti-mouse Alexa 633 (1:200) |
| T-cells | CD3 | Mouse | BD Pharmingen (557706) | 1:50 | Alexa 647 | - |
| eosinophils | SIGLEC8 | Mouse | Biolegend (347102) | 1:50 [0.5 mg/mL] | Alexa 660 | - |

GUIDELINES

### *Excitation and filter settings*

Label-free imaging was performed with femtosecond pulsed 910 nm excitation and de-scanned emissions were collected with custom band-passes of 435-465 nm for collecting SHG and 500- 550 for collecting auto-fluorescence. Spectral confocal imaging was performed with sequential scanning at each z frame for 4 excitations with 4 band-passes each giving 16 total channels. The configuration and scan settings are given in Table 2.

### *Protocol Quality Control metrics*

1. Antibody validation: All primary and secondary antibodies are validated either by CLIA or GUDMAP citation, or by internal validation.
2. Negative controls: When tissue is available, secondary only controls will be included.
3. Tissue qualification:

a.A low resolution image of a 12 µm section of the entire biopsy labeled with PAS is collected (dissecting microscope, 6 µm resolution) and evaluated for tissue morphology and sample integrity.

b.The entire biopsy is imaged in 3D prior to labeling using multiphoton excitation to collect second harmonic and autofluorescence images (910 nm excitation, 430-460 nm and 460-660 nm emissions, respectively). Second harmonic images are used to identify/quantify the degree of fibrosis and along with tissue autofluorescence, to characterize tissue structure.

c.The entire biopsy is imaged in 2D using confocal microscopy prior to labeling.(A mosaic of confocal images of a single focal plane collected from the middle of the tissue section, using the 16 channel protocol used to image labeled tissue).These images are used to

characterize levels of background tissue autofluorescence in each channel to identify potentially problematic regions and to inform the process of spectral deconvolution of the labeled tissue.
4. Microscope image collection: Prior to collection of labeled tissues, the performance of the microscope system is verified by quantitative analysis of images collected from a slide containing with 0.1 µm and 4 µm TetraSpeck fluorescent microspheres (Blue – 360 nm excitation, 430 nm emission, Green – 505 nm excitation, 515 nm emission, Orange – 560 nm excitation, 580 nm emission and Dark Red – 660 nm excitation, 680 nm emission).By exciting the fluorescence of the microspheres with each of the four laser lines, and collecting the fluorescence in the four detectors, this procedure provides a sensitive test of the integrated performance of the microscope. Performance is quantified as signal-to-noise in the images of each of the four fluorophores in the 4 µm beads. Departures in signal-to-noise or resolution exceeding 1 standard deviation of the previously established values would be grounds to delay image collection until the microscope deficiency has been remediated.

**Table 4. QC metrics scan settings**

| Metric | Excitation (nm) | Laser power (%) | Detector | Voltage | Gain (%) | Offset | Bandpass (nm) | Line average | Line summation |
|---|---|---|---|---|---|---|---|---|---|
| PSF | 488 | 3 | PMT2 | 690 | NA | -0.18 | 493-600 | 4 | 1 |
| SNR | 405 | 10 | HyD1 | NA | 74 | NA | 410-483 | 1 | 1 |
| SNR | 488 | 0.5 | PMT2 | 650 | NA | -0.18 | 493-575 | 1 | 1 |
| SNR | 552 | 4 | HyD3 | NA | 50 | NA | 575-633 | 1 | 1 |
| SNR | 638 | 0.2 | PMT4 | 800 | NA | -0.18 | 643-776 | 1 | 1 |

5. Digital image analysis:Digital image analysis is used to (1) distinguish 8 fluorescent probes in the 16-channel confocal image volumes (spectral deconvolution), (2) segment individual nuclei in the resulting image volumes and (3) quantify the amount of fluorescence associated with each nucleus in each of the 8 channels.

a. Spectral deconvolution is validated by analysis of 16 channel images of beads labeled each of the fluorescent probes in specific proportions. The number of each type of bead is determined using spectral deconvolution, which is judged valid if the relative proportions of the different beads fall within 10% of the known proportions.

b.The accuracy of nuclear segmentation is quantified as an F1 score (composite of precision and recall) obtained from 5 random fields (quantified relative to manually annotated).Automated nuclear segmentation is judged valid if F1 scores are greater or equal than 0.8

c.Linearity in image collection is ensured by automatic elimination of regions of signal saturation (voxel values exceeding 4095) and detector dropout (voxel values of 0).

MATERIALS

| NAME | CATALOG # | VENDOR |
|---|---|---|
| DAPI Primary Antibody | D1306 | Thermofisher |
| Phalloidin Primary Antibody | O7466 | Thermofisher |
| THP Primary Antibody | AF5144 | R&D Systems |
| AQP1 Primary Antibody | sc-9878 | Santa Cruz Biotechnology |
| MPO Primary Antibody | ab9535 | Abcam |
| CD68 Primary Antibody | M0876 | Dako |
| CD3 Primary Antibody | 557706 | BD Biosciences |

| NAME | CATALOG # | VENDOR |
|---|---|---|
| Siglec-8 Primary Antibody | 347102 | BioLegend |
| Secondary Antibodies | | |
| Phosphate buffered saline pH 7.4 (PBS) | | |
| PBST (PBS & 0.1% Triton X-100) | 807423 | MP Biomedicals |
| PBSTS (PBST & 10% NDS) | 017-000-121 | Jackson Immunoresearch |
| 4% PFA | | |
| 24 or 48 flat well bottom culture dishes | | |
| Fluoromount | F4680 | Sigma |
| Glass slides | 3017 | |
| Coverglass #1.5 22X22mm | 12-541-B | Thermo Fisher Scientific |
| Elmers Rubber Cement | | |
| Silicone grease | | |
| CML Latex Beads | C37253 | Thermofisher |

STEPS MATERIALS

| NAME | CATALOG # | VENDOR |
|---|---|---|
| Phosphate buffered saline pH 7.4 (PBS) | | |
| PBSTS (PBST & 10% NDS) | 017-000-121 | Jackson Immunoresearch |
| PBST (PBS & 0.1% Triton X-100) | 807423 | MP Biomedicals |
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| PBST (PBS & 0.1% Triton X-100) | 807423 | MP Biomedicals |
| Fluoromount | F4680 | Sigma |
| Glass slides | 3017 | |
| Coverglass #1.5 22X22mm | 12-541-B | Thermo Fisher Scientific |
| Coverglass #1.5 22X22mm | 12-541-B | Thermo Fisher Scientific |

## Label Free Imaging of Tissue

1 Mount sample on uncharged slide in PBS using prepositioned piece of spacer tape to support coverslip.

2 Gently push coverslip to the sample and seal with rubber cement dispensed from a syringe and 16 G needle.

3 Configure the microscope for multiphoton excitation at 910 nm for detecting SHG and auto-fluorescence (AF) (Table 2).

SP8 confocal scan-head mounted upright DM6000 microscope
Confocal Microscope

**Leica n/a**
Custom motorized stage coupled to a femtosecond pulsing MaiTai DeepSee laser (Spectra Physics, Santa Clara, CA) with EOM modulation for multiphoton excitation and a solid state laser launch providing 405 nm, 488 nm, 552 nm and 635 nm excitation. Imaging is performed with 25x 0.95 NA or 20x 0.75 NA Mimm objective with water immersion for label-free imaging or Leica immersion oil RI=1.51 with a 20x 0.75 NA Mimm objective for spectral confocal imaging. Detectors on the SP8 include two HyD detectors and two photo-multiplier tube (PMT) detectors ordered by wavelength, HyD1, PMT2, HyD3, PMT4. The SP8 is controlled by and linear unmixing is performed in Leica LASX software v. 3.5.2 build 18963.

4 Configure emission path by opening confocal pinhole and adjust the bandpasses on 2 to 4 detectors to collect SHG (~455 nm) and AF as desired (refer to “Excitation and filter settings”). Use PMTs to avoid detector “overloading” and data loss.

5 Configure x, y and z limits to cover the entire tissue. Scan in x and y at 0.5-1 µm/pixel. Sample in z with 1-2 um steps.

6 Perform scans with standard galvanometers set to 400 Hz, and averaging by line 2x.

7 Fluorescence lifetime imaging is under development and may be included at this step.

## Staining of Tissue with Fluorescent Small Modules and by Immunofluoresence

8 Recover specimen by removing rubber cement and gently lifting coverslip.

Lifting the coverslip may be easier if done after or while soaking the slide in PBS.

9 Washing: Sample is washed 1x in PBS > **00:10:00**

Phosphate buffered saline pH 7.4 (PBS)

10 Blocking: Remove wash and block non-specific binding sites with PBSTS 4–8 hours at **Room temperature** on rocking platform.

PBSTS (PBST & 10% NDS)
by Jackson Immunoresearch
Catalog #: 017-000-121

11 Indirect primary antibody staining (Table 1): Incubate tissue in **200 µl** staining solution for all the indirect detection primary antibodies 8-16 hours at **Room temperature** on rocking platform.

12 Washing: Wash sample with **2 ml** PBST 2 times for a total of **06:00:00** .

PBST (PBS & 0.1% Triton X-100)
by MP Biomedicals
Catalog #: 807423

13 Blocking: Remove wash and block non-specific binding sites with PBSTS **04:00:00** to **08:00:00** at **Room temperature** on rocking platform.

PBSTS (PBST & 10% NDS)
by Jackson Immunoresearch
Catalog #: 017-000-121

14 Indirect secondary antibody staining (Table 1): Incubate tissue in **200 µl** staining solution for all the indirect detection secondary antibodies **08:00:00** to **16:00:00** at **Room temperature** on rocking platform.

15 Washing: Wash sample with **2 ml** PBST twice for a total of **06:00:00** .

PBST (PBS & 0.1% Triton X-100)
by MP Biomedicals
Catalog #: 807423

16 Blocking: Remove wash and block non-specific binding sites with PBSTS **04:00:00** to **08:00:00** at **Room temperature** on rocking platform.

17 Directly conjugated antibody staining (Table 1): Incubate tissue in **200 µl** staining solution for all the direct detection primary antibodies **08:00:00** to **16:00:00** at **Room temperature** on rocking platform.

18 Washing: Wash sample with **2 ml** PBST twice for a total of **06:00:00** .

PBST (PBS & 0.1% Triton X-100)
by MP Biomedicals
Catalog #: 807423

19 Mount tissue with Prolong Glass or Fluoromount on pre-cleaned sides with silicone grease as needed to support coverslip.

Fluoromount
by Sigma
Catalog #: F4680

Glass slides
Catalog #: 3017

Coverglass #1.5 22X22mm
by Thermo Fisher Scientific
Catalog #: 12-541-B

20 Seal cover slip to slide with rubber cement applied with a syringe.

21 Configure the microscope for spectral confocal imaging as described in Table 2 below.

**Table 2. Tissue scan settings**

| Channel | Sequence | Excitation (nm) | laser power (%) | detector | voltage | gain (%) | offset | bandpass (nm) | line average | line summation |
|---|---|---|---|---|---|---|---|---|---|---|
| SHG | NA | 910* | 18 | PMT2 | 650 | NA | 0.1 | 439-465 | 2 | 1 |
| AF | NA | 910* | 18 | PMT4 | 700 | NA | 0.1 | 473-497 | 2 | 1 |
| | | | | | | | | | | |
| 1 | 1 | 405 | 8 | HyD1 | NA | 100 | NA | 410-430 | 2 | 1 |
| 2 | 1 | 405 | 8 | PMT2 | 700 | NA | 0.5 | 430-450 | 2 | 1 |
| 3 | 1 | 405 | 8 | HyD3 | NA | 20 | NA | 450-470 | 2 | 1 |
| 4 | 1 | 405 | 8 | PMT4 | 650 | NA | 0.5 | 470-490 | 2 | 1 |
| 5 | 2 | 488 | 1 | HyD1 | NA | 100 | NA | 500-509 | 2 | 1 |
| 6 | 2 | 488 | 1 | PMT2 | 700 | NA | -0.5 | 510-519 | 2 | 1 |
| 7 | 2 | 488 | 1 | HyD3 | NA | 50 | NA | 520-530 | 2 | 1 |
| 8 | 2 | 488 | 1 | PMT4 | 700 | NA | -0.5 | 530-540 | 2 | 1 |
| 9 | 3 | 552 | 0.5 | HyD1 | NA | 10 | NA | 570-590 | 2 | 1 |
| 10 | 3 | 552 | 0.5 | PMT2 | 700 | NA | -0.5 | 590-610 | 2 | 1 |
| 11 | 3 | 552 | 0.5 | HyD3 | NA | 30 | NA | 610-630 | 2 | 1 |
| 12 | 3 | 552 | 0.5 | PMT4 | 700 | NA | -0.5 | 631-651 | 2 | 1 |
| 13 | 4 | 638 | 2 | HyD1 | NA | 30 | NA | 643-664 | 2 | 4 |
| 14 | 4 | 638 | 2 | PMT2 | 700 | NA | -0.5 | 664-685 | 2 | 4 |
| 15 | 4 | 638 | 2 | HyD3 | NA | 30 | NA | 686-706 | 2 | 4 |
| 16 | 4 | 638 | 2 | PMT4 | 700 | NA | -0.5 | 706-726 | 2 | 4 |

*Multiphoton.

The settings for laser power, detector, voltage, gain and offset may vary based on the microscope configuration.

22 Configure x, y and z limits to cover the entire tissue. Scan in x and y at 0.5-1 µm/pixel. Sample in z with **1 µm** steps.

23 Perform scans with standard galvanometers set to 400 Hz. The multispectral 16 channels are configured as indicated in Table 2 with 4 sequences of 4 detectors.

Preparation of Bead Slide for Reference Spectra

24 Incubate **20 µl** of CML latex beads (ThermoFisher, cat# C37253) with **100 µg** **0 x g** of fluorophore conjugated secondary overnight.

Reference spectra for DAPI and Oregon Green are collected from tissue labeled only with DAPI or Oregon Green. The remaining reference spectra from fluorophores outlined in Table 1 are collected from single-fluorophore labeled latex beads.

25 Wash the beads after centrifuging at **1000 x g, Room temperature 00:00:30** . Repeat twice. Dilute and mix individually labeled beads in MM (Prolong Glass or Fluoromount) such that 5-10 beads of each individually labeled beads is visible in a field size of 500 x 500 µm.

26 Beads are mounted under #1.5 coverglass and cured for **48:00:00** to **96:00:00**

Coverglass #1.5 22X22mm
by Thermo Fisher Scientific
Catalog #: 12-541-B

## Genearating Matrix for Linear Unmixing

27 Scan slide-mounted beads labeled with known fluorophores with settings given in the table below for channels 1-16.

**Table 3. Reference beads scan settings**

| Channel | Sequence | Excitation (nm) | Laser power (%) | Detector | Voltage | Gain (%) | Offset | Bandpass (nm) | Line average | Line summation |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 405 | 8 | HyD1 | NA | 100 | NA | 410-430 | 2 | 1 |
| 2 | 1 | 405 | 8 | PMT2 | 700 | NA | 0.5 | 430-450 | 2 | 1 |
| 3 | 1 | 405 | 8 | HyD3 | NA | 20 | NA | 450-470 | 2 | 1 |
| 4 | 1 | 405 | 8 | PMT4 | 650 | NA | 0.5 | 470-490 | 2 | 1 |
| 5 | 2 | 488 | 1 | HyD1 | NA | 100 | NA | 500-509 | 2 | 1 |
| 6 | 2 | 488 | 1 | PMT2 | 700 | NA | -0.5 | 510-519 | 2 | 1 |
| 7 | 2 | 488 | 1 | HyD3 | NA | 50 | NA | 520-530 | 2 | 1 |
| 8 | 2 | 488 | 1 | PMT4 | 700 | NA | -0.5 | 530-540 | 2 | 1 |
| 9 | 3 | 552 | 4 | HyD1 | NA | 90 | NA | 570-590 | 2 | 1 |
| 10 | 3 | 552 | 4 | PMT2 | 750 | NA | -0.5 | 590-610 | 2 | 1 |
| 11 | 3 | 552 | 4 | HyD3 | NA | 90 | NA | 610-630 | 2 | 1 |
| 12 | 3 | 552 | 4 | PMT4 | 750 | NA | -0.5 | 631-651 | 2 | 1 |
| 13 | 4 | 638 | 8 | HyD1 | NA | 90 | NA | 643-664 | 2 | 1 |
| 14 | 4 | 638 | 8 | PMT2 | 750 | NA | -0.5 | 664-685 | 2 | 1 |
| 15 | 4 | 638 | 8 | HyD3 | NA | 30 | NA | 686-706 | 2 | 1 |
| 16 | 4 | 638 | 8 | PMT4 | 770 | NA | -0.5 | 706-726 | 2 | 1 |

Data collected is subjected to linear unmixing with Leica LASX software

28 Select “Process” -> “Dye Separation” -> “Channel Dye Separation”

29 Select regions of interest including beads intensity and select “Add” to add to spectra.

30 Select Rescale to “Per Channel.”

31 Select “Load” to load a saved matrix. The saved matrix will be displayed near the bottom of the window.

32 Select “Apply”.

## Applying Unmixing Matrix for Linear Unmixing

33 In the LASX software select image to unmix.

Once a matrix file is generated (.sdm) it may be used again to apply collected images.

34 Select “Process” -> “Dye Separation” -> “Automatic Dye Separation”

35 Set fluorescent dyes to “8.”

36 Set Rescale to “Per Channel.”

37 Select “Load” to load a saved matrix. The saved matrix will be displayed near the bottom of the window.

38 Select “Apply”.

## Stitiching/Merging of Unmixed Confocal Volumes

39 Select images to merge.

Following unmixing, the confocal dataset is merged with LEICA LASX software.

40 Select “Process” -> “Mosaic Merge”

41 Check “Autostitching”, “Smooth” overlap and “Linear Blending”.

42 Select “Apply”.

## Cytometric analysis of stitched and merged unmixed confocal volumes

43 All 3D cytometry is performed with the ImageJ plugin Volumetric Tissue Exploration and Analysis (VTEA). The settings for processing, segmentation and gating are given in analysis files. As software improves, new versions of VTEA may be used.