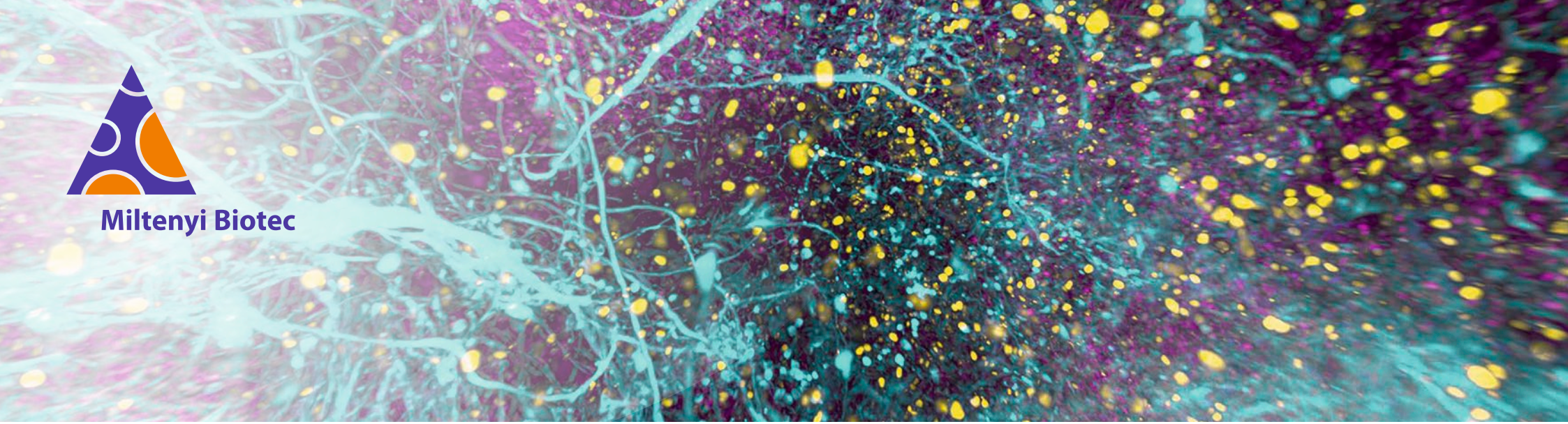

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Tips and tricks

Tissue clearing and sample preparation

Overview

Tissue clearing allows for 3D visualization and imaging of entire opaque tissues. Numerous tissue clearing protocols were published that can be attributed to the following main classes: aqueous-based, hydrogel-based, and organic solvent–based protocols. Organic solvent–based protocols are considered as fast and efficient.

Miltenyi Biotec's MACS® Clearing Kit was developed as a non-toxic variant of organic solvent–based tissue clearing. To support users of organic tissue clearing we compiled this list of tips and tricks. Covering common problems and pitfalls, it describes potential causes and recommended solutions to overcome these issues.

Refer to www.miltenyibiotec.com for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide.
Visit www.miltenyibiotec.com/support for local Miltenyi Biotec Technical Support contact information.

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Tissue preparation and fixation

Incident	Potential cause	Recommended solution
Incomplete fixation (under-fixation) Possible observations: • Perfusion fixation is performed but the tissue (like liver) is not white/bright after perfusion. • Inside of the organ is degraded, i.e., autofluorescence is very hazy, anatomical structure or cellular positions visible in autofluorescence are not distinguishable or are non-existent (observable under a microscope). • Tissue starts degrading or losing parts during or after fixation.	• Insufficient perfusion or post-fixation immersion perfusion is missing.	• If under-fixation is noticed early enough, an additional fixation step in the very beginning would help. No salvaging is possible after organ is cleared; improve for the next experiment.
	• Wrong fixative concentration.	• Use recommended concentration, e.g., 4% PFA.
	• Fixative is expired.	• Check fixative expiration date prior to use. In doubt, use only fresh fixative.
	• Wrong fixative has been used.	• 4% PFA is recommended, make sure that the antibodies are tested to bind after methanol fixation; some epitopes (like filaments epitopes) are damaged by methanol.
	• Insufficient fixative volume; not enough active fixative is available; tissue was not fully immersed.	• Use enough volume of the fixative, make sure that the tissue is fully immersed in the fixative.
	• Fixative temperature.	• Not a severe issue; shorten the fixation process at room temperature as much as possible and perform it at 4 °C; keep in mind that you would need more time for fixation at 4 °C.
	• Too short fixation time.	• Increase the incubation time, but make sure you avoid over-fixation.
Over-fixation Possible observations: • Organ shrinkage. • Antibody binds non-specifically. • Very high autofluorescence, loss of endogenous GFP. • Insufficient antibody penetration on the same incubation time.	• General.	• If organ is over-fixed, no salvaging is possible; improve for the next experiment.
	• Too high fixative concentration / too long fixation time.	• Decrease the fixative concentration or incubation time.
	• pH was not optimal.	• Normally for PFA, pH is adjusted to 7.2–7.4; for the endogenous GFP preservation fixative pH = 9, dilute with HCl or NaOH for pH adjustment.
	• Temperature is too high.	• Perform fixation at 4 °C and use cold fixative.

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Tissue preparation and fixation

Incident	Potential cause	Recommended solution
Pigmentation/blood residue Possible observations: • Dark residues in the tissue, observable by eye. • Shadow artifacts during light sheet imaging.	• Perfusion was not done, or it is done inefficiently.	• If possible, do perfusion for the next experiment. If perfusion is not possible, depigmentation* might help to decolorize the area.
	• Too fast perfusion (can also introduce rupture artifacts!).	• Improve perfusion by using a slower perfusion solution flow.
	• Perfusion solutions like heparin, PBS, EDTA are not able to efficiently remove the extra blood or the pigments which are not related to blood.	• Try decolorization/depigmentation* using protocols with e.g. H_2O_2.
	• Perfusion is not possible due to age, or the tissue is already fixed.	• Try decolorization/depigmentation* by adding a chemical bleaching step using protocols with e.g. H_2O_2.
	• Tissue-specific properties: pigmented tissues like skin, melanocytes, eyes.	• Perform depigmentation*. If possible, use a sample from a younger animal to have fewer pigments; remove the pigmented tissue if it is not necessary.
	• Markers from medical operations, sample positioning marks, tumor cell tracers, contrast agents, tattoo pigments.	• Physically remove marks if the area is not necessary for imaging.
Agarose embedding of the sample failed Possible observation: • Tissue falls out of the agarose/phytagel.	• The surface of the organ was wet when embedding in agarose was done.	• Dry the organ carefully with only a piece of tissue (not completely!) to avoid changes in concentration due to water content.
	• Temperature of the agarose or its concentration are not optimal.	• Pay attention to the optimal temperature (not too hot, as a rule of thumb, the bottle can be touched by hand without hurting) and concentration of the agarose (1.5%) or phytagel (1%); cover it completely if organ is prone to falling out; re-embed it until it stays in.
Tissue is dried out Possible observations: • Rupture in the tissue, yielding artifacts during imaging. • Tissue sticks to the side of the tube during incubation steps. • Very high autofluorescence.	• Solution volumes are too low.	• Make sure the sample is fully immersed in the respective liquids, store it in buffer after fixation.
	• Too fast dehydration.	• No salvaging possible; imaging can be done but might yield results with artifacts; restart and make sure that the sample is floating, is fully immerse, and does not stick to the tube; slow down the rehydration.

*In this document, the term "depigmentation" is used synonymously with "decolorization" and "chemical bleaching".

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Labeling with antibodies

Incident	Potential cause	Recommended solution
Strong staining on the surface of tissue or staining gradient Possible observations: • Bright "crust" on the rim of the tissue during imaging. • Strong intensity signals on the surface, growing weaker towards tissue center.	• Insufficient antibody incubation.	• Increase the incubation time according to the tissue size.
	• Insuffcient permeabilization.	• Increase time for incubation with permeabilization solution (sample specific). • Refresh permeabilization solution; increase solution volume according to the tissue size. • Improve permeabilization by an additional dehydration/rehydration series using 2% Tween®-20 in ethanol prior to staining.
	• Insufficient penetration or diffusion of secondary antibody.	• Use conjugated primary antibodies to reach a homogeneous labeling and penetration in the sample and to reduce the overall protocol time in parallel. • Increase incubation time for the secondary antibody or try out different secondary antibody. • Optimize the permeabilization (see "Insufficient permeabilization").
	• High antibody concentration.	• Optimize antibody concentration via titration and consider lower concentrations. For Miltenyi Biotec antibodies, refer to their recommended dilution; for other providers include dilution ranges given for IHC.
	• Insufficient antibody amount, depletion of antibody by target.	• Optimize antibody concentration via titration and consider higher concentrations. • For Miltenyi Biotec antibodies, refer to their recommended dilutions; for other providers include dilution ranges given for IHC.
	• Insufficient blocking.	• Consider a longer blocking incubation; increase blocking serum volume.
	• Antibody aggregates on sample's surface.	• If the aggregates are present in antibody stock solution, centrifugation (e.g. 10,000×g for 10 min) or filtration might help. • If penetration is not causing this issue, reduce the antibody concentration.
	• Antibody is too large or unsuited, and cannot penetrate deep into the sample.	• Try other antibodies if available.
	• Over-fixation.	• See "Over-fixation" section.
	• A natural matter of high abundance of antigens on the surface of the tissue, for example, abundance of astrocytes and their markers in comparison to other glia or neurons.	–
	• The antibody does not stain the region of interest but other regions on the surface.	• Increase the antibody incubation time; increase antibody concentration and optimize the permeabilization (see "Insufficient permeabilization").

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Labeling with antibodies

Incident	Potential cause	Recommended solution
Weak staining or no staining Possible observations: • No apparent signal is visible. • Only autofluorescence is visible.	• Insufficient fixation.	• See "Under-fixation" section.
	• Wrong fixative.	• Use 4% PFA.
	• Insufficient permeabilization.	• Optimize the permeabilization (see "Strong staining on the surface of tissue or staining gradient → Insufficient permeabilization").
	• Antibody is not suitable.	• Try other antibodies if available.
	• Degradation of the epitopes during fixation, depigmentation*, or clearing. • H_2O_2 depigmentation* can affect epitopes (for example mouse CD31).	• Tissue is old; under-fixation happened; see "Under-fixation" section above. Other clearing methods might be suitable.
	• Light exposure, photobleaching.	• Avoid light exposure throughout sample preparation process. Consider using Miltenyi Biotec's 3D-IF antibodies conjugated to highly photostable Vio® Dyes.
	• Marker is not expressed on the sample.	• Check if the absence of the staining or marker has a biological relevance or is due to technical issues. • Include a positive control for tissue staining in parallel to your staining.
	• Antibody/reagents are too old.	• Check the expiry date of the product before use.
	• High background is masking the staining signal.	• Check the "High background" section below.
	• Primary antibody labeling resulted in a weak signal.	To amplify the signal different staining strategies can be pursued: • Using a primary non-conjugated antibody + secondary antibody. • Using a polyclonal antibody to bind more epitopes per target protein. • Increase the antibody concentration if non-specific binding is not an issue.
	• Quenching of GFP fluorescence or other fluorescent proteins.	• Adapt the protocol by adjusting dehydration agent and pH to preserve the GFP signal, or use anti-GFP antibodies to boost the signal.

*In this document, the term "depigmentation" is used synonymously with "decolorization" and "chemical bleaching".

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Labeling with antibodies

Incident	Potential cause	Recommended solution
High background signal	• Over-fixation.	• See "Over-fixation" section.
	• Dried-out tissue.	• See "Tissue is dried out" section.
	• Insufficient washing steps.	• Add more or longer washing steps.
	• Antibody concentration is too high.	• Use the recommended concentration; titrate antibody down.
	• Tissue-specific issues related to non-specific antibody binding or stickiness.	• Test non-specific antibody binding using an isotype control antibody. • If antibody penetration is not cause of the issue, non-specific binding might be a feature of this particular antibody/sample combination.
	• Insufficient blocking.	• Optimize blocking see "Strong staining on the surface of tissue or staining gradient → Insufficient blocking. • Change antibody if it still produces background.
	• Autofluorescence.	• Consider depigmentation* protocols which might reduce autofluorescence (e.g. melanin in skin). • Sample the tissue from younger animal which tend to have less autofluorescence-producing pigments. • Physically remove the autofluorescent sample parts, e.g., skin, etc.
Scattered fluorescent signal on the tissue Possible observations: • Scattered bright spots on the tissue.	• Aggregates from the antibody solution.	• If the aggregates are present in antibody stock solution, centrifugation (e.g. 10,000×g for 10 min) or filtration might help.
	• Buffer contamination.	• Filter the buffer or simply use fresh buffers.
	• Air bubbles.	• Not a real fluorescent signal, rather ambient light is reflected in all channels. • Gently shake the tissue in its solution; remove the bubbles using forceps or a desiccator (vacuum extraction of air bubbles); extract air bubbles, e.g., from lungs using insulin syringe by suction or by injection of liquid; depending on the situation.
	• Necrotic areas.	• Choose bright antibodies that exceed the background signal; exclude this area from imaging; cut away necrotic regions, if possible, exclude the area from the image analysis.

*In this document, the term "depigmentation" is used synonymously with "decolorization" and "chemical bleaching".

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Tissue clearing

Incident	Potential cause	Recommended solution
Tissue is not cleared Possible observations: • Blurry signal/autofluorescence. • Sample appears foggy macroscopically.	• Also see "Pigmentation / blood residue" section.	
	• Over-fixation.	• See "Over-fixation" section.
	• Insufficient permeabilization.	• Optimize the permeabilization (see "Strong staining on the surface of tissue or staining gradient → Insufficient permeabilization").
	• Insufficient tissue clearing or dehydration solution volume.	• Make sure the solution volume is high enough to cover the tissue and is also sufficient for the dehydration/clearing chemical processes.
	• Insufficient incubation time.	• Increase the incubation time relative to the sample size, see Miltenyi Biotec's tissue clearing protocols.
	• Washing steps interfered.	• Always make sure you include long enough washing steps, with sufficient volume of washing buffers.
	• Refractive index (RI) is altered.	• Make sure not to contaminate the clearing solution with other substances. Use fresh clearing solution. The sample might carry a bit of ethanol after dehydration. If the clearing solution volume is low, then the ethanol mixing with clearing solution might lead to RI mismatch.
	• Clearing reagents are expired.	• Check the shelf life dates.
	• Temperature.	• Increase temperature (to about 28 °C) if possible. Verify if antibody staining resists higher temperature during clearing step.
	• Dehydration was too short.	• If dehydration was too short and minimal residual water remains in sample, it becomes blurry (not fully white). A second round of incubation in clearing solution can be helpful to render the sample transparent. Also see "Tissue turns white during clearing". • Increase dehydration time and/or volume.
	• Insufficient delipidation.	• Optimize the permeabilization (see "Strong staining on the surface of tissue or staining gradient → Insufficient permeabilization"). Increase the incubation time with ethanol series. • Increase solution volume according to the tissue size.

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Tissue clearing

Incident	Potential cause	Recommended solution
Tissue turns white during clearing Possible observations: • After transfer to clearing solution, organ turns white.	• The tissue is not sufficiently dehydrated.	• Not salvageable if sample contains substantial residual water, improve parameters as below for the next experiments. • If dehydration was too short and minimal residual water remains in sample, it becomes blurry (not fully white). A second round of incubation in clearing solution can be helpful to render the sample transparent. • Consider adding multiple or longer dehydration steps. • Volumes of dehydration or clearing solution are insufficient for the size of the sample. • Increase the dehydration duration. • If the organ has floated up during dehydration or clearing (they do after transfer from 100% ethanol to clearing solution), gently shake the sample after transfer to the clearing solution. Make sure it can sink down; if it doesn't, one part of the organ turns white. • Make sure that the sample does not create streaks in the ethanol series or clearing solution anymore before imaging or transfer to next tube. If streaks persist, repeat the current step and exchange for fresh ethanol or clearing solution.
	• Ethanol contains water.	• Use fresh ethanol bottle. • We recommend adding activated/dehydrated molecular sieves (3 Å, 4–8 mesh, Sigma, 208574-1-KG) to stored 2.5 L bottles of pure ethanol. **Note:** Make sure you let sieves cool down after activation to prevent potential ignition/explosion of ethanol solution.

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Imaging

Incident	Potential cause	Recommended solution
Blurry spots	• RI mismatch or insufficient delipidation.	• Follow the source of blurriness. Based on the tissue type and your observations above-mentioned points can be helpful.
	• Burned tissue from cauterization.	• Try not to burn the tissue; cut away the burned part. This burned tissue is not salvageable.
	• Air bubbles stuck in the sample.	• The emission light can be deflected and lost, see "Scattered fluorescent signal on the tissue" section above.

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▸ **miltenyibiotec.com/3D-IF-antibodies**

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Browse our protocols based on the MACS® Clearing Kit to get started right away. You will find optimized step-by-step protocols for various tissue types.

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