Protocol: Fibrinogen – iDisco Human

Citation: Merlini M, Rafalski VA, Rios Coronado PE, Gill TM, Ellisman M. Muthukumar G, Subramanian KS, Ryu JK, Syme CA, Davalos D, Seeley WW, Mucke L, Nelson RB, Akassoglou K. Fibrinogen induces microglia-mediated spine elimination and cognitive impairment in an alzheimer's disease model. **Neuron** 2019; 101: 1099-1108.

iDISCO protocol for processing of human brain

IMPORTANT: Because of the lack of a species barrier, handle all human tissue using the appropriate personal protective lab clothing and by performing all steps in a biosafety cabinet as set out by your institutional guidelines on biosafety.

Primary antibodies

NAME	VENDOR & catalog #	DILUTION OF STOCK
Guinea pig anti-human CD31/PECAM-1	Synaptic Systems; 351004	1:100
Rabbit anti-human β amyloid	IBL-America; 18584	1:100
Sheep anti-human fibrinogen	US Biological; F4200-06	1:100

Secondary antibodies

NAME	VENDOR & catalog #	DILUTION OF STOCK
Alexa488-conjugated donkey anti-guinea pig	Jackson ImmunoResearch; 706-545-148	1:200
Alexa647-conjugated donkey anti-rabbit	Jackson ImmunoResearch; 711-605-152	1:200
Cy3-conjugated donkey anti-sheep	Jackson ImmunoResearch; 713-165-147	1:200

Human brain tissue samples: *post mortem* time interval, fixation, and storage

The protocol has been successful for iDISCO processing of human brain tissue obtained within a *post mortem* time interval of 6–15 h, fixed in formalin for 72 h, and subsequent storage in PBS + 0.05% sodium azide at 4 °C.

Sectioning of the human brain tissue samples

Cut human brain tissue blocks into 1.5–2-mm thick sections with a razor blade or scalpel.

Pretreatment, incubation, and staining procedures

The iDISCO protocol for processing of human brain follows all steps as outlined in the detailed step-by-step protocol of the Tessier-Lavigne laboratory (http://idisco.info) with the amendments described under "Amendment 1", "Amendment 2", and "Amendment 3" below.

IMPORTANT: The methanol pretreatment protocol is required for the antibodies and human brain tissue described in this protocol.

IMPORTANT: Perform all primary and secondary antibody incubation steps using 2-mL Eppendorf tubes. All other steps are performed using 5-mL Eppendorf tubes to ensure sufficient washing, pretreatment, and clearing of the samples.

Amendment 1: Immunolabeling incubation times

- Permeabilization solution: 36 h
- Blocking solution: 48 h
- Primary antibody incubation: 72 h
- Secondary antibody incubation: 72 h

Amendment 2: Bleaching in 5% hydrogen peroxide in methanol, step 5 of "Sample Pretreatment with Methanol"
Incubate the samples for 36 h in 5% hydrogen peroxide in methanol at 4 °C.

Amendment 3: Clearing in dichloromethane, step 3 of "Clearing"
Incubate the samples in 100% dichloromethane twice for 30 min.

Image acquisition

The human brain iDISCO-processed samples were imaged using sequential confocal imaging. Details of the microscope, lasers, and optics are detailed below.

IMPORTANT: Due to the relatively high photon scattering in human brain, especially white matter, it is highly recommended to excite Alexa488-labeled antigens with a two-photon laser as described below. The Cy3- and Alexa647-labeled antigens can be imaged with conventional one-photon lasers.

- Microscopy setup: Olympus FluoView 1000 MPE platform with an Olympus BX61WI microscope with integrated one-photon and two-photon laser lines
- One-photon lasers used in this protocol: 543 nm HeNe gas laser to excite Cy3-labeled secondary antibodies and 635 nm diode laser to excite Alexa647-labeled secondary antibodies
- Two-photon laser used in this protocol: Spectra-Physics MaiTai DeepSee *e*HP, 690–1040 nm Ti:Sapphire laser, tuned at 940 nm, to excite Alexa488-labeled secondary antibodies
- Fluorescence emission from 543- and 635-nm laser-excited Cy3 and Alexa647, respectively, were separated by a 640-nm dichroic mirror and passed through 560–620-nm (Cy3) and 655–755-nm (Alexa647) emission filters mounted before the detector
- Fluorescence emission from two-photon laser-excited Alexa488 secondary antibodies was passed through a 495–540-nm emission filter mounted before the detector
- Z-stacks were acquired in sequential imaging mode at 1 µm step resolution, 4.0 µs pixel dwell time, and 512 × 512 or 1024 × 1024 pixel resolution

Post-acquisition image processing

- Software used: FIJI/ImageJ
- The combined Alexa488, Cy3, and Alexa647 image z-stack files were loaded as separate z-stacks in FIJI
- Images were converted to 8 bit grey scale images and were denoised using the "Remove outliers" plugin with radius set at 2.0 pixels and threshold set at 50
- The Alexa488 image stack (CD31 signal) was subtracted from the Cy3 image stack (fibrinogen signal) to ensure removal of any potential non-specific, false-positive fluorescence signals from lipofuscin and other non-fibrinogen deposits
- The processed image z-stacks were combined as pseudo-colored RGB stacks and were processed with the "Unsharp mask" plugin with radius/sigma set between 3.0 and 5.0 and mask weight at $1/10^{th}$ of the radius/sigma, e.g., radius/sigma = 4.0 and mask weight = 0.4
- The RGB stacks were processed into 3D volume projections using the "3D Viewer" plugin followed by 360° rotation recording to yield a .tiff 3D volume projection image file