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# Sample preparation and imaging for large scale 3D spectral confocal imaging of tissues V.1

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Tarek M. El-Achkar<sup>1</sup>, Michael Ferkowicz<sup>1</sup>

<sup>1</sup>Indiana University School of Medicine, Indianapolis, Indiana

KPMP

Tech. support email: [info@kpmp.org](mailto:info@kpmp.org)



Tarek M. El-Achkar

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**Protocol status:** Working

**We use this protocol and it's working**

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## 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  $\mu\text{m}$  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**

Metri c	Excita tion (nm)	Lase r powe r (%)	Detec tor	Volta ge	Gain (%)	Offse t	Band pass (nm)	Line avera ge	Line summ ation

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

### MATERIALS

- ☒ DAPI Primary Antibody **Thermofisher Catalog #D1306**
- ☒ Phalloidin Primary Antibody **Thermofisher Catalog #O7466**
- ☒ THP Primary Antibody **R&D Systems Catalog #AF5144**
- ☒ AQP1 Primary Antibody **Santa Cruz Biotechnology Catalog #sc-9878**
- ☒ MPO Primary Antibody **Abcam Catalog #ab9535**
- ☒ CD68 Primary Antibody **Dako Catalog #M0876**
- ☒ CD3 Primary Antibody **Becton Dickinson (BD) Catalog #557706**
- ☒ Siglec-8 Primary Antibody **BioLegend Catalog #347102**
- ☒ Secondary Antibodies
- ☒ Phosphate buffered saline pH 7.4 (PBS)
- ☒ PBST (PBS & 0.1% Triton X-100) **MP Biomedicals Catalog #807423**
- ☒ PBSTs (PBST & 10% NDS) **Jackson ImmunoResearch Laboratories, Inc. Catalog #017-000-121**
- ☒ 4% PFA
- ☒ 24 or 48 flat well bottom culture dishes
- ☒ Fluoromount **Merck MilliporeSigma (Sigma-Aldrich) Catalog #F4680**
- ☒ Glass slides **Catalog #3017**
- ☒ Coverglass #1.5 22X22mm **Thermo Fisher Scientific Catalog #12-541-B**
- ☒ Elmers Rubber Cement
- ☒ Silicone grease
- ☒ CML Latex Beads **Thermofisher Catalog #C37253**

### STEP MATERIALS

- ☒ Phosphate buffered saline pH 7.4 (PBS)
- ☒ PBSTs (PBST & 10% NDS) **Jackson ImmunoResearch Laboratories, Inc. Catalog #017-000-121**
- ☒ PBST (PBS & 0.1% Triton X-100) **MP Biomedicals Catalog #807423**
- ☒ PBSTs (PBST & 10% NDS) **Jackson ImmunoResearch Laboratories, Inc. Catalog #017-000-121**
- ☒ PBST (PBS & 0.1% Triton X-100) **MP Biomedicals Catalog #807423**
- ☒ PBST (PBS & 0.1% Triton X-100) **MP Biomedicals Catalog #807423**
- ☒ Fluoromount **Merck MilliporeSigma (Sigma-Aldrich) Catalog #F4680**
- ☒ Glass slides **Catalog #3017**

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

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

## Protocol materials

⊗ 4% PFA

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

⊗ Coverglass #1.5 22X22mm **Thermo Fisher Scientific Catalog #12-541-B**

⊗ CD3 Primary Antibody **Becton Dickinson (BD) Catalog #557706**

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

⊗ Glass slides **Catalog #3017**

⊗ MPO Primary Antibody **Abcam Catalog #ab9535**

⊗ DAPI Primary Antibody **Thermofisher Catalog #D1306**

⊗ 24 or 48 flat well bottom culture dishes

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

⊗ PBSTs (PBST & 10% NDS) **Jackson ImmunoResearch Laboratories, Inc. Catalog #017-000-121**

⊗ Coverglass #1.5 22X22mm **Thermo Fisher Scientific Catalog #12-541-B**

⊗ Silicone grease

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

⊗ Fluoromount **Merck MilliporeSigma (Sigma-Aldrich) Catalog #F4680**

⊗ Phosphate buffered saline pH 7.4 (PBS)

⊗ Phosphate buffered saline pH 7.4 (PBS)

⊗ Secondary Antibodies

⊗ Phalloidin Primary Antibody **Thermofisher Catalog #O7466**

⊗ CML Latex Beads **Thermofisher Catalog #C37253**

⊗ PBSTs (PBST & 10% NDS) **Jackson ImmunoResearch Laboratories, Inc. Catalog #017-000-121**

⊗ CD68 Primary Antibody **Dako Catalog #M0876**

⊗ PBSTs (PBST & 10% NDS) **Jackson ImmunoResearch Laboratories, Inc. Catalog #017-000-121**

⊗ Glass slides **Catalog #3017**

⊗ Elmers Rubber Cement

⊗ THP Primary Antibody **R&D Systems Catalog #AF5144**

⊗ Siglec-8 Primary Antibody **BioLegend Catalog #347102**

⊗ Fluoromount **Merck MilliporeSigma (Sigma-Aldrich) Catalog #F4680**

⊗ Coverglass #1.5 22X22mm **Thermo Fisher Scientific Catalog #12-541-B**

⊗ AQP1 Primary Antibody **Santa Cruz Biotechnology Catalog #sc-9878**

 Phosphate buffered saline pH 7.4 (PBS)

 PBSTs (PBST & 10% NDS) **Jackson ImmunoResearch Laboratories, Inc. Catalog #017-000-121**

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

 PBSTs (PBST & 10% NDS) **Jackson ImmunoResearch Laboratories, Inc. Catalog #017-000-121**

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

 Fluoromount **Merck MilliporeSigma (Sigma-Aldrich) Catalog #F4680**

 Glass slides **Catalog #3017**

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

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

## 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).

### Equipment

<b>SP8 confocal scan-head mounted upright DM6000 microscope</b>	NAME
Confocal Microscope	TYPE
Leica	BRAND
n/a	SKU
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 25× 0.95 NA or 20× 0.75 NA Mimm objective with water immersion for label-free imaging or Leica immersion oil RI=1.51 with a 20× 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.	SPECIFICATIONS

- 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  $\mu\text{m}/\text{pixel}$ . Sample in z with 1-2  $\mu\text{m}$  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 Immunofluorescence

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

### Note

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) **Jackson ImmunoResearch Laboratories, Inc. Catalog #017-000-121**

- 11 Indirect primary antibody staining (Table 1): Incubate tissue in 200  $\mu\text{L}$  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) **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) **Jackson ImmunoResearch Laboratories, Inc. Catalog #017-000-121**

14 Indirect secondary antibody staining (Table 1): Incubate tissue in  200  $\mu$ L 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) **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  $\mu$ L 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) **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 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #F4680**

 Glass slides **Catalog #3017**

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

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

## Large-scale 3D Spectral Confocal Imaging

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.

### Note

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  $\mu\text{m}/\text{pixel}$ . Sample in z with  $\pm 1 \mu\text{m}$  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  $20 \mu\text{L}$  of CML latex beads (ThermoFisher, cat# C37253) with  $100 \mu\text{g}$  of fluorophore conjugated secondary overnight.

### Note

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  $1000 \times g$ , 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  $500 \times 500 \mu\text{m}$ .
- 26 Beads are mounted under #1.5 coverglass and cured for 48:00:00 to 96:00:00
-  Coverglass #1.5 22X22mm Thermo Fisher Scientific Catalog #12-541-B

## Generating 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

**Note**

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.

### Note

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".

## Stitching/Merging of Unmixed Confocal Volumes

39 Select images to merge.

### Note

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.