3DISCO

1. Animal Perfusion and tissue preparation

Timing: 30-60 min per mouse + post-fixation (a few hours to overnight).

1.1 Anesthetize the animal with triple combination of metedominim 0,5mL + midazolam 5mL + fentanyl 1mL. The dosage depends on animal's weight. You can also use the anesthesia method of your choice such as ketamine (80-200 mg/kg) and xylazine (7-20 mg/kg) or 2.5% avertin (0.5 ml/25 grams body weight IP).

1.2 Wait a few minutes for anesthesia to take a complete effect.

1.3 Pinch the toe and tail of the animal to make sure that the animal is fully anesthetized.

1.4 Perfuse the animal first at room temperature with 0.1 M Phosphate Buffer Saline (PBS) for 5-10 min until the blood is completely removed from the tissue.

1.5 Switch the perfusion to ice cold fixative solution: 4% PFA (paraformaldehyde, MORPHISTO cod. 11762.01000) in 0.1 M PBS and continue perfusion with 4% PFA for 20-30 min at a speed of 3 ml/min with a peristaltic pump or (50-100mm Hg pressure on Leica perfusion one system).

1.6 Dissect the organ/s of interest carefully without damaging, e.g. avoid puncturing and squeezing the tissue that is being dissected.

1.7 Post-fix the organs in 4% PFA for a few hours or overnight at 4°C. Avoid long post-fixation because PFA might quench the signal or increase the autofluorescence overtime especially for GFP channel (which is a lesser problem in red and far red channels).

1.8 Remove the extra tissue surrounding the organs (connective tissue, meninges or dura matter) in a petri dish filled with PBS.

1.9 Wash the organs 2-3 times with PBS at room temperature just before starting the clearing procedure.

2. Tissue clearing

Timing: 2-3 hours for small organs and 1-4 days for large organs.

Notes: The fluorescent labeling of the tissues by transgene expression, viral transfection, dye tracing or antibody labeling should be completed before clearing. All tissue clearing steps are performed at room temperature. The clearing solutions: THF (tetrahydrofuran, SIGMA cod.186562), DBE (dibenzylether, SIGMA cod. 33630), BABB (1 part benzyl alcohol, SIGMA cod.24122 and 2 part benzyl benzoate, SIGMA cod. B6630) and DCM (dichloromethane, SIGMA cod. 270997) are toxic and inhalation (conduct experiments in properly ventilated fume hood) and direct contact with skin should be avoided (use nitrile gloves).

2.1 Prepare 50% (vol/vol), 70%, and 80% THF dilutions in distilled water in separate glass bottles as follows: For 50% THF, e.g. mix 25 ml THF and 25 ml of distilled water in a glass bottle with volume of larger than 50 ml.

2.2 Mix solutions by gently shaking the bottle for a 1-2 min.

2.3 Label the glass bottle clearly.

2.4 Securely close the bottles and keep the working solutions in a dark cabinet. For the best results, do not use the same working solutions longer than 1-2 weeks. Therefore, order the clearing solutions as the smallest volume available to be able use fresh stock reagents.

2.5 Fill 5mL eppendorf with 4-5mL of the first clearing solution, 50% THF, and transfer the organs from PBS into the eppendorf to start clearing.

2.6 Securely close the eppendorfs with their lids and use a rotator (e.g. a wheel stirrer) for stirring.
2.7 Use aluminum foil to cover and keep the eppendorfs in dark. Start the rotator to stir the samples in the eppendorfs for the indicated time (Table) at a constant speed.
2.8 Remove the clearing solution and add the next one in the protocol when the time in the table is completed.
2.9 Collect the clearing waste into glass waste containers that are kept in a hood.
2.10 Repeat previous step for each clearing solution in the protocol until the end. Use a new pasteur pipette when a new clearing solution is added (e.g. changing from THF to DBE).

Notes: At the final clearing step with BABB or DBE, the incubation times can be extended or shortened until the samples become completely transparent in visual light (or yellowish/transparent if it is a very large tissue like brain).

2.11 Keep the cleared organs in the final clearing solution at all times including the imaging steps.

3. Preparing cleared organs for imaging
Timing: 5-15 min.

Notes: Image the cleared organs as soon as a complete clearing is achieved. To this end, follow the next steps to prepare the samples for light-sheet microscopy (e.g. ultramicroscopy) or confocal / multi photon microscopy imaging.

Notes: Cleared organs will lose their fluorescence strength over time. For imaging, various fluorescent microscopy techniques can be used as long as proper handling of the organs in the clearing solution is achieved.

3.1. For light-sheet microscopy
3.1.1 Place or mount the sample appropriately.
3.1.2 Fix the cleared organ by manually turning the screw of the sample holder.
3.1.3. Dip the sample into the imaging chamber (preferably made of glass) of the light-sheet microscope that is filled with BABB or DBE, whichever was used at the final clearing step.
3.2 For multi photon / confocal microscopy
3.2.1 Mount the sample on an imaging slide (or an imaging chamber) with the final imaging solution to be able to image with a multi photon or confocal microscopy, which typically use oil or water immersed objectives.
3.2.2 Use silicone gel (OBI cod. 4799656) to make a border around the tissue.
3.2.3 Fill the pool with BABB or DBE
3.2.4 Place the cleared sample in the middle of the pool and cover it with a cover glass.
3.2.5 Press the cover glass until it is completely sealed by the silicone gel and touches at the surface of the cleared organ, which will allow reaching the maximum imaging depth by confocal / multi photon microscopy.

Notes: This ensures that no clearing solution will be spilled during the imaging. Avoid dipping the imaging lens directly into clearing solution, which can harm the lens unless it is resistant to BABB/DBE or has a protective cover.

4. Imaging cleared organs

Timing: 15-45 min.

4.1 Collect a z-scan covering the entire cleared tissue (if the used lens allows) at the best resolution that the microscope can deliver.

4.2 Zoom in on the regions of interest to collect higher resolution images.

	Small tissues (e.g. spinal cord, hippocampus, cortex...)	brain
50% THF	30min	1h
70% THF	30min	1h
80% THF	30min	1h
100% THF	3 x 30min	1h, o/n, 1h
DCM	20 min	1h
BABB or DBE	≥ 15min	≥ 30min

3DISCO literature

Ertürk A., Becker K., Jährling N., Mauch C.P., Hojer C.D., Egen J.G., Hellal F., Bradke F., Sheng M. & Dodt H.U. Three-dimensional imaging of solvent-cleared organs using 3DISCO. Nature protocols. 2012 Oct

Dodt H.U., Leischner U., Schierloh A., Jährling N., Mauch C.P., et al. (2007) Ultramicroscopy: three-dimensional visualization of neuronal networks in the whole mouse brain (2007) Nat Meth 4(4): 331–336.

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What you should know about – iDISCO

Simon Merz

Immunolabeling-enabled three-dimensional imaging of solvent-cleared organs

The iDISCO protocol [1] was originally developed on the basis of the 3DISCO [2] clearing method, but it allows volume immunostaining prior to the clearing process.
This note provides some considerations before establishing the protocol. More general information concerning the method and troubleshooting are available at idisco.info.

When should be used this method?
In general: If the autofluorescence is sufficient to visualize the structure of interest, it is enough to clear the sample without prior immunostaining. Furthermore, if one would like to preserve endogenous fluorescence (e.g. eGFP), 3DISCO clearing can be performed, which yields results in 3-4 days. However, quenching of GFP fluorescence by methanol during the iDISCO protocol can be overtaken by the application of the well-established anti-GFP antibodies that gives the opportunity to shift the detected fluorescent signal to the far-red detection spectrum. This dramatically increases the signal-to-noise ratio. If the project requires immunolabeling of structures or co-labelling experiments within several mm to cm thick tissue and three-dimensional data is needed, then, iDISCO is one very suitable protocol

Advantages and limitations of iDISCO:
Compared to the preparation, staining and imaging of 1000 sections of one tissue volume and the eventual three-dimensional reconstruction, iDISCO can save a lot of working time and resources. However, the amount of antibody needed to stain a cubic centimeter of tissue is still high. Furthermore, the method depends strongly on the specific antigens and the penetration capacity of the antibodies. In some cases specific adaptations of the protocol may be needed to to achieve good and reliable results. Spending time here is a good investment.

Need to know and prepare for:
The Authors of the iDISCO paper share a list of validated antibodies (idisco.info) that work best under the published circumstances. If no antibody against the target of choice is among these or different type of tissue is applied, screening for those antibodies that compatible with the methanol pretreatment (immunostaining on methanol pre-treated cryo-sections), that penetrate the tissue best and that yield the best signal is suggested. If the application of an antibody that is not compatible with methanol is unavoidable, there is also a variation of the protocol for that, albeit its application is limited to small embryos or thinner samples). Keep in mind that diffusion is also limited by the amount of antigen present. Antibodies against highly abundant antigens like GFAP will diffuse rather poorly.

The general parameters defining penetration depths beside antibody properties are the harshness of tissue permeabilisation (prolong MeOH steps and/or lower temperatures) and the duration of antibody incubation at 37 °C (long incubations of 14d+ are possible, depending on antibody integrity, as shown by [3]).

If a ring-like staining on the surface of the sample is noticed, the reduction of the primary antibody concentration may be a solution. For further trouble shooting, please visit idisco.info

Concerning fluorophores, the motto is: The farther red is the better. Imaging in the green (eGFP) or even in the blue (DAPI) spectrum is not advisable due to high tissue autofluorescence and low signal-to noise ratio. Fluorophores like Alexa dyes work well with iDISCO and are stable in DBE for a long time.

The DBE clearing itself is, following a second dehydration, an easy and fast process. It can happen that the sample exhibits a yellowish color upon DBE contact. This does not impede imaging and, in most cases, diminishes after a bit longer storage in DBE. Also note that samples become really hard during the iDISCO clearing process, which makes them easier to handle (still can be cut easily with a razer). Finally, samples can be imaged preferably by a light sheet microscope or by a multi-photon setup. Keep in mind that lots of data is produced and must be processed (computers with high amount of RAM and with adequate imaging software e.g. Imaris are needed).

1. Renier, N., et al., *iDISCO: A Simple, Rapid Method to Immunolabel Large Tissue Samples for Volume Imaging.* Cell. 159(4): p. 896-910.
2. Erturk, A., et al., *Three-dimensional imaging of solvent-cleared organs using 3DISCO.* Nat Protoc, 2012. 7(11): p. 1983-95.
3. Belle, M., et al., *A simple method for 3D analysis of immunolabeled axonal tracts in a transparent nervous system.* Cell Rep, 2014. 9(4): p. 1191-201.

iDISCO+

Recommendations for sample handling
The following are given as a general guideline and may vary for specific applications.

Sample type	Incubation time (n)	Solution volume
Embryonic:		
E10-E11 embryo	1d	1.6mL
E12 embryo	2d	1.6mL
E13-E14 embryo	3d	1.6mL
E15-16 embryo	4d	4mL
E18 head	4d	4mL
Adult organ	4d	1.6mL
Adult brain:		
hindbrain + cerebellum	3d	1.6mL
cut hemisphere	5d	1.6mL
Whole brain	7d	4.5mL

To insure the best staining and imaging conditions we recommend trimming the sample to a size most relevant for the specific biological question.

Add .02% NaN3 to all stock solutions to prevent microbial growth.

Buffers
PTx.2 (1L)
- 100mL PBS 10X
- 2mL TritonX-100

PTwH (1L)
- 100mL PBS 10X
- 2mL Tween-20
- 1mL of 10mg/mL Heparin stock solution

Permeabilization Solution (500mL)
- 400mL PTx.2
- 11.5g of Glycine
- 100mL of DMSO

Blocking Solution (50mL)
- 42mL PTx.2
- 3mL of Donkey Serum
- 5mL of DMSO

GERMAN FIXATIVE FOR iDISCO+

Chemical	1L	2L
NaH_2PO_4 x 1H2O Na_2HPO_4 x 2H2O	3.3 g 28.08 g Ad 400 ml **MilliQ water**	6.6 g 56.16 g Ad 800 ml **MilliQ water**
Picric acid solution (**SIGMA, P6744-1GA**)	176 ml, filter	352 ml, filter
PFA (**Carl-Roth, 0335.3**)	40 g ad 250 ml 56 ^{0}C **MilliQ water**, filter	80 g ad 500 ml 56 ^{0}C **MilliQ water**, filter

Buffer, filtered picric acid and filtered PFA solutions must be mixed, filled up till 1 or 2 liters with additional **MilliQ water**, must be filtered again and then pH must be adjusted to 7.4. Keep in +4^{0}C until application. Do not keep longer than one-two weeks.

Bench Protocol

Sample Collection

Embryo:

1. Collect E10.5-E16.5 mouse embryos in ice-cold Leibovitz L-15 or PBS.
2. Keep on ice for 5min to drain blood from umbilical cord.
3. Fix in 1xPBS/4%PFA at 4°c, o/n with shaking.
4. Fix at room temperature (RT) 1h (optional).
5. Wash in PBS with shaking: RT 30min x 3times.

Can store in PBS with .02% NaN_3 at 4°.

Adult:

1. Anesthetize the mouse.
2. Perfuse with 20mL PBS.
3. Perfuse with 20mL 4%PFA/PBS.
4. Dissect the brain/organ and trim to the appropriate size.
5. Fix in 1xPBS/4%PFA at 4°c, o/n with shaking, then RT 1h.
6. Wash in PBS with shaking: RT 30min x 3times.

Can store in PBS with .02% NaN_3 at 4°.

Sample Pretreatment with Methanol

⚠ Before staining make sure antibodies are compatible with methanol pretreatment. Instruction on how to do so are listed on the last page.

1. Dehydrate with methanol/H2O series: 20%, 40%, 60%, 80%, 100%; 1h each.
2. Wash further with 100% methanol for 1h and then chill the sample at 4°c.
3. Bleach in chilled fresh 5%H2O2 in methanol (1 volume 30% H_2O_2 to 5 volumes MeOH), overnight at 4°c.
4. Rehydrate with methanol/H2O series: 80%, 60%, 40%, 20%, PBS; 1h each at RT.
5. Wash in **PTx.2** RT 1h x2 at RT.

⚠ Centrifuging secondary antibody solution at 20000g for 10 minutes can prevent formation of precipitates in the sample. Alternatively, you can syringe-filter the solution at 0.2µm.

Alternative Pretreatment (<1mm (adult) or embryos only)

1. Wash fixed samples in **PTx.2**, RT 1h x2.
2. Incubate in 1xPBS/0.2%TritonX-100/20%DMSO, 37°C, o/n.
3. Incubate in 1xPBS/0.1%Tween-20/0.1%TritonX-100/0.1%Deoxycholate/0.1%NP40/20%DMSO, 37°C o/n.
4. Wash in **PTx.2**, RT 1h x 2.

Perform all steps in closed tubes. Fully fill tubes to prevent oxidation.

Immunolabeling

1. Incubate samples in **Permeabilization Solution**, 37°C n/2 days (max. 2 days)
2. Block in **Blocking Solution**, 37 °, n/2 days (max. 2 days).
3. Incubate with primary antibody in **PTwH**/5%DMSO/3% Donkey Serum, 37°, n days.
4. Wash in **PTwH** for 4-5 times until the next day.
5. Incubate with secondary antibody in **PTwH**/3% Donkey Serum, 37°, n days.
6. Wash in **PTwH** for 4-5 times until the next day.

Read the safety data sheet (MSDS) for the use of DCM and DBE. Incubations and storage can be done

Clearing

1. Dehydrate in methanol/H2O series: 20%, 40%, 60%, 80%, 100%, 100%; 1hr each at RT. Can be left optionally overnight at RT at this point.
2. 3h incubation, with shaking, in 66% DCM / 33% Methanol at RT
3. Incubate in 100% DCM (Sigma **270997-12X100ML**) 15 minutes twice (with shaking) to wash the MeOH.
4. Incubate in DiBenzyl Ether (DBE, Sigma **108014-1KG**) (no shaking). The tube should be filled almost completely with DBE to prevent the air from oxidizing the sample. Before imaging, invert the tube a couple of time to finish mixing the solution.

Imaging

Light sheet microscope

The use of a light sheet microscope (e.g. LaVision Biotec.) is recommended. The sample is ready to be directly imaged in the microscope chamber filled with DBE. Light sheet allows fast imaging of a large field of view, with very deep optical penetration and limited photobleaching.

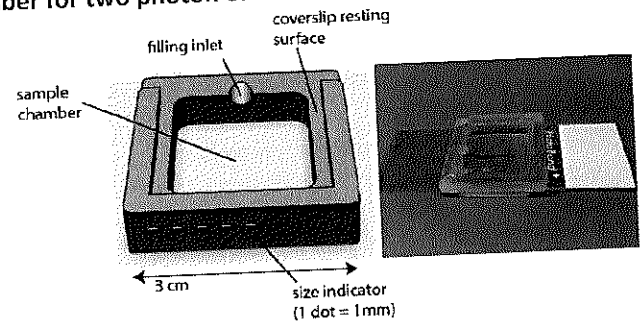
A chamber can also be built up using dental epoxy if access to a 3D printer in not available. Also, online 3D printing services can provide a good alternative. **http://www.3dhubs.com** is a good way to

Scanning microscope (confocal or 2-photon)

Upright confocal and 2-photon microscopes can image the sample with a depth up to the working distance of the objective at high resolution. A chamber has to be built to confine the DBE and protect the microscope. To build an imaging chamber:

1. The chamber (Script provided at iDISCO.info) can be 3D printed with Visijet M3 Crystal resin, which is resistant to DBE.
2. Secure the chamber to the microscope slide with Kwik-sil epoxy (VWR). This epoxy does not permanently bond so that sample and spacers can be recovered after imaging. Make sure there are no gaps that the DBE can leak through.
3. Place a drop of epoxy in the center of the chamber and place sample on drop.
4. Close the chamber by gluing a coverslip to the spacer with the epoxy. Do not glue over filling inlet.
5. Fill the chamber with DBE with a pipet through the filling inlet.
6. Plug the inlet with epoxy.

Chamber for two photon or confocal microscope



Antibody validation

To assess the methanol compatibility of untested antibodies, we recommend doing the following:

1) Collect 20µm frozen sections of the PFA fixed tissue of interest on superfrost slides. Floating vibratome sections also work.
2. Incubate the sections for 3h at least in 100% methanol at room temperature
3. Rehydrate in PBS directly and proceed with the immunostaining normally. Use non methanol treated slides as a positive control.

If the antibody yields a good signal to noise ratio, the antibody is then compatible with the methanol treatment and should work in whole-mount. If the signal is strongly diminished after the methanol treatment, one can use the non-methanol protocol, or test alternative antibodies against the target protein.

Antibody Concentration and Choice

Antibody concentration is the most important parameter to optimize for a successful staining.

- If you notice a "ring" background around the edges of the sample, the antibody is causing a non-specific staining, indicating that the concentration is too high. This will also cause poor diffusion. Reduce the concentration.
- If you don't have any staining deep in the sample AND don't have a ring background, the antibody is depleted by the antigens and the concentration should be increased. If increasing the concentration leads to the formation of a ring background, or is not economically viable, another reference should be used, or the antigen is too concentrated for whole-mount labeling.
- If you have a weak staining at the center, but still visible, the incubation time should be increased.
- Never use antibodies raised in mouse on mouse tissue, as the secondary IgGs will bind to the endogenous mouse IgG in the vasculature.
- It is very often a good investment to spend time screening antibodies from different vendors for a given antigen. Not all antibodies are made equal!
- Secondary antibody concentrations are less critical, but concentrations should be within the same range as for the primary IgG concentrations.

Questions?

You can visit http://www.idisco.info to get the latest public updates on the protocol and validated antibodies.

Reagents list

We use the following reagents for iDISCO+. Most reagents may be replaced by similar products from other vendors, but those replacement should be carefully considered. It is critical to use very high quality organic solvents to prevent oxidation of the sample during clearing.

We use double distilled water (MilliQ system).

Reagent	Reference
PBS 10X	Ambion AM9624
Triton-X100	Sigma X100-500ML
Tween-20	Sigma P9416-100ML
DMSO	Fisher D128-4
Sodium Azide	58032-100G
Donkey Serum	Jackson Immunoresearch 017-000-121
Glycine	Sigma G7126-500G
Heparin	Sigma H3393-50KU
Methanol	Fisher A412SK-4
Hydrogen Peroxide 30%	Sigma 216763-100ML
DiChloroMethane	Sigma 270997-12X100ML
DiBenzylEther	Sigma 108014-1KG or 3KG
ParaFormAldehyde 16%	EMS 15710-S

Consumables and hardware

Reagent	Reference
Tubes (small samples)	Eppendorf 2mL
Tubes (large samples)	Eppendorf 5mL
Orbital shaker	VWR rotating mixer
Hybridization oven	VWR 5420 With carousel 47746-112

iDISCO literature

Cell Rep. 2014 Nov 20;9(4):1191-201. A Simple Method for 3D Analysis of Immunolabeled Axonal Tracts in a Transparent Nervous System. Belle M, Godefroy D, Dominici C, Heitz-Marchaland C, Zelina P, Hellal F, Bradke F, Chédotal A

Cell. 2014 Nov 6;159(4):896-910. iDISCO: A Simple, Rapid Method to Immunolabel Large Tissue Samples for Volume Imaging. *Renier N, Wu Z, Simon DJ, Yang J, Ariel P, Tessier-Lavigne M.*

Cell. 2017 Mar 23;169(1):161-173.e12. doi: 10.1016/j.cell.2017.03.008., Tridimensional Visualization and Analysis of Early Human Development., Belle M, Godefroy D, Couly G, Malone SA, Collier F, Giacobini P, Chédotal A.

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We thank Alain Chedotal for providing this protocol.

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We thank Csaba Adori for providing the GERMAN FIXATIVE FOR iDISCO+ protocol.

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uDISCO

1. Animal Perfusion and tissue preparation

Timing: 30-60 min per mouse + post-fixation (a few hours to overnight).

1.1 Anesthetize the animal with triple combination of metedominim 0,5mL + midazolam 5mL + fentanyl 1mL. The dosage depends on animal's weight. You can also use the anesthesia method of your choice such as ketamine (80-200 mg/kg) and xylazine (7-20 mg/kg) or 2.5% avertin (0.5 ml/25 grams body weight IP).

1.2 Wait a few minutes for anesthesia to take a complete effect.

1.3 Pinch the toe and tail of the animal to make sure that the animal is fully anesthetized.

1.4 Perfuse the animal first at room temperature with 0.1 M Phosphate Buffer Saline (PBS) (100–125 mm Hg pressure on Leica perfusion one system) for 5-10 min until the blood is completely removed from the tissue.

1.5 Switch the perfusion to 4% PFA (paraformaldehyde, MORPHISTO cod. 11762.01000) in 0.1 M PBS with the same pressure and continually perfuse for 20-30 min.

1.6 Dissect the organ/s of interest carefully without damaging, e.g. avoid puncturing and squeezing the tissue that is being dissected.

1.7 Post-fix the organs in 4% PFA for a few hours or overnight at 4°C. Avoid long post-fixation because PFA might quench the signal or increase the autofluorescence overtime especially for GFP channel (which is a lesser problem in red and far red channels).

1.8 Remove the extra tissue surrounding the organs (connective tissue, meninges or dura matter) in a petri dish filled with PBS.

1.9 Wash the organs 2-3 times with PBS at room temperature just before starting the clearing procedure.

2. Tissue clearing

Timing: 2 days for small organs such as spinal cord and 3-4 days for large organs such as dissected brain.

Notes: The fluorescent labeling of the tissues by transgene expression, viral transfection, dye tracing or antibody labeling should be completed before clearing. All tissue clearing steps are performed at room temperature. The clearing solutions: tert-butanol (SIGMA cod. 360538), BABB (1 part benzyl alcohol, SIGMA cod.24122 and 2 part benzyl benzoate, SIGMA cod. W213802), DCM (dichloromethane, SIGMA cod. 270997) and diphenyl ether (DPE) (Alfa Aesar, A15791) are toxic and inhalation (conduct experiments in properly ventilated fume hood) and direct contact with skin should be avoided (use nitrile gloves).

2.1 Prepare 30% (vol/vol), 50%, 70%, 80%, 90% and 96% tert-butanol dilutions in distilled water in separate glass bottles as dehydration solutions. BABB-D4, prepared by mixing BABB with DPE at a ratio of 4:1 and adding 0.4% vol DL-alpha-tocopherol (Vitamin E) (Alfa Aesar, A17039). BABB-D15, prepared by mixing BABB with DPE at a ratio of 15:1 and adding 0.4% vol vitamin E.

Tips: In passive clearing, BABB-D4 preserved the fluorescence signal better, while BABB-D15 gave better transparency.

2.2 Mix solutions by gently shaking the bottle for a 1-2 min.
2.3 Label the glass bottle clearly.
2.4 Securely close the bottles and keep the working solutions in a dark cabinet. For the best results, do not use the same working solutions longer than 1-2 weeks. Therefore, order the clearing solutions as the smallest volume available to be able use fresh stock reagents.
2.5 Fill 5 mL eppendorf with 4-5 mL of the first clearing solution, 30% tert-butanol, and transfer the organs from PBS into the eppendorf to start clearing.

Tips: All the clearing steps should be finished under 35 °C with gentle shaking

2.6 Securely close the eppendorfs with their lids and use a rotator (e.g. a wheel stirrer) for stirring.
2.7 Use aluminum foil to cover and keep the eppendorfs in dark. Start the rotator to stir the samples in the eppendorfs for the indicated time (Table) at a constant speed.
2.8 Remove the clearing solution and add the next one in the protocol when the time in the table is completed.
2.9 Collect the clearing waste into glass waste containers that are kept in a hood.
2.10 Repeat previous step for each clearing solution in the protocol until the end. Use a new pasteur pipette when a new clearing solution is added (e.g. changing from tert-butanol to DCM).

Notes: At the final clearing step with BABB-D, the incubation times can be extended or shortened until the samples become completely transparent in visual light (or yellowish/transparent if it is a very large tissue like brain).

2.11 Keep the cleared organs in the final clearing solution at all times including the imaging steps.

3. Preparing cleared organs for imaging
Timing: 5-15 min.

Notes: Image the cleared organs as soon as a complete clearing is achieved. To this end, follow the next steps to prepare the samples for light-sheet microscopy (e.g. ultramicroscopy) or confocal / multi photon microscopy imaging.
Notes: Cleared organs will lose their fluorescence strength over time. For imaging, various fluorescent microscopy techniques can be used as long as proper handling of the organs in the clearing solution is achieved.

3.1. For light-sheet microscopy
3.1.1 Place or mount the sample appropriately.
3.1.2 Fix the cleared organ by manually turning the screw of the sample holder.
3.1.3. Dip the sample into the imaging chamber (preferably made of glass) of the light-sheet microscope that is filled with BABB-D, whichever was used at the final clearing step.
3.2 For multi photon / confocal microscopy
3.2.1 Mount the sample on an imaging slide (or an imaging chamber) with the final imaging solution to be able to image with a multi photon or confocal microscopy, which typically use oil or water immersed objectives.
3.2.2 Use silicone gel (OBI cod. 4799656) to make a border around the tissue.
3.2.3 Fill the pool with BABB-D

3.2.4 Place the cleared sample in the middle of the pool and cover it with a cover glass.

3.2.5 Press the cover glass until it is completely sealed by the silicone gel and touches at the surface of the cleared organ, which will allow reaching the maximum imaging depth by confocal / multi photon microscopy.

Notes: This ensures that no clearing solution will be spilled during the imaging. Avoid dipping the imaging lens directly into clearing solution, which can harm the lens unless it is resistant to BABB-D or has a protective cover.

4. Imaging cleared organs

Timing: 15-45 min.

4.1 Collect a z-scan covering the entire cleared tissue (if the used lens allows) at the best resolution that the microscope can deliver.

4.2 Zoom in on the regions of interest to collect higher resolution images.

	Small tissues (e.g. spinal cord, hippocampus, cortex...)	brain
30% tert-butanol	4 hours	Overnight
50% tert-butanol	4 hours	10 hours
70% tert-butanol	Overnight	Overnight
80% tert-butanol	4 hours	10 hours
90% tert-butanol	4 hours	Overnight
96% tert-butanol	Overnight	10 hours
100% tert-butanol	-	Overnight
DCM	-	50-70 min
BABB-D4 or BBAB-D15	≥ 2 hours	≥ 3 hours

uDISCO literature

Nat Methods. 2016 Aug 22. Shrinkage-mediated imaging of entire organs and organisms using uDISCO. *Pan C, Cai R, Quacquarelli FP, Ghasemigharagoz A, Lourbopoulos A, Matryba P, Plesnila N, Dichgans M, Hellal F, Ertürk A.*

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ECi Clearing

Sample Pre-Treatment

- For antibody staining and perfusion see protocol "Blood vessel staining via i.v.-Injection"
- Caution: This method should not be combined with organic fluorescent dyes (FITC, PE, ...) but Alexa dyes!
- For better image quality organs should always be perfused!
- Lung preparation: collapsed lungs have to be filled with 800µl of 0.75% low melting/low gelling agarose (SeaPrep™ Agarose, Lonza, Cat# 50302) for expanding the organ to its physiological shape
- Lymph node preparation: small organs like lymph nodes have to be embedded in 1% low melting/low gelling agarose
- Gut preparation: flush out the feces with PBS via a syringe. Embed washed gut pieces (1cm length) in 1% low melting/low gelling agarose.

Sample Preparation

- If necessary (small) organs are embedded in 1% low melting-low gelling agarose (dissolved in H_2O) or 1% Phytagel (dissolved in H_2O) after perfusion with 5 mM EDTA/PBS and 4% PFA/PBS
- after perfusion with 4% PFA/PBS organs have to be additionally fixed in 4% PFA/PBS at 4-8°C (incubation times see table 1)
- Fixed organs are dehydrated in EtOH with adjusted pH = 9.00 (incubation times and concentrations see table 1) at 4-8°C, gently shaking!
- Dehydrated organs are transferred into ECi and incubated at room temperature (!) until they become transparent

Incubation times for fixation, dehydration and clearing of multiple organs

Organ	4 % PFA fixation	30 % EtOH pH = 9.0	50 % EtOH pH = 9.0	70 % EtOH pH = 9.0	2x 100 % EtOH	ECi
Brain	2 h	+ 2 % Tween20 12 h	+ 2 % Tween20 12 h	+ 2 % Tween20 12 h	+ 2 % Tween20 12 h	2 h
Lymph node*[1]	1 h	-	2 h	2h	2 h	15 min
Calvaria	2 h	-	4 h	4 h	4 h	15 min
Lung*[2]	2 h	-	4 h	4 h	4 h	30 min
Heart	2 h	-	4 h	4 h	4 h	2 h
Kidney	2 h	-	4 h	4 h	4 h	2 h
Liver	2 h	-	4 h	4 h	4 h	2 h
Spleen	2 h	-	4 h	4 h	4 h	2 h
Gut	2 h	-	4 h	4 h	4 h	1 h
Bones	4 h	-	12 h	12 h	12 h	6 h

*[1] embedded in 1% agarose/Phytagel (dissolved in H_2O)
*[2] filled with 0.75% agarose/Phytagel (dissolved in H_2O)

Practical considerations

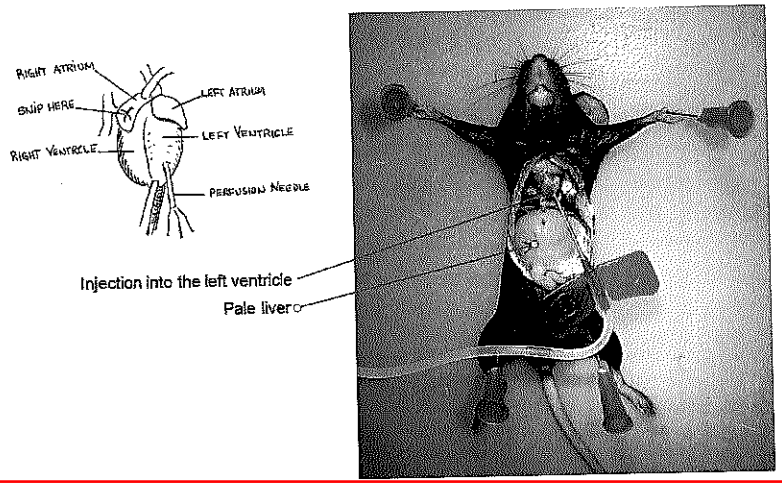
- Listed incubation times are optimized for short-term handling and indicate the minimum-required incubation times for tissue fixation and dehydration
- Incubation times for tissue dehydration via Ethanol can be extended if necessary (flexible working schedule, e.g. overnight incubation)
- Incubation times for fixation via 4% PFA should not be extended, as this might increase tissue autofluorescence. Furthermore, extended PFA-fixation hardens the tissue and might impair diffusion of ethanol trough the tissue. Caution: Non-optimal tissue dehydration results in non-optimal clearing results (milky tissue appearance)!
- Store cleared samples in glas- or polypropylene-tubes as organic solvents can decompose polystyrene and polyethylene!

Materials/Reagents

Type/Name	Company	Serial/Cat-Number	Purpose/Remarks
Low gelling – low melting agarose, SeaPrep™ Agarose Or Phytagel™	Lonza Sigma	50302 P8169-100G	Lung preparation: 0.75% in H_2O Embedding: 1% in H_2O Lung preparation: 0.75% in H_2O Embedding: 1% in H_2O
4 % PFA/PBS, pH = 7.4			
Ethanol			pH adjustment to 9.0 with NaOH/HCl According to organ-specific protocol: 30%, 50% and 70%. NOT 100%!
Tween20	Carl Roth GmbH + Co. KG	9127.1	
Ethyl cinnamate (ECi)	Sigma Aldrich	112372	

Blood vessel staining via i.v.-Injection

1) Narcotize the mouse with 1.5 - 2 % Isoflurane (Forene 100 %, Abbott GmbH & Co. KG) in the Fluovac Narkosesystem of Havard Apparatus.
2) Inject (i.v.) 10 µg/mouse CD31 + 10 µg Sca1 + 110 µl PBS (total volume 150 µl) with a 1 ml Insulin syringe into the retro bulbar Plexus
3) Sacrifice the mouse via CO_2 20 min after antibody-injection
4) Perfuse the mouse with cold 5 mM EDTA/PBS until the liver gets bright (ca. 15 ml)
5) Perfuse the mouse with cold 4% PFA/PBS (10-15 ml)
6) Retrieve the samples (bones/lung/brain/...) and fix them in 4 % PFA/PBS at 4-8 °C for 4h **(If you don't perform a perfusion the bones have to be fixed in 4% PFA/PBS over night at 4-8°C!)**
7) After fixation the samples (bones/lung/brain/...) have to be transferred to PBS/Clearing solutions/embedding media...
8) The Fluorescence of the fixed organs is stable for month



ECi literature

J Am Soc Nephrol. 2016 Aug 3. Fully Automated Evaluation of Total Glomerular Number and Capillary Tuft Size in Nephritic Kidneys Using Lightsheet Microscopy. Klingberg A, Hasenberg A, Ludwig-Portugall I, Medyukhina A, Männ L, Brenzel A, Engel DR, Figge MT, Kurts C, Gunzer M.

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Embedding organic solvent-cleared Samples

Durable, cleared samples embedded in clear resin

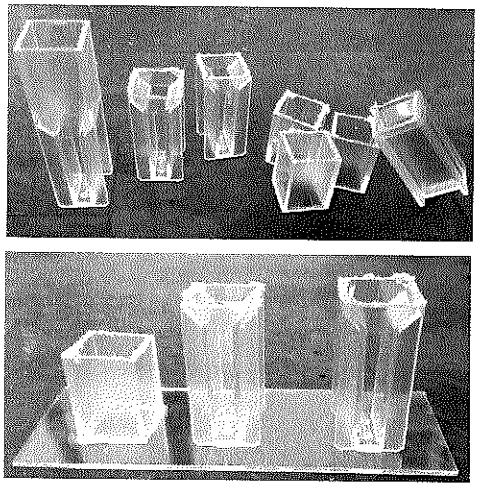
In order to protect the samples, we sought an optically clear resin/glue without autofluorescence to ensheath the sample, protect it from physical stress like scratching and pressure while being mounted and from surrounding liquids that are not equal to DBE used for clearing the samples.

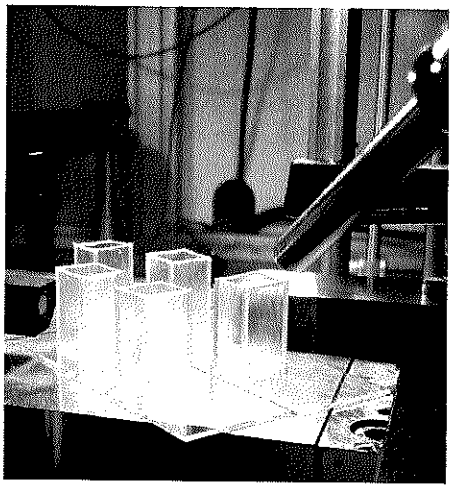
Two alternatives were tested: UV-glue (DYNMAX; OP-4-20632) and BioDur resin (E12/E1; www.biodur.de)

Things to know before you start

Molds

- UV-cuvettes out of special plastic are used as molds (XK26.1 Roth; ask specifically for 'Spezialkunststoff').
- **NEVER USE MOLDS OUT OF POLYSTYRENE/STYROL (PS)** → hardening BioDur becomes clouded and milky
- Saw the cuvettes into halves, leaving you with a bigger, rectangular part and the small measurement part
- Glue the rectangular part upside down (uncut side down) with UV-glue onto a glass coverslide (TIP: place glue along the outer side of the cuvette to avoid contact of glue and embedding material)
- For small samples (preferred and better to handle), use lower measurement part of cuvette. It can also be glued onto a coverslide or placed securely in a tube holder.
- Use Filter 4 of UV-source at gluing station

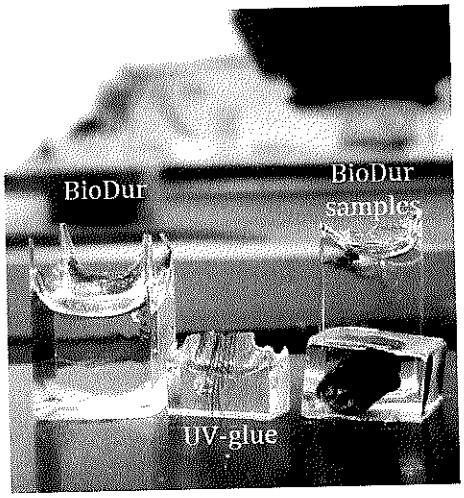




UV glue

Procedure

- Dry DBE-cleared sample briefly, than position it in mold
- Fill up mold with UV-glue
- Readjust sample to the correct position
- Harden glue for 5-10 min with UV light
- Break mold, recover embedded sample



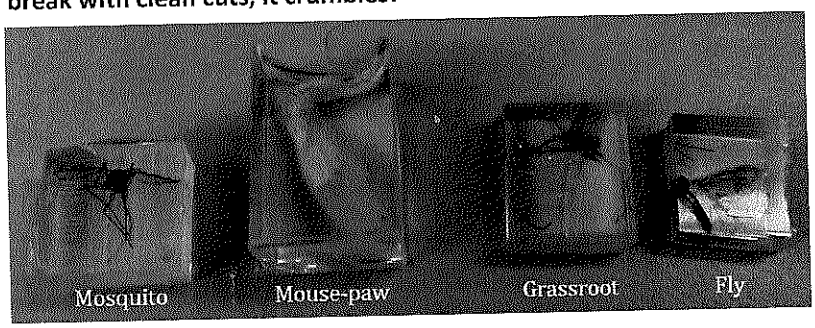
Pros and Limitations

- Due to the hardening process being UV-dependent, no fluorescent dyes can be used within the samples
- Glue comes in small tubes and is relatively expensive.
- Operated with air pressure, limiting the flow rate, which can lead to long filling times of the molds
- The fast hardening process allows for immediate imaging

BioDur

Procedure

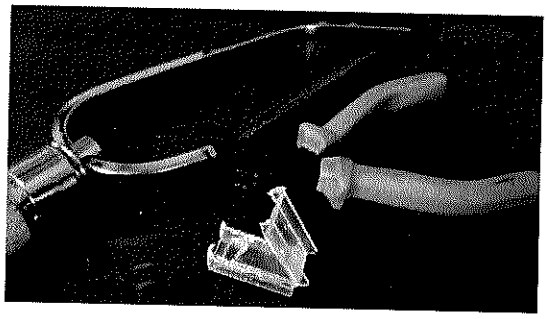
- Make sure E12 is not crystallized, but clear. If crystallization occurred, heat E12 up (50 – 70°C). Heating the canister can take up to one day. If you plan on more embeddings in the future, fill some 15ml Falcon tubes with 6ml E12 and store them at RT.
- With the samples at the ready, mix E12 (60%) with E1 (40%; shake well). Avoid air bubbles. Mix well by pipetting up and down until you no longer see refraction anomalies ('Schlieren').
- Fill molds to desired height. Consider the width of a normal sample holder.
- Place sample into mixture with forceps or scalpel.
- Place molds on radiator. Be aware that samples may drift upwards or downwards within the resin. Control and readjust samples after 2h.
- Apply parafilm to keep water out (BioDur turns white)
- Hardening takes up to 3 days; BioDur shows no autofluorescence and is very durable.
- Beware of two things: **If left in DBE, resin gets sticky and can be deformed!** It can also attach to other samples and destroy them in the process (separation difficult). **BioDur also does not break with clean cuts, it crumbles!**

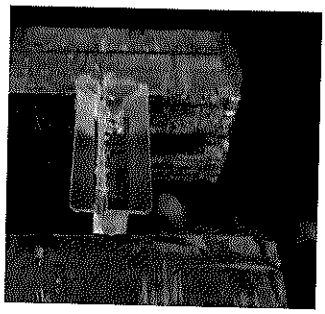


Breaking the mold

Procedure

- Once you are sure that the resin is completely hardened, cut along the side of the cuvette. Do not scratch the resin! Repeat this step on the other side. Take a cutting plier and crack open one side of the cuvette. Now you can cut the resin to size while still in the mold to avoid scratches. Cut the lower part and the upper part and recover sample. But beware, **cut surfaces are opaque and cannot be polished!**





Things to consider

Molds

- Do NOT use molds out of polystyrene (PS) because BioDur becomes cloudy

Samples

- If you are interested in imagery of already tested samples embedded in BioDur, view PPP about *Marketingsamples*. There is also a PPP about the improved onion stained with propidium iodide.
- I also cleared a human molar tooth nerve (provided by Heinrich) which can be found in the sample box embedded in BioDur and stained with PI.
- I also tried to produce a BioDur samples with fluorescent TetraSpec beads. Beads were detectable but very few in number. In order to embed them in BioDur, they need to be transferred to 100% EtOH. Thermo Fisher stated, that beads swell while in organic solvent and release the fluorophores. This results in drastically reduced bead numbers of altered size in the resin sample. I inquired whether PS beads are more stable (also at Phosphorex Inc. (info@phosphorex.com)), which they are not. At the moment, there seem no good solutions producing BioDur bead-samples.

Clearing

- Problematic samples were bigger insects with an exoskeleton of black chitin (bigger flies, locust head etc) and larger plants with dense tissue (Lilly of the valley). In the case of chitin, very long incubation times (2 months or longer) result in partial clearing. In the case of plants, I also tried a clearing protocol based on urea which yielded worse results than DBE.

Agarose cubes / Phyta Gel cube

- Boil 1% low melting agarose (conventional agarose may also work) in A. dest.
- Fill the agarose solution into a Petri dish (approx. 0.5 -0.8 cm)
- Wait until it is cooled down for cutting out small cubes (approx.1x1 cm edge length)
- Transfer a maximum of 5 cubes into 20 ml of 50% MetOH for 2.5 h at RT
- 70% for 2.5 h at RT
- 100% for 2.5 h at RT
- 100% over night at RT
- 100% DBE for 2.5 h at RT
- 100% DBE until usage in the fridge

If the cubes start shrinking and get milky whitish as soon as you transfer them into the DBE the dehydration with MetOH was not completed. In that case incubate for a longer time in a larger volume of 100% MetOH.

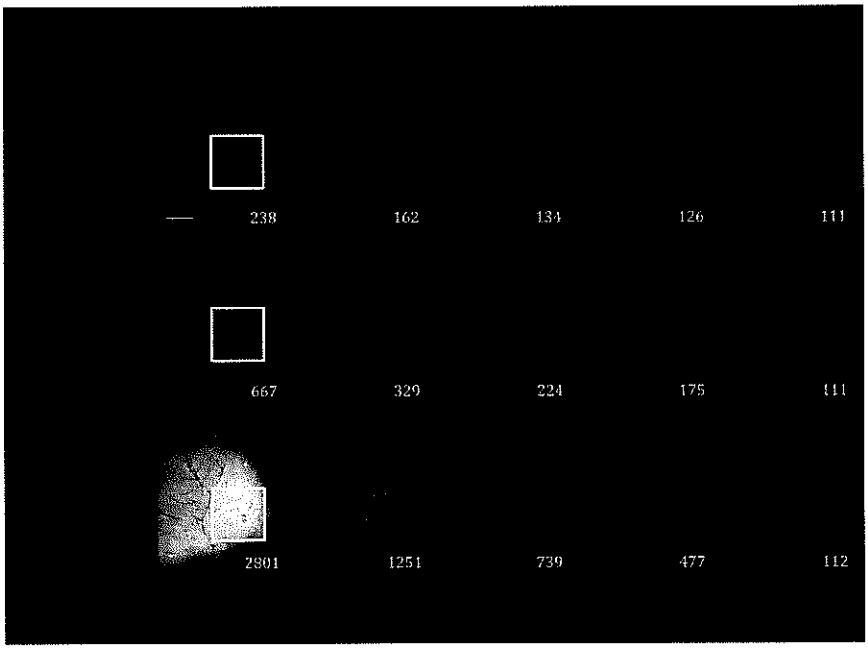
Extended dehydration of cubes with 100% MetOH does impair the quality of the clearing. It promotes a better result.

DBE (Benzylether)
ECi (Ethyl cinnamate)
MetOH (Methanol)

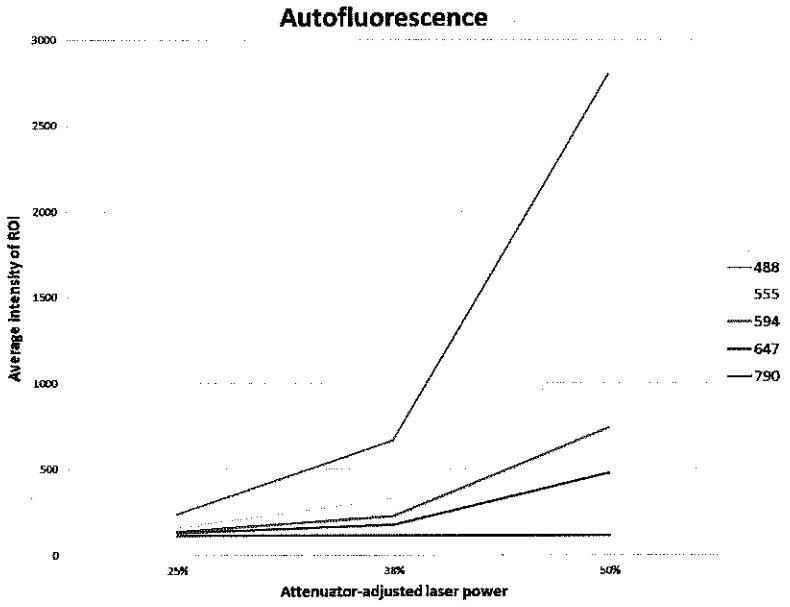
reagents	sample size	
	5- 10 mm	15 mm
50% (vol/vol) THF or EtOH	1 h	1 h
70% (vol/vol) THF or EtOH	1 h	1 h
80% (vol/vol) THF or EtOH	1 h	1 h
100% (vol/vol) THF or MetOH	2 x 1 h	1 h, overnight
DBE or ECi	2 x 1 h	2 x 1 h

The clearing can be done using DBE or ECi. If the MetOH or THF is not completely dry agarose cubes will not be cleared sufficiently. They will get milky and shrink. It might be useful to fill small aliquots of MetOH in bottles with 3Å molecular sieve.

Autofluorescence



- Stable parameters: 200ms illumination; 50% laser power; 2x optical magnification; NA: 0,027; sheet width: 3152; 1660 µm deep in tissue;
- Displayed boundaries (pixel values): 0 – 4000
- White # displays average intensity of rectangular in each image (16bit)
- Scale bar depicts 1.5 mm
- Sample: 1 cm^3 of THF/DBE cleared (3DISCO) formaldehyde fixed turkey breast



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