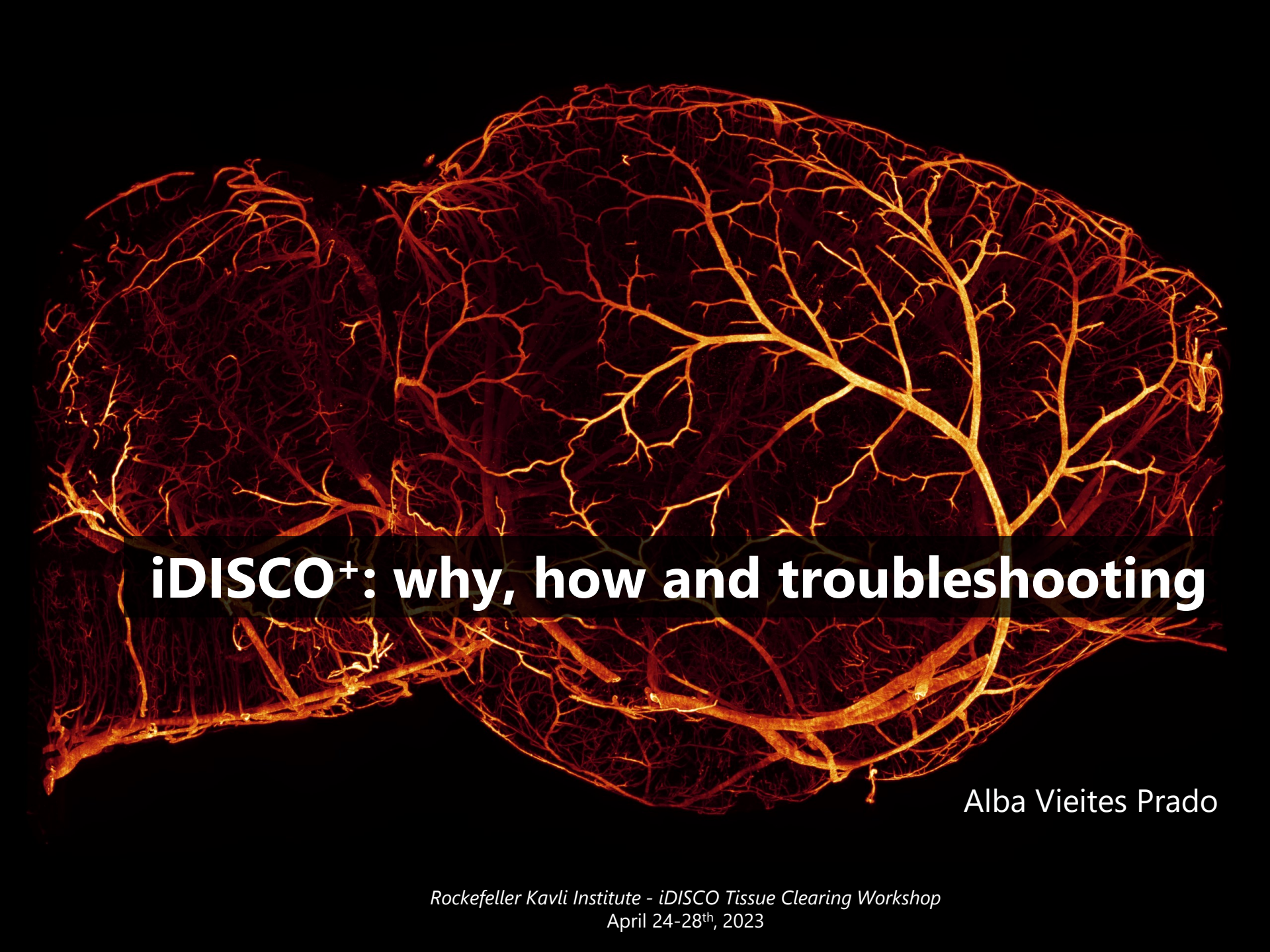
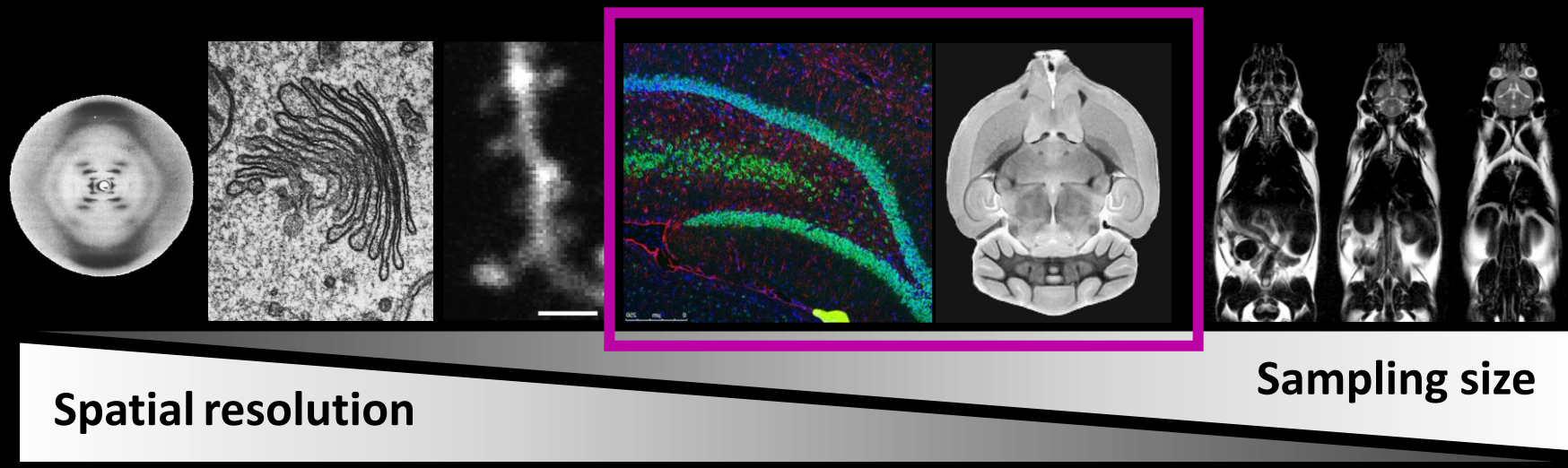


iDISCO^{+}: why, how and troubleshooting

Alba Vieites Prado

Rockefeller Kavli Institute - iDISCO Tissue Clearing Workshop
April 24-28th, 2023

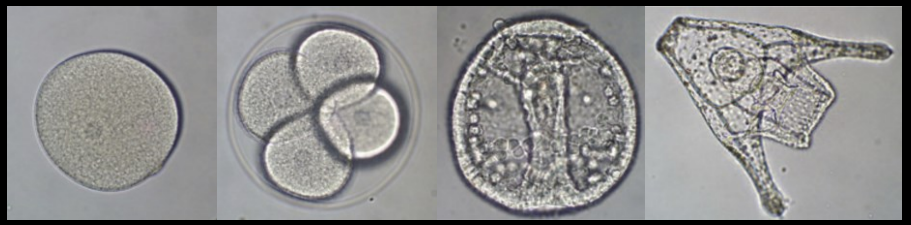
Scales in Biology



How to study cm-large tissues with cellular resolution?

Transparency & model organisms

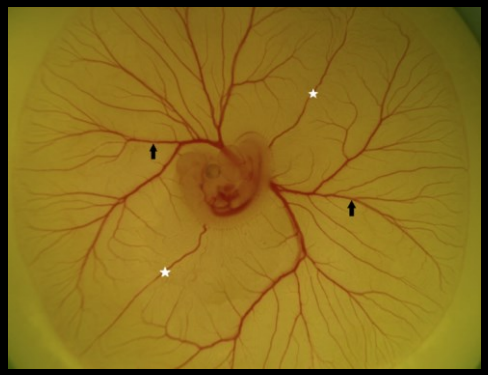
Early development in the sea urchin
(Cebra-Thomas)



C. elegans
(Mark Leaver)

Ascidians
(Christian Gloor)

Zebrafish larvae
(Hill, M.A. Embryology Zebrafish Development)



Chorioallantoic vasculature in the chicken
(Kennedy et al. 2022)

Chicken embryo
(Schoenwolf G. 2018)

Transparency & model organisms

Sea urchin
(Ann-Cutting)

C. elegans
(Mark Leaver)

Zebrafish

Ascidians
(Christian Gloor)

Chick

Mouse embryo

Tissue clearing, an old concept that has just recently developed

Spalteholz 1914

Über das Durchsichtigmachen von menschlichen und tierischen Präparaten und seine theoretischen Bedingungen

Nebst Anhang:

Über Knochenfärbung

Von

Dr. med. et LL. D. Werner Spalteholz

a. o. Professor an der Universität Leipzig

Zweite, erweiterte Auflage

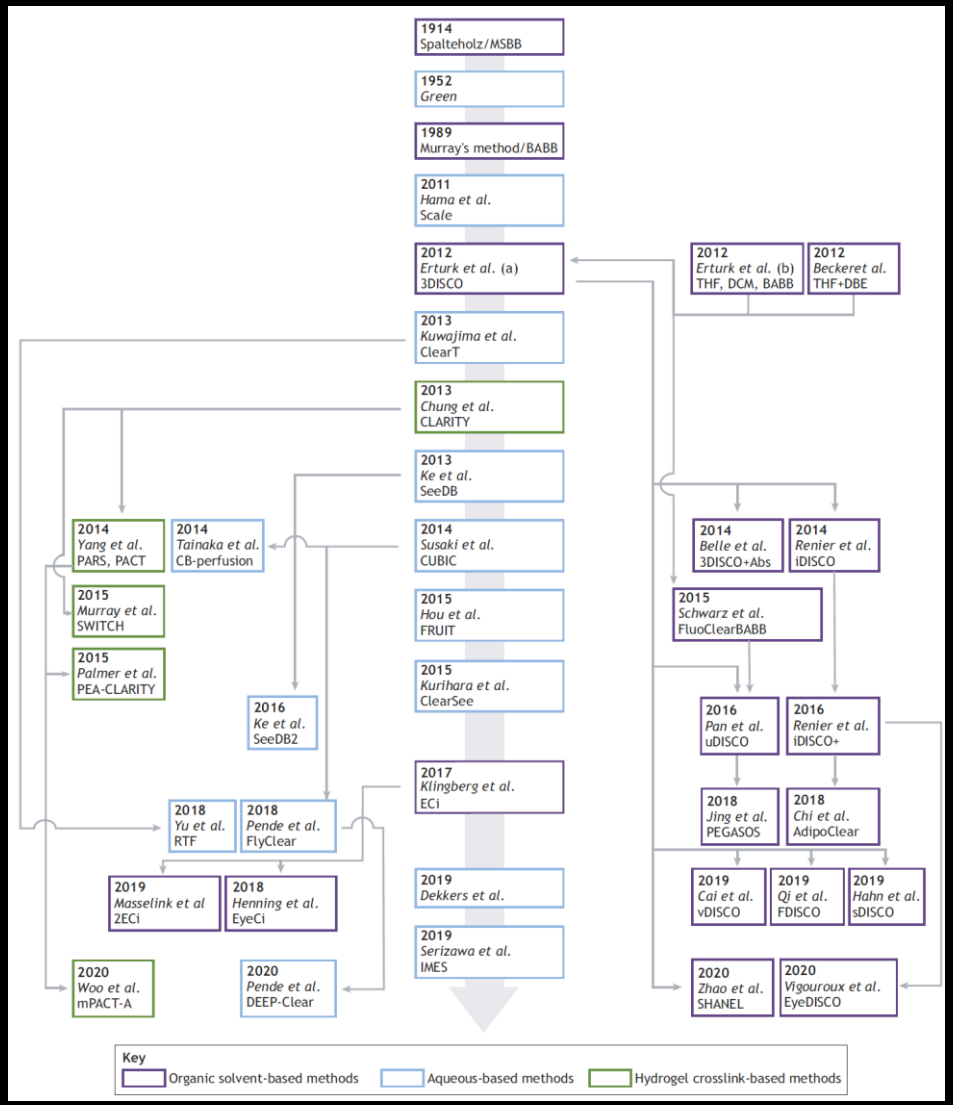
LEIPZIG

VERLAG VON S. HIRZEL

1914

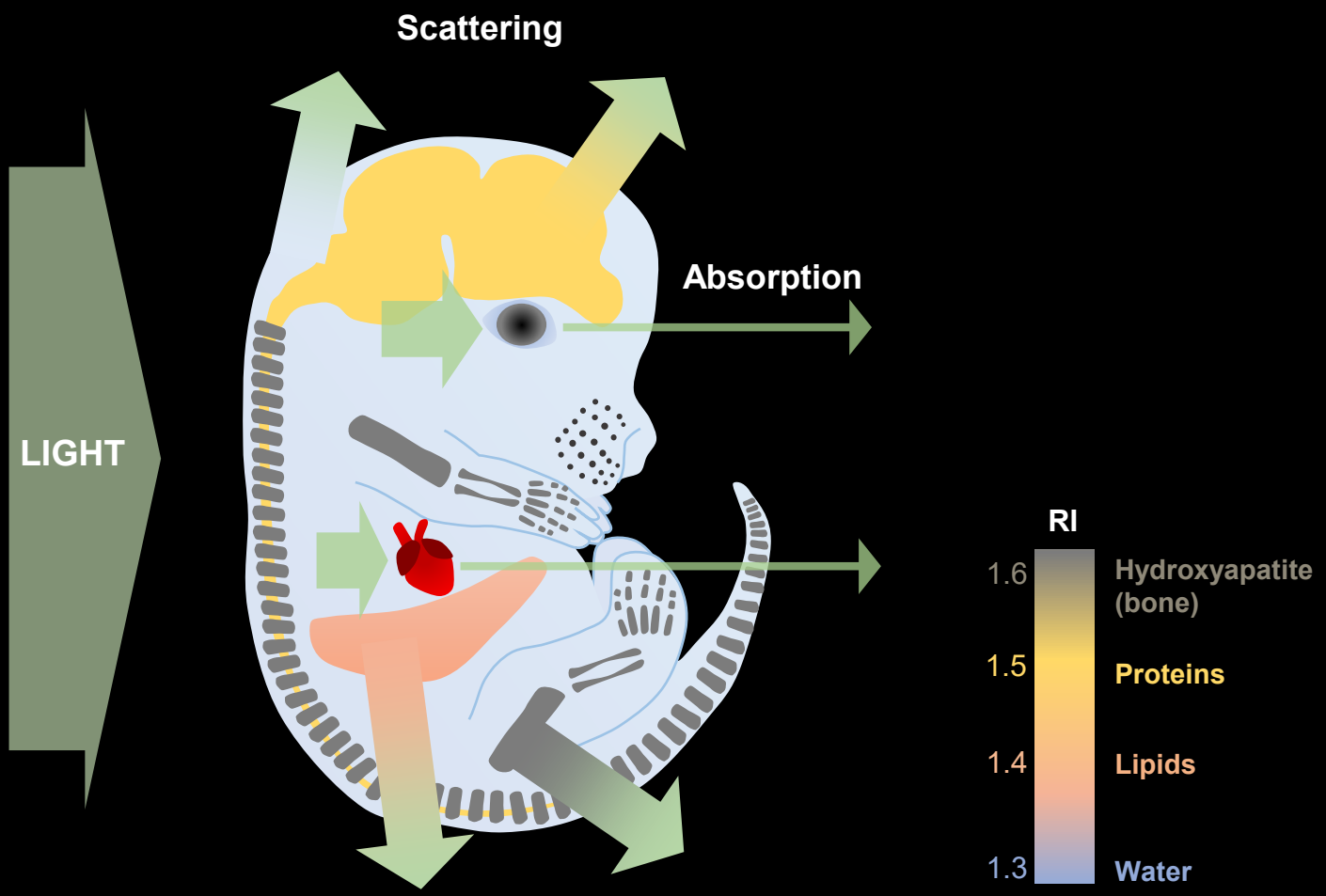
"About the transparency of human and animal preparations and its theoretical conditions"

Tissue-clearing methods used in developmental studies (2021)



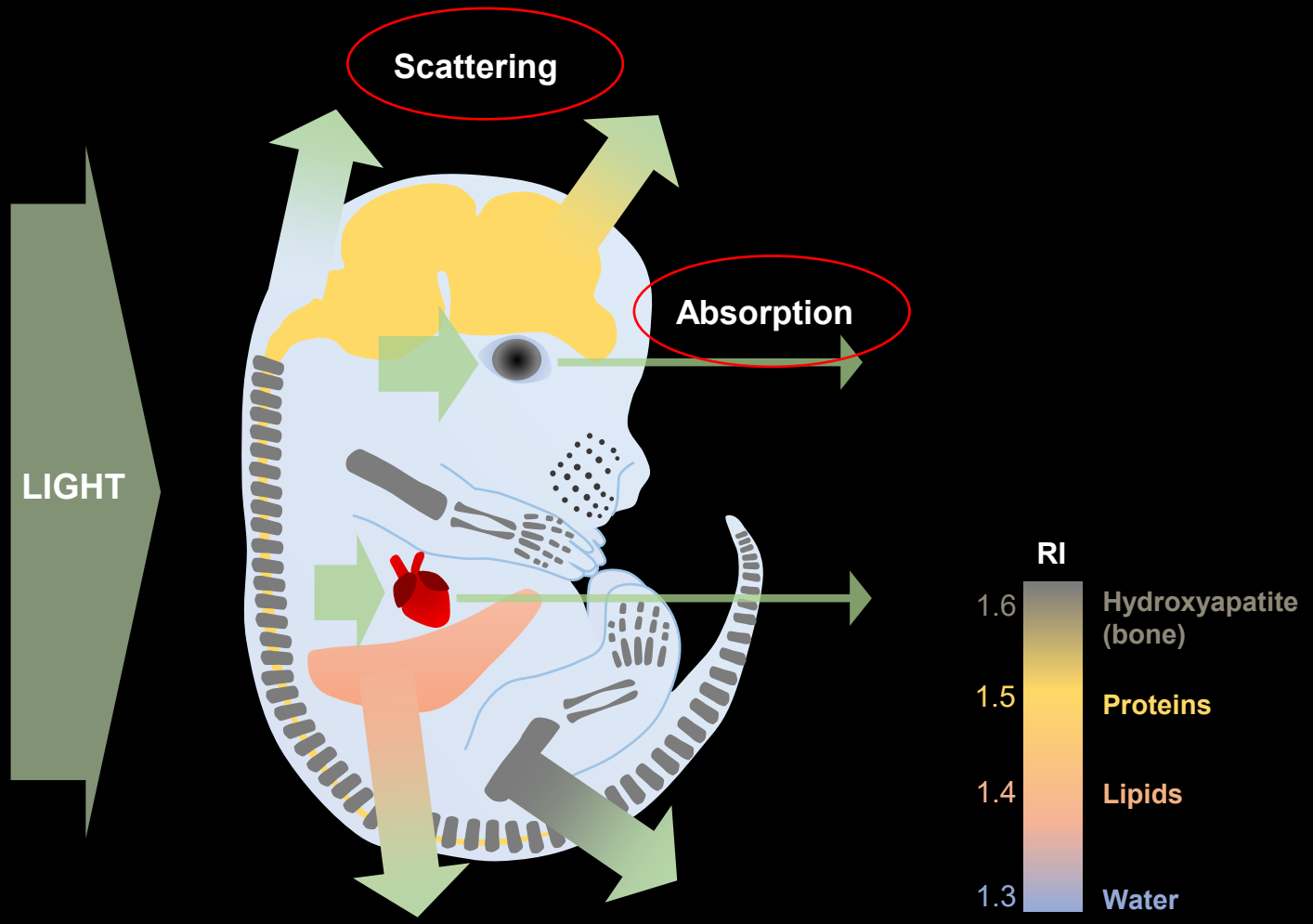
Vieites-Prado A, Renier R. Development 2021

Many methods for tissue clearing, same principle: RI homogenization



Biological samples contain a mixture of Refractive Index (RI) and pigments that perturb the light path

Many methods for tissue clearing, same principle: RI homogenization



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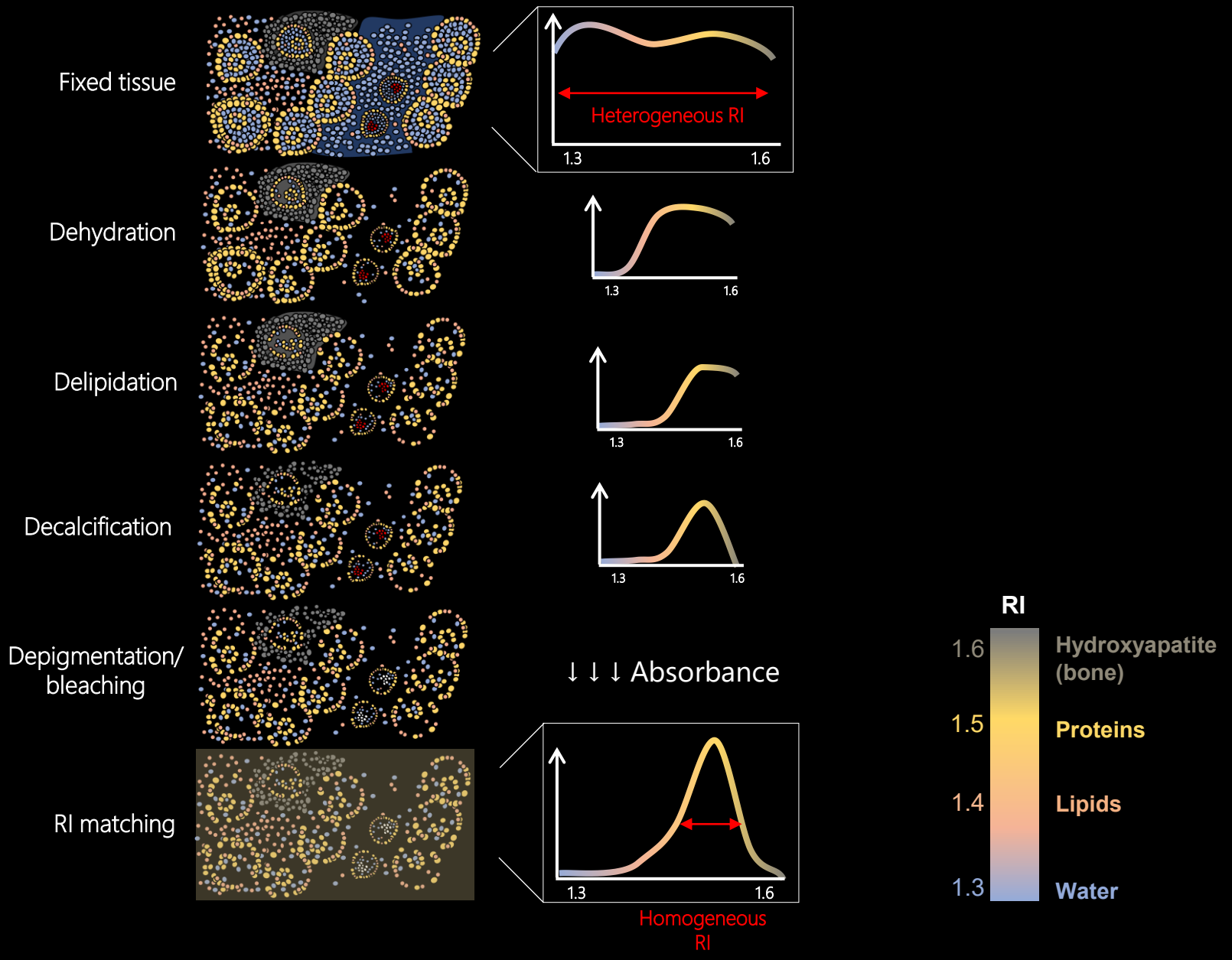
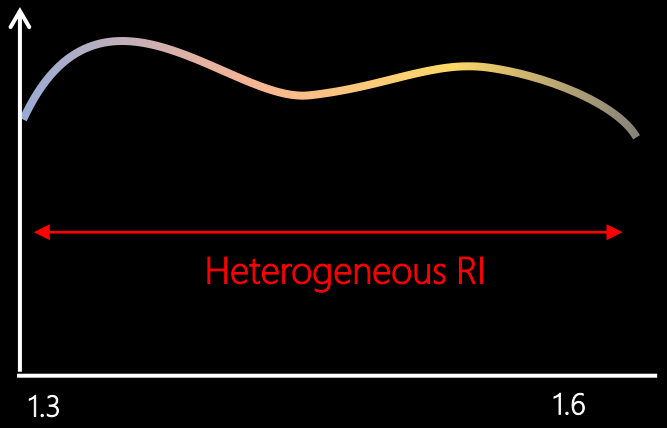
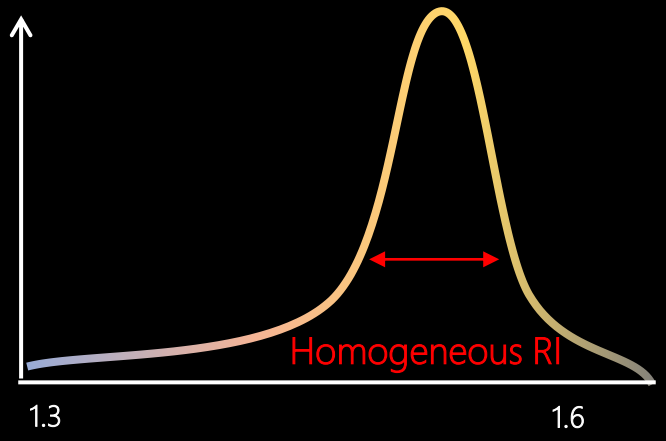


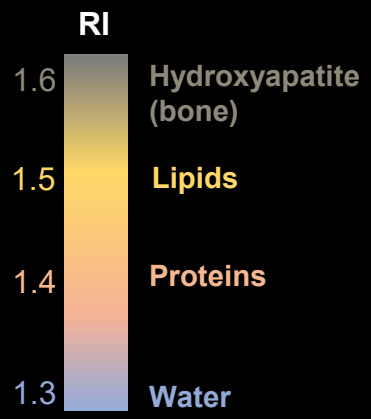
Many methods for tissue clearing, same principle: RI homogenization

Raw biological tissues



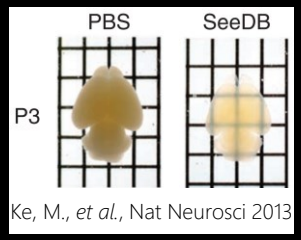
After tissue clearing





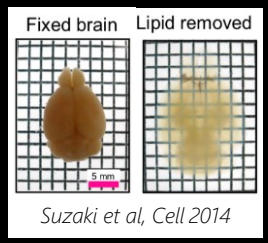
Four families of tissue clearing protocols

SeeDB



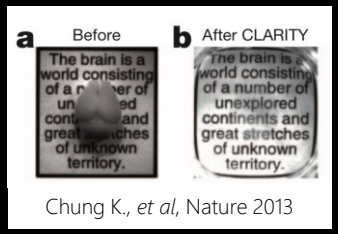
Ke, M., *et al.*, Nat Neurosci 2013

CUBIC



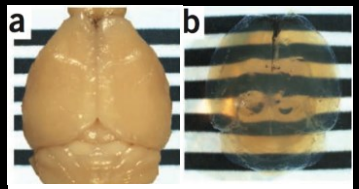
Suzaki et al, Cell 2014

CLARITY



Chung K., *et al*, Nature 2013

3DISCO



Erturk A., *et al*, Nat Med 2012

Direct RI Equilibration

SeeDB

Ke, M., *et al.*,
Nat Neurosci 2013

FRUIT
SeeDB2G/S
TDE
OptiClear
FluoClear/RapidClear
…

Hyper-hydration

CUBIC

Suzaki, EA., *et al.*,
Cell 2014

Scale U/A2
Scale S
CUBIC-X
CUBIC Cancer
CUBIC 1,2,3,4
…

Protein-Gel Crosslinking

CLARITY

Chung, K., *et al.*,
Nature 2013

PARS/PACT
Switch
SHIELD
ExM
…

Organic Solvents

DISCO

Ertürk, A., *et al.*,
Nat. Med. 2012

FluoBABB
iDISCO+
ECi
PEGASOS
uDISCO
vDISCO
…

Protein fluorescence preservation

Scatter-free imaging

How to choose a clearing method?

1. Sample size

2. Sample type

3. Signal type

4. Imaging modality

How to choose a clearing method?

1. **Sample size**
   - Brain slice < 1mm/embryos – Refractive index matching
   - Mid-size samples – Classic methods (DISCO, CUBIC, Clarity)
   - Large samples (>2cm– uDISCO, vDISCO, perfused CUBIC)
2. **Sample type**
3. **Signal type**
4. **Imaging modality**

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How to choose a clearing method?

1. **Sample size**
2. **Sample type**
   - Sample age
     - Embryo
     - Young tissue
     - Adult tissue
   - Sample complexity
     - Whole-organ
     - Whole-organism
3. **Signal type**

   Ex. 3DISCO, iDISCO, CUBIC, FluoClearBABB, etc
4. **Imaging modality**

How to choose a clearing method?

1. **Sample size**
2. **Sample type**
   - Sample age
     - Embryo
     - Young tissue
     - Adult tissue
   - Sample complexity
     - Whole-organ
     - Whole-organism
3. **Signal type**

Ex. iDISCO^{+}, CLARITY, 2ECi

4. **Imaging modality**

How to choose a clearing method?

1. Sample size

2. Sample type

Tissue clearing methods are modular, adapt the chosen protocol to the features of your sample by adding:

- Pigment bleaching
- Decalcification
- Permeabilization

3. Signal type

4. Imaging modality

How to choose a clearing method?

1. Sample size

2. Sample type

3. **Signal type**
   - Endogenous XFP (Ex. All aqueous)
   - Lipophilic tracer (Ex. ClearT2, RTF, FRUIT)
   - Immunolabeling (Ex. iDISCO^{+})

4. Imaging modality

How to choose a clearing method?

1. Sample size

2. Sample type

3. **Signal type**
   - Endogenous XFP (Ex. All aqueous)
   - Lipophilic tracer (Ex. ClearT2, RTF, FRUIT)
   - Immunolabeling (Ex. iDISCO^{+})

4. Imaging modality

How to choose a clearing method?

1. Sample size

2. Sample type

3. **Signal type**
   - Endogenous XFP (Ex. All aqueous)
   - Lipophilic tracer (Ex. ClearT2, RTF, FRUIT)
   - Endogenour XFP+Immunolabeling (Ex. iDISCO^{+})

4. Imaging modality

How to choose a clearing method?

1. **Sample size**

2. **Sample type**

3. **Signal type**

4. **Imaging modality**
   - Confocal (Ex. RI matching methods)
   - Light-sheet (Ex. Any method)
   - 2 Photon microscopy (Ex. Any method?)

How to choose a clearing method?

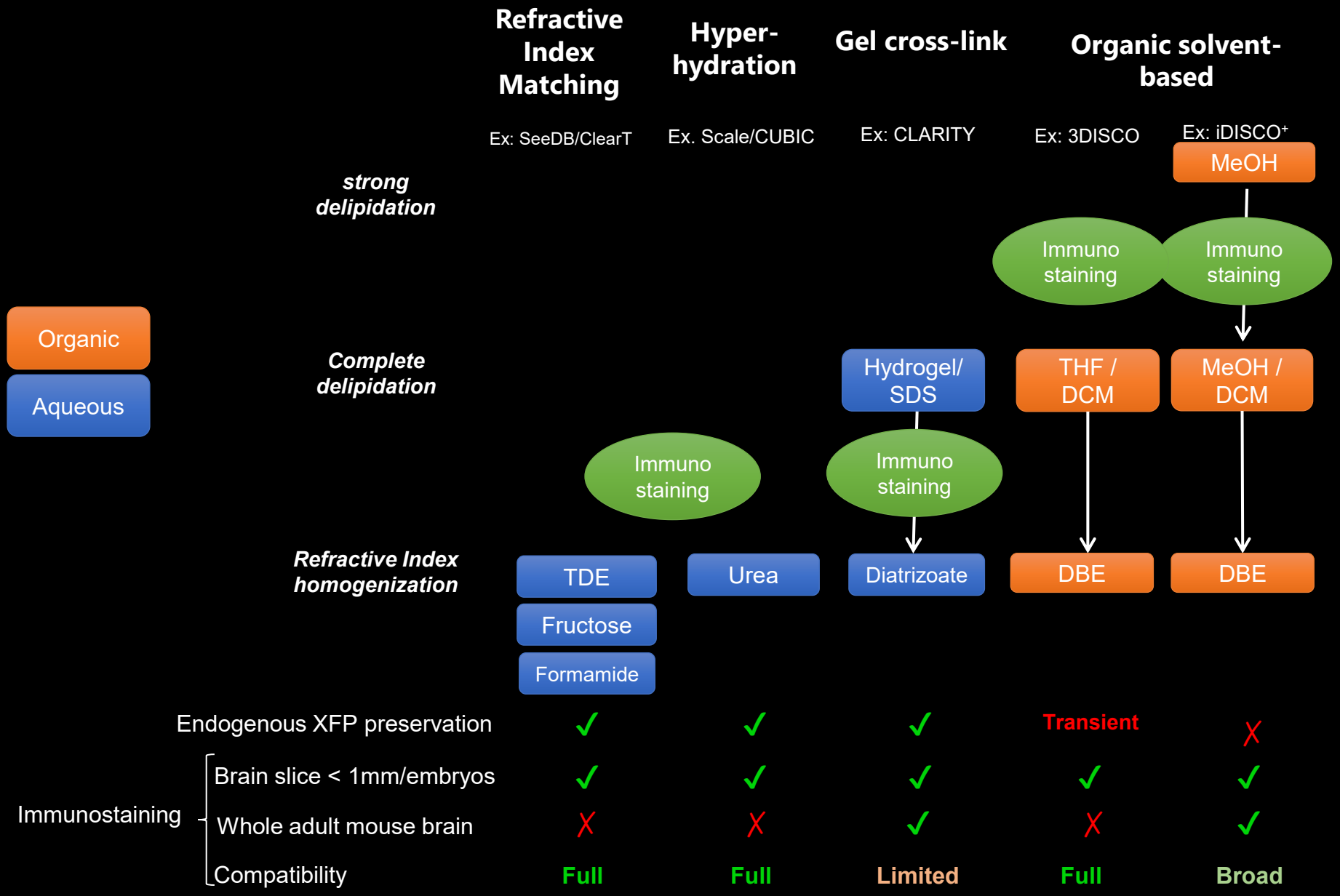
1. **Sample size**

2. **Sample type**

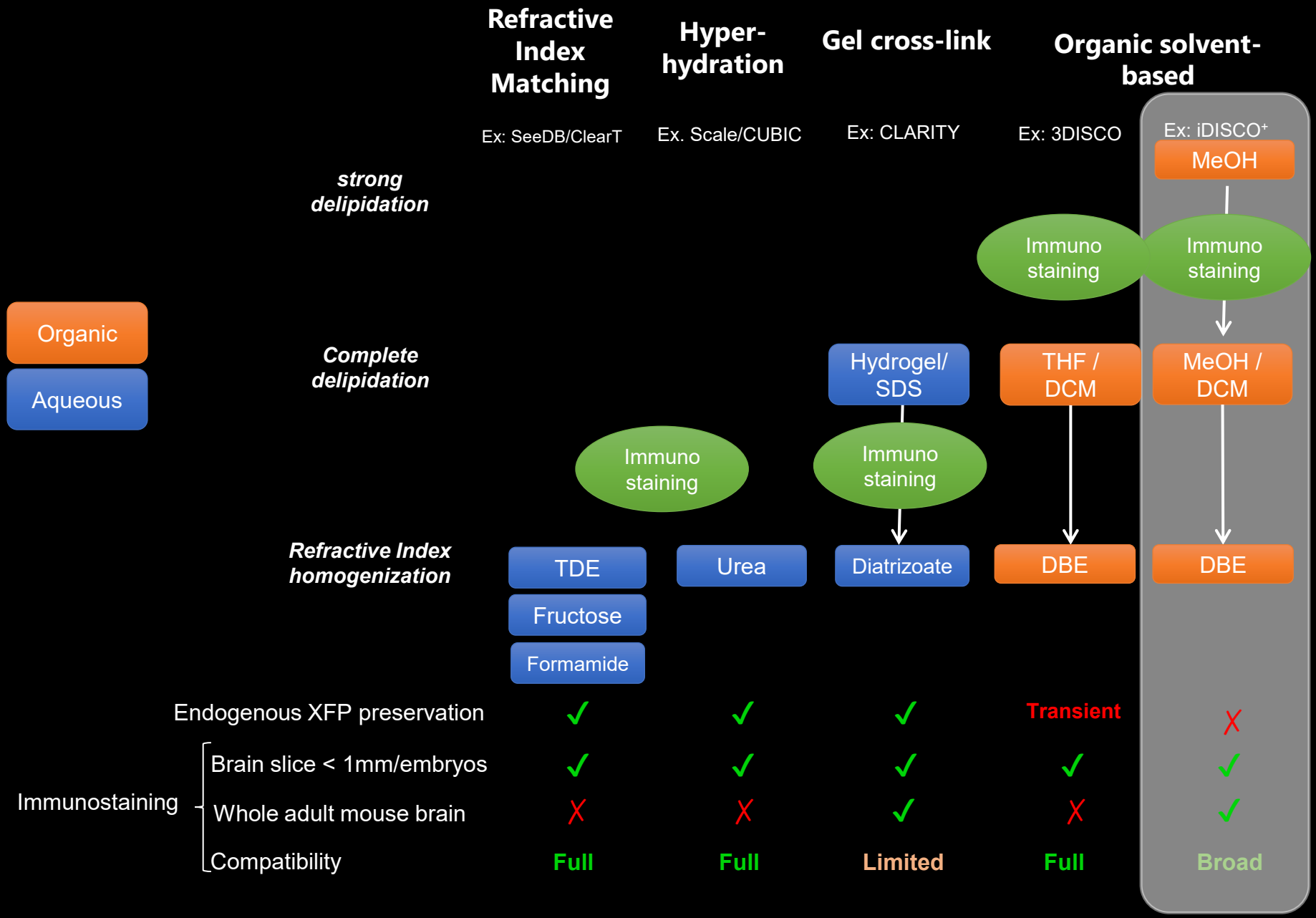
3. **Signal type**

4. **Imaging modality**
   - Confocal (Ex. RI matching methods)
   - Light-sheet (Ex. Any method)
   - 2 Photon microscopy (Ex. Any method?)

Summary: selecting a clearing method

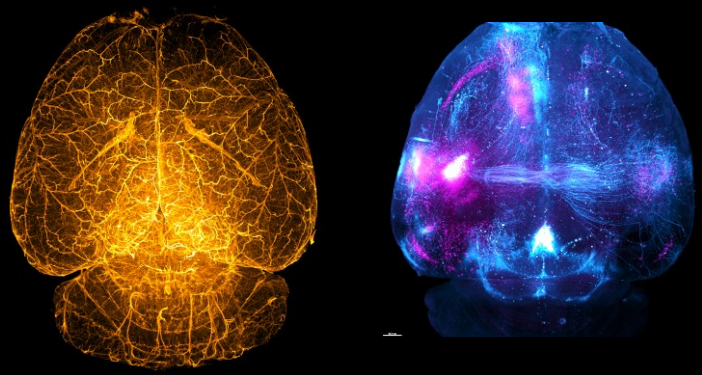


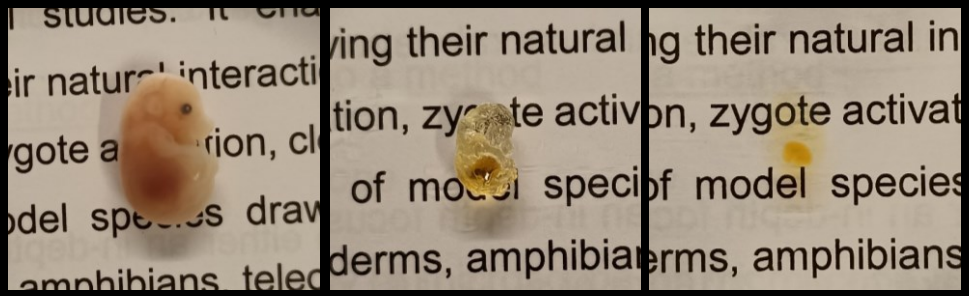
Summary: selecting a clearing method

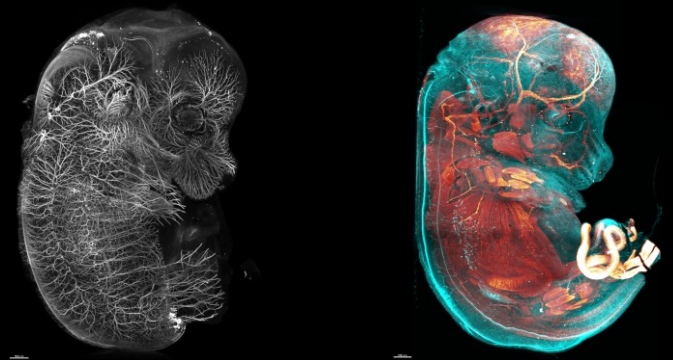


iDISCO+

A combined method for IMMUNOLABELING and CLEARING large complex tissues



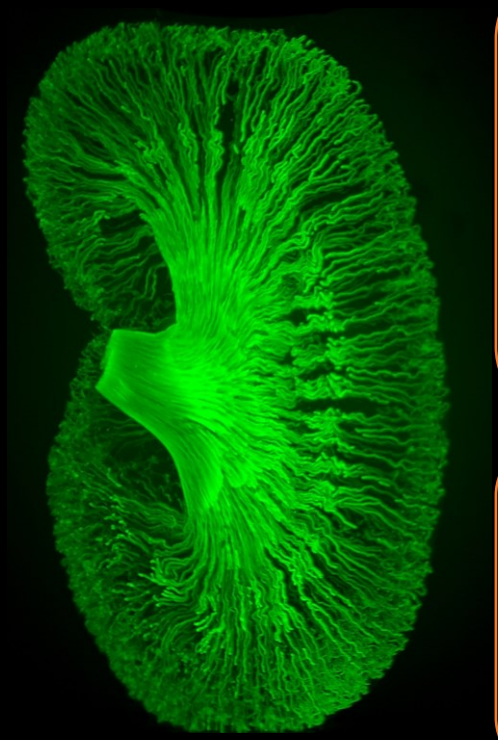


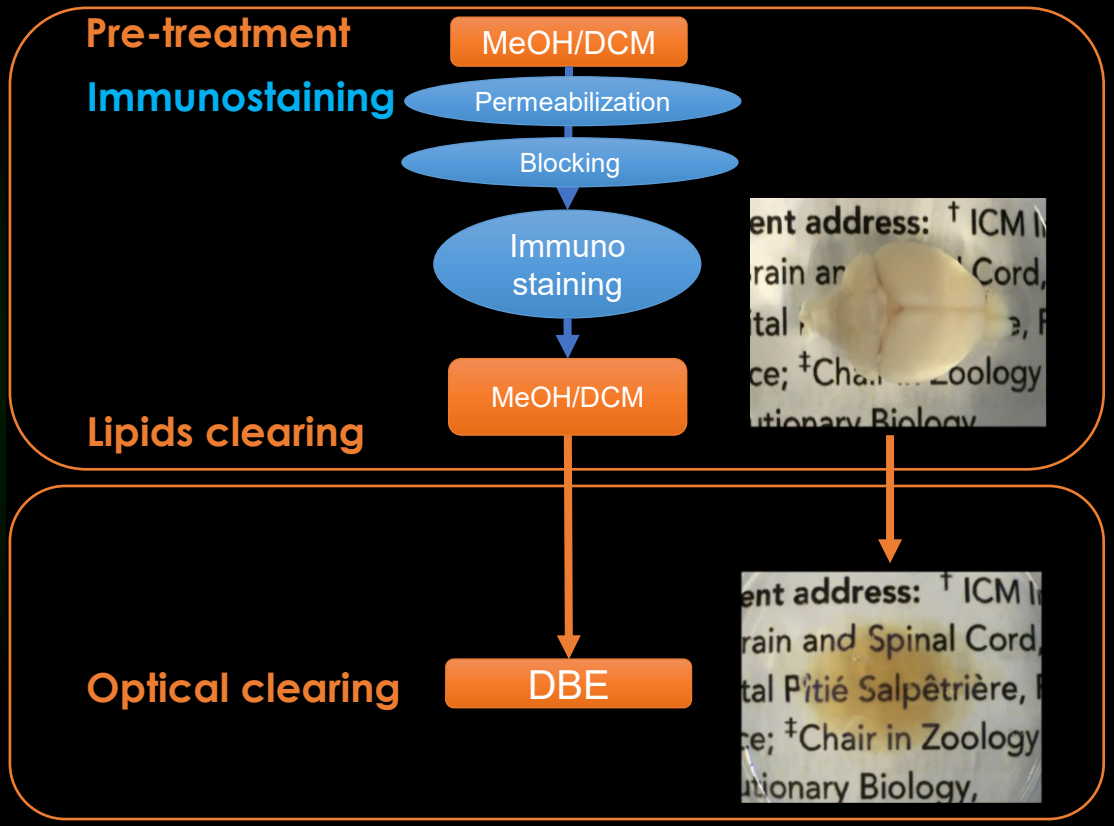


iDISCO+

A combined method for IMMUNOLABELING and CLEARING large complex tissues

*Enhanced **whole-mount immunolabeling protocol** + **3DISCO clearing** =* **iDISCO**





Renier, Wu, et al. Cell 2014

iDISCO+ overview

Day « 0 » - Sample preparation

Days 1 to 3 – Delipidation and bleaching

Day 3 – Permeabilization

Day 4 – Blocking

Day 5 – Antibody incubation

Day 15 – Washes in PBS-TwH

Day 16 – Antibody incubation

Day 24 – Washes in PBS-TwH

Day 25 – Dehydration

Day 26 – Refractive Index matching

iDISCO+ overview

Day « 0 » - Sample preparation

Days 1 to 3 – Delipidation and bleaching

Day 3 – Permeabilization

Day 4 – Blocking

Day 5 – Antibody incubation

Day 15 – Washes in PBS-TwH

Day 16 – Antibody incubation

Day 24 – Washes in PBS-TwH

Day 25 – Dehydration

Day 26 – Refractive Index matching

Aqueous

Organic

Day « 0 »: Sample preparation

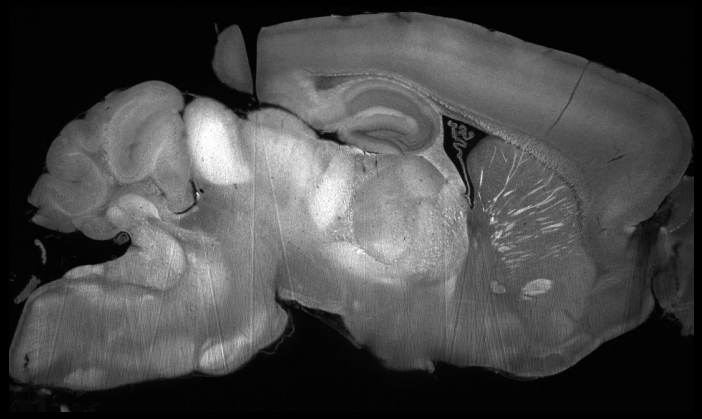
Perfusion (PBS + 4% PFA, or 4%PFA alone)
Postfixation (PFA 4% RT)
Washes in PBS - 3 washes x 15min (aprox.) in PBS - RT

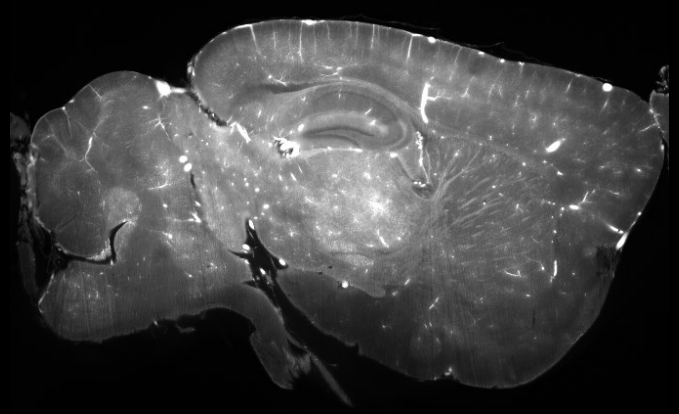
Day « 0 »: Sample preparation

Perfusion (PBS + 4% PFA, or 4%PFA alone)
Postfixation (PFA 4% RT)
Washes in PBS - 3 washes x 15min (aprox.) in PBS - RT

***What can go wrong?**

1. Overfixation (↑ tissue autofluorescence)



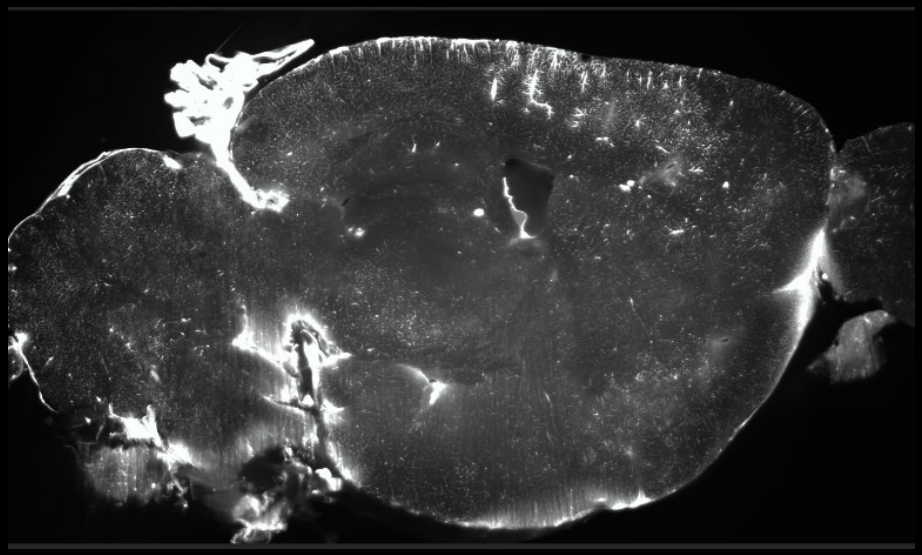


Day « 0 »: Sample preparation

Perfusion (PBS + 4% PFA, or 4%PFA alone)
Postfixation (PFA 4% RT)
Washes in PBS - 3 washes x 15min (aprox.) in PBS - RT

***What can go wrong?**

2. Partial perfusion (specially problematic if using Mouse 1Abs:
↑ cross-reactivity in between blood IgGs and anti-Mouse 2ºAbs)



Day « 0 »: Sample preparation

Perfusion (PBS + 4% PFA, or 4%PFA alone)
Postfixation (PFA 4% RT)
Washes in PBS - 3 washes x 15min (aprox.) in PBS - RT

***What can go wrong?**

3. Fungi contamination, if get this, do not continue with the protocol

IMPORTANT:
All aqueous buffers of the protocol need to be supplemented with 0,01% NaN_3!

Day 1: MeOH dehydration

MeOH 20% ≥ 1 hour
MeOH 40% ≥ 1 hour
MeOH 60% ≥ 1 hour
MeOH 80% ≥ 1,5 hours
MeOH 100% ≥ 1 hour
MeOH 100% ≥ 1,5 hours
MeOH 33%/DCM 66% over night RT

***Ex. for adult whole-brain, to be adapted depending on the sample size**

*What can go wrong?

1. Partial dehydration due to short steps of dehydration:

   High autofluorescence in the center of the brain
   Anisotropic shrinkage (short steps of dehydration)

Day 1: MeOH dehydration

MeOH 20% ≥ 1 hour
MeOH 40% ≥ 1 hour
MeOH 60% ≥ 1 hour
MeOH 80% ≥ 1,5 hours
MeOH 100% ≥ 1 hour
MeOH 100% ≥ 1,5 hours
MeOH 33%/DCM 66% over night RT

***Ex. for adult whole-brain, to be adapted depending on the sample size**

*What can go wrong?

2. Brain can blow up! Skip initial MeOH dehydration in brains <P7)

Day 2: MeOH rehydration and permeabilization

MeOH 100% x 2 long washes along the day
MeOH 80%/H_2O_2 20% (5 to 6% final concentration) over night 4ºC

***What can go wrong?**

1. Nothing!!

Tubes could eventually open due to O2 pressure: ensure they are correctly closed.

Day 3: MeOH washes and bleaching

MeOH 60% ≥ 1,5 hours
MeOH 40% ≥ 1 hour
MeOH 20% ≥ 1 hour
PBS
PBS-Tx
Permeabilization solution – 37ºC over night

***What can go wrong?**

1. Nothing really

Day 4: Blocking

Blocking solution – 37°C over night

***What can go wrong?**

1. Nothing really

Day 5: 1°Ab incubation

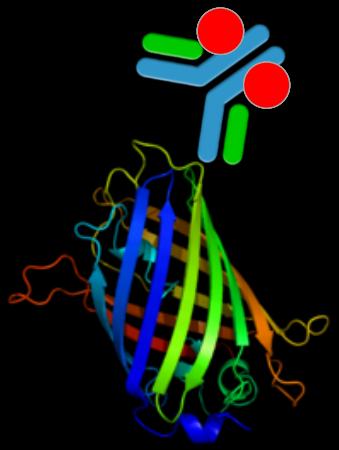
Antibody diluted in blocking solution – variable duration – 37°C

Ex. adult mouse brain 10 days, P3 brain 5 days, 1mm section 2 days, etc.

***What can go wrong?**

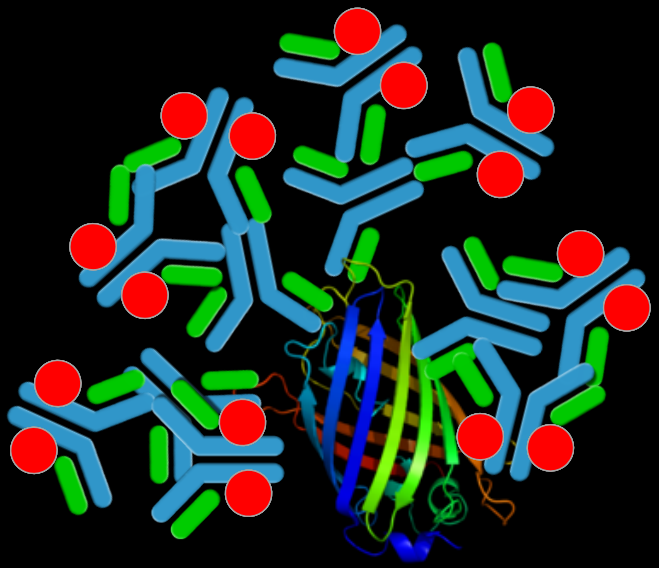
1. Signal amplification should be optimized with the labeling strategy

- Signal amplification

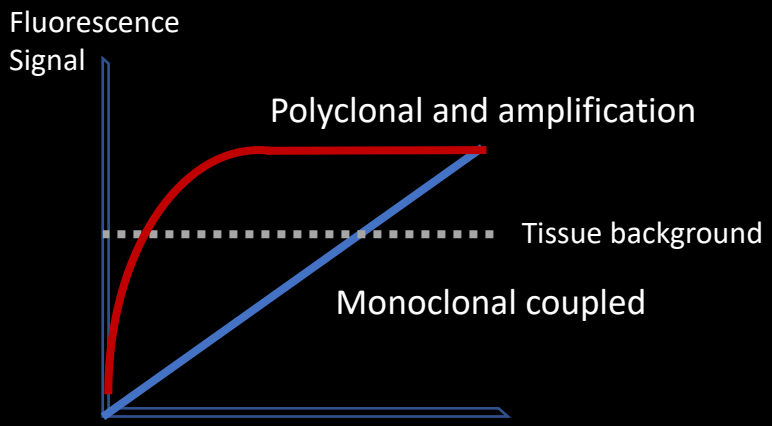


Monoclonal coupled

Monoclonal secondary Ab amplification



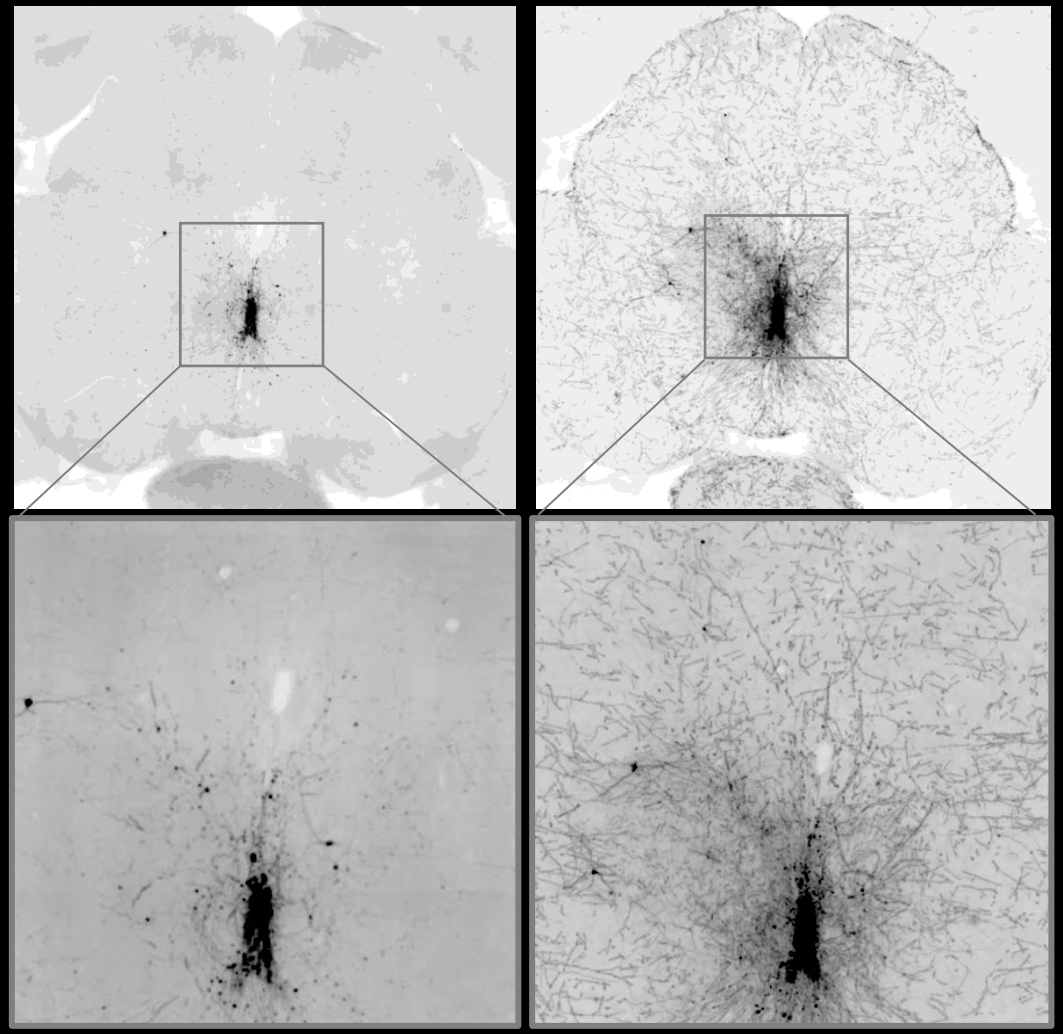
Polyclonal secondary Ab amplification

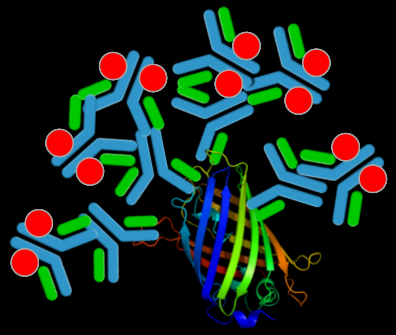


Immunolabeling with **2-stages polyclonals** to amplify **sparse antigens**

GFP endogenous

GFP immuno



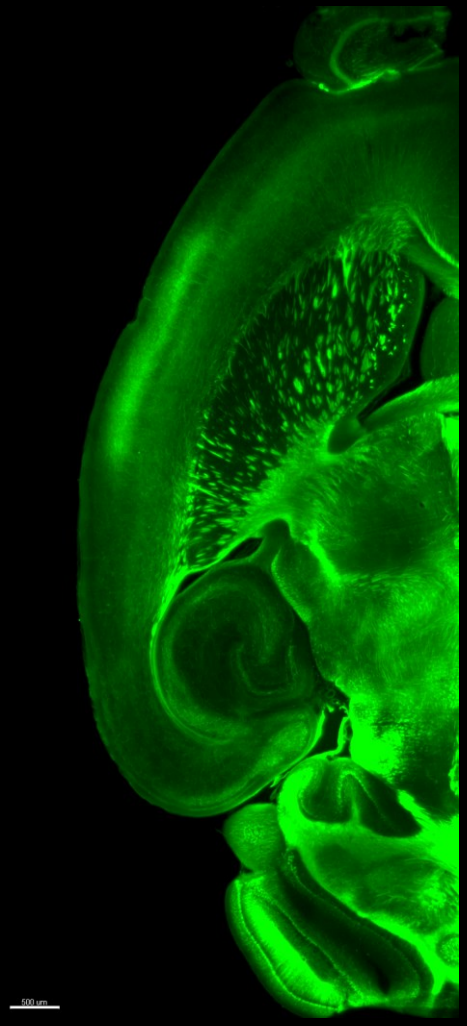


Polyclonal
secondary Ab amplification

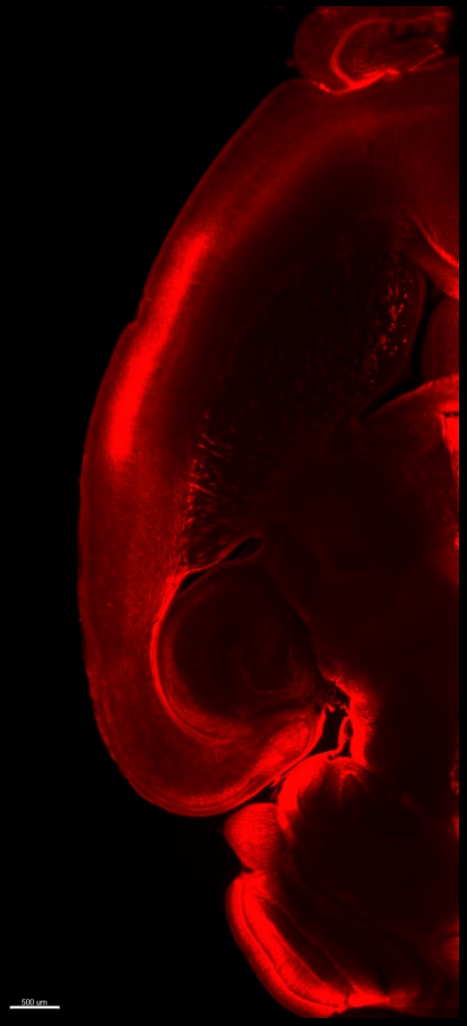
CART-cre :: AAV1-lsl-hSyn-eGFP

Immunolabeling with **conjugated mAb** to amplify **dense antigens**

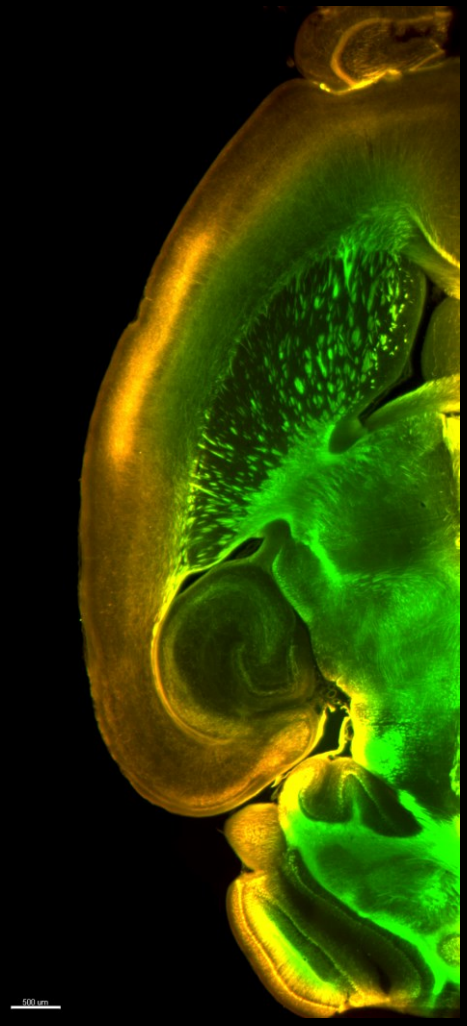
Conjugated primary

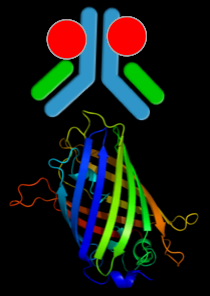


Conjugated secondary



Overlap





Direct immunodetection enables optimal diffusion

Day 5: 1°Ab incubation

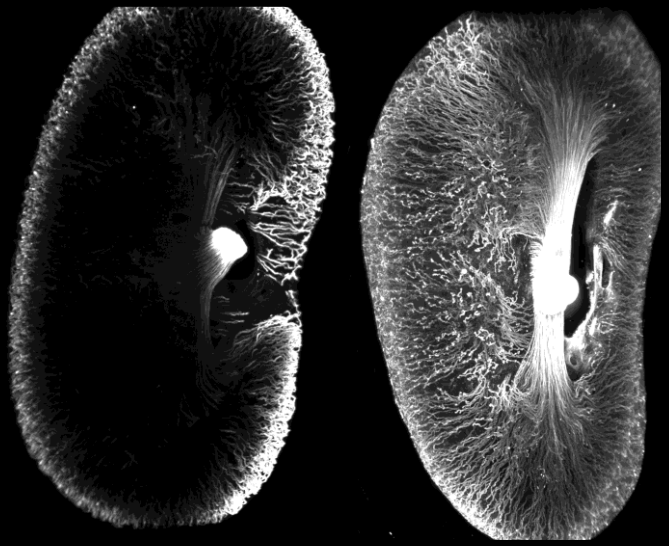
Antibody diluted in blocking solution – variable duration – 37°C

Ex. adult mouse brain 10 days, P3 brain 5 days, 1mm section 2 days, etc.

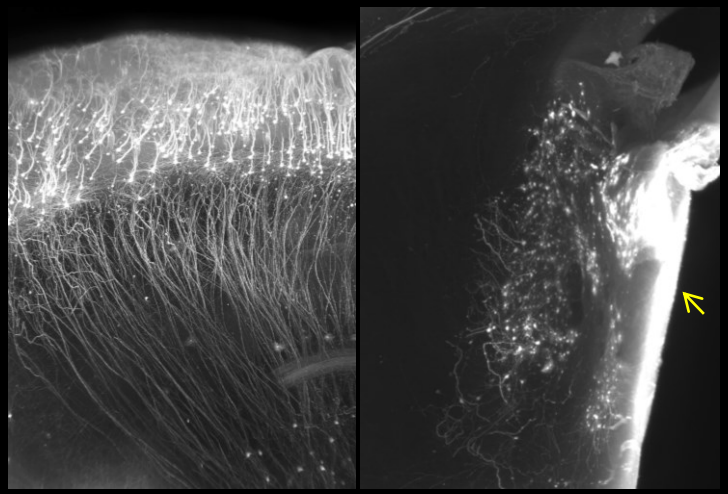
*What can go wrong?

2. Antibody concentration

"antibody depletion when concentration is too low



"ring background' when concentration is too high



[IgG] < [antigen] | Working range | Saturating [IgG]

Day 5: 1°Ab incubation

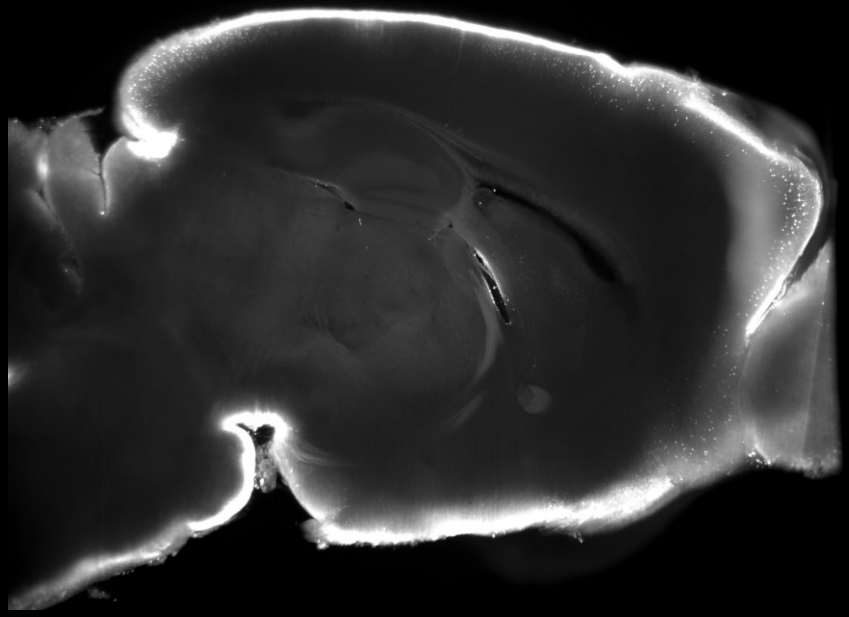
Antibody diluted in blocking solution – variable duration – 37°C

Ex. adult mouse brain 10 days, P3 brain 5 days, 1mm section 2 days, etc.

*What can go wrong?

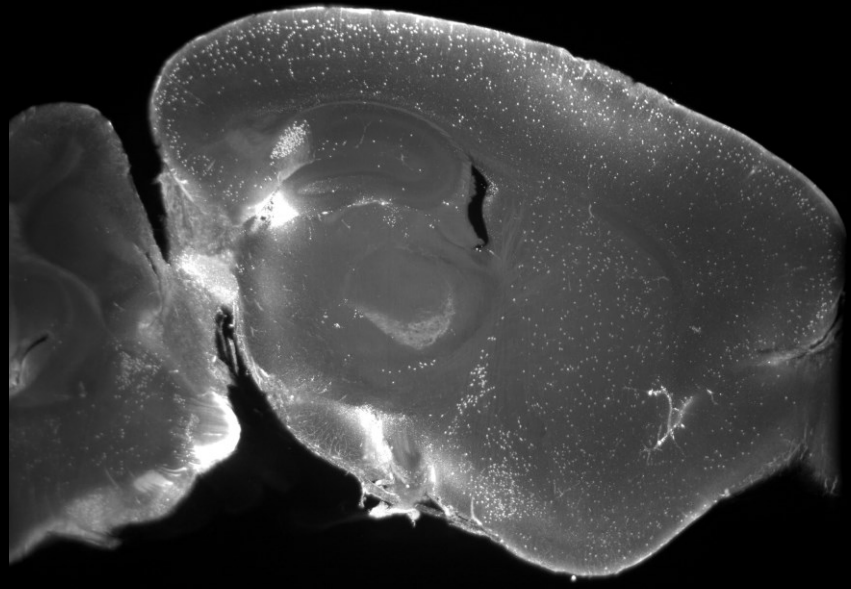
3. Antibody reference

Novus chicken anti-Cherry/tdTomato at 1/500th

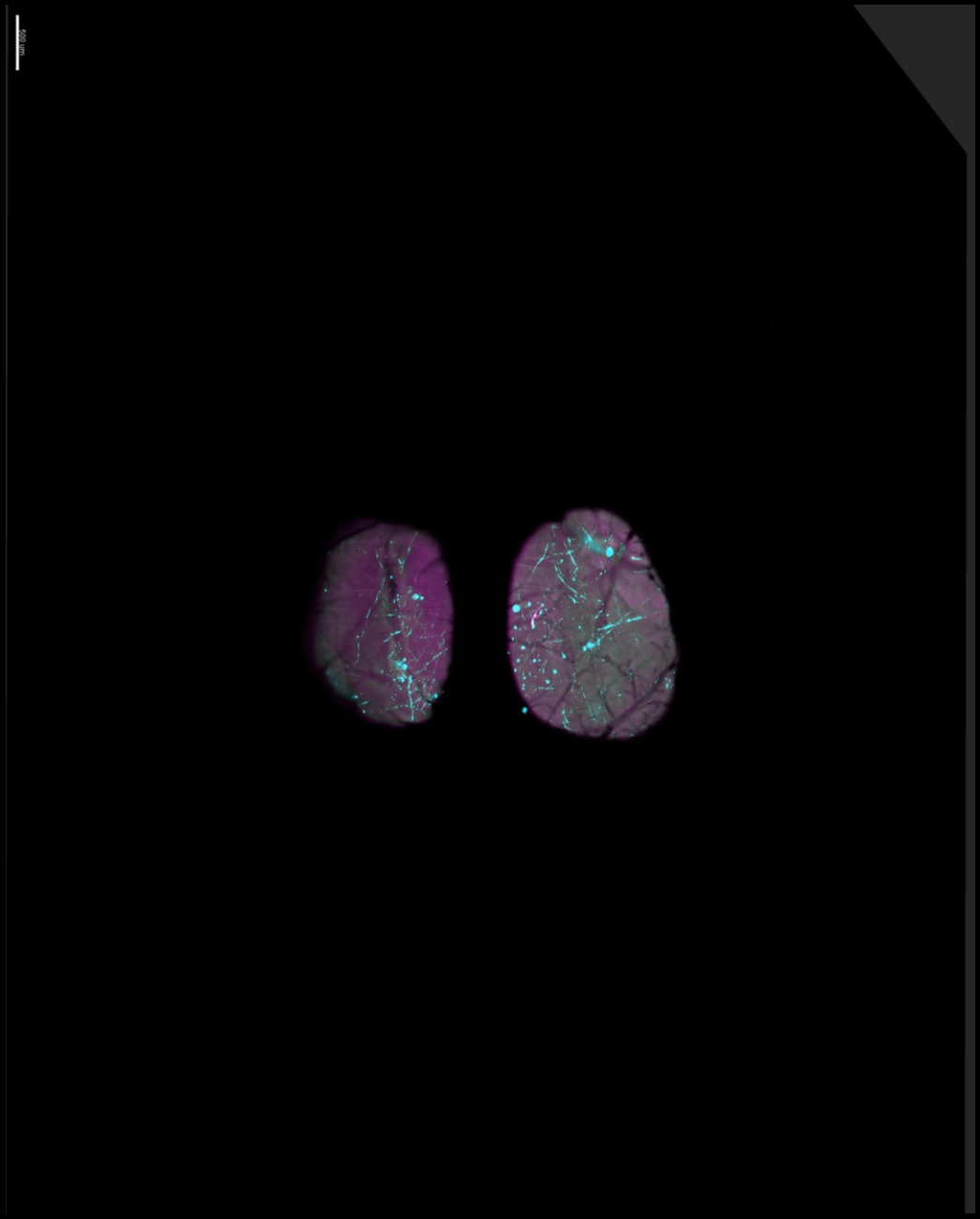


Ring surface background

Rockland rabbit anti-RFP/tdTomato at 1/500th



complete diffusion


cFOS
Autofluorescence

Day 15: washes

Washes in PBS-TwH at RT

***What can go wrong?**

Really nothing

Day 16: 2° Ab incubation (if necessary)

Antibody diluted in blocking solution – variable duration – 37°C

Ex. adult mouse brain 8 days, P3 brain 4 days, 1mm section 1 day, etc.

***What can go wrong?**

Much less critical than 1°Ab, just avoid this step and use 1° conjugated Abs for dense antigens

Day 26: washes

Washes in PBS-TwH at RT

***What can go wrong?**

Really nothing

Day 27: dehydration in MeOH

MeOH 20% ≥ 1 hour
MeOH 40% ≥ 1 hour
MeOH 60% ≥ 1 hour
MeOH 80% ≥ 1,5 hours
MeOH 100% ≥ 1 hour
MeOH 100% overnight – RT

***Ex. for adult whole-brain, to be adapted depending on the sample size**

*What can go wrong?

1. Short incubations will lead to incomplete dehydration and poor transparency

Day 28: RI matching (« clearing »)

MeOH 33%/DCM 66% - 3 hours – RT
DCM 15 to 30min 2x
DBE

***What can go wrong?**

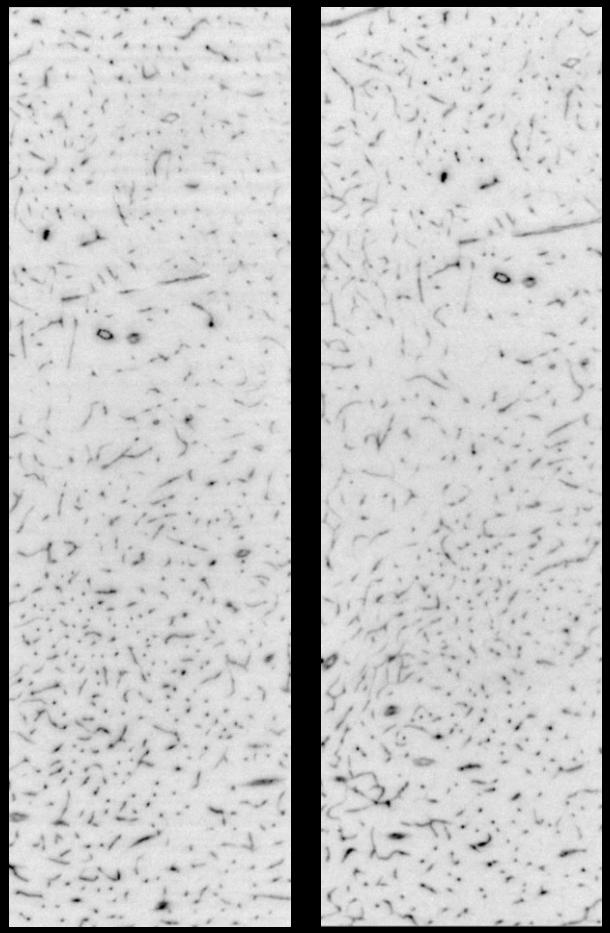
1. If there is any trace of water, the samples will look partially opaque

Day before imaging: RI matching with Eci

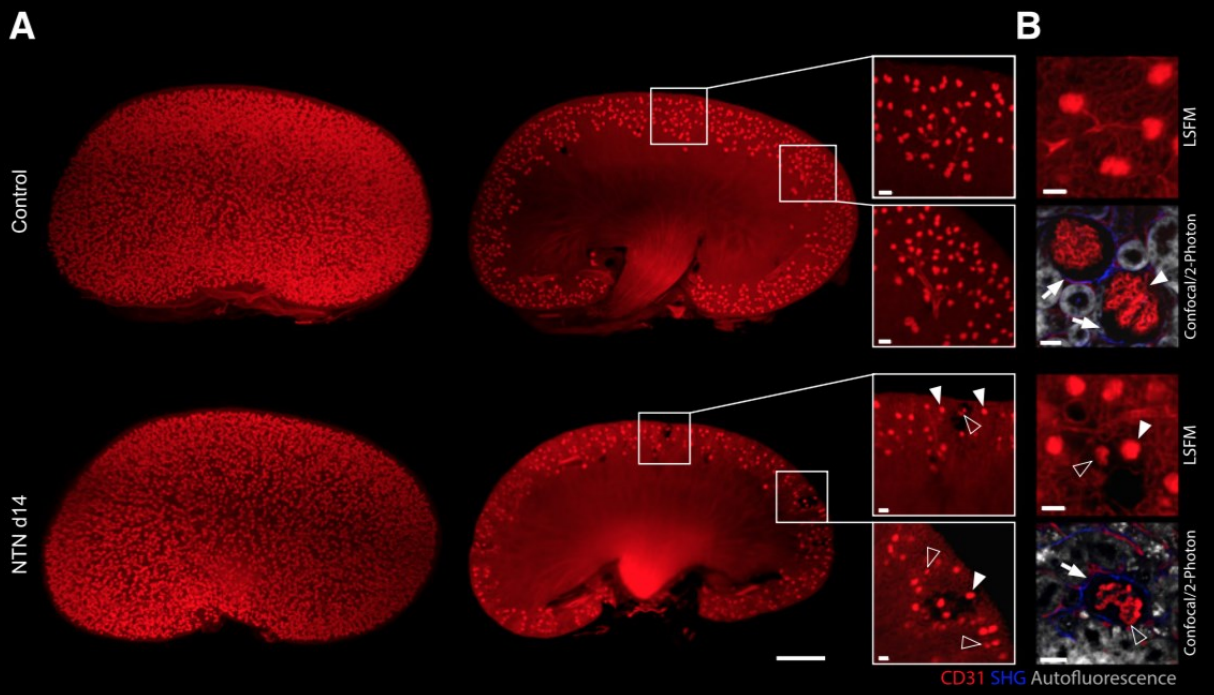
Eci – overnight RT

DBE

Ethyl cinnamate



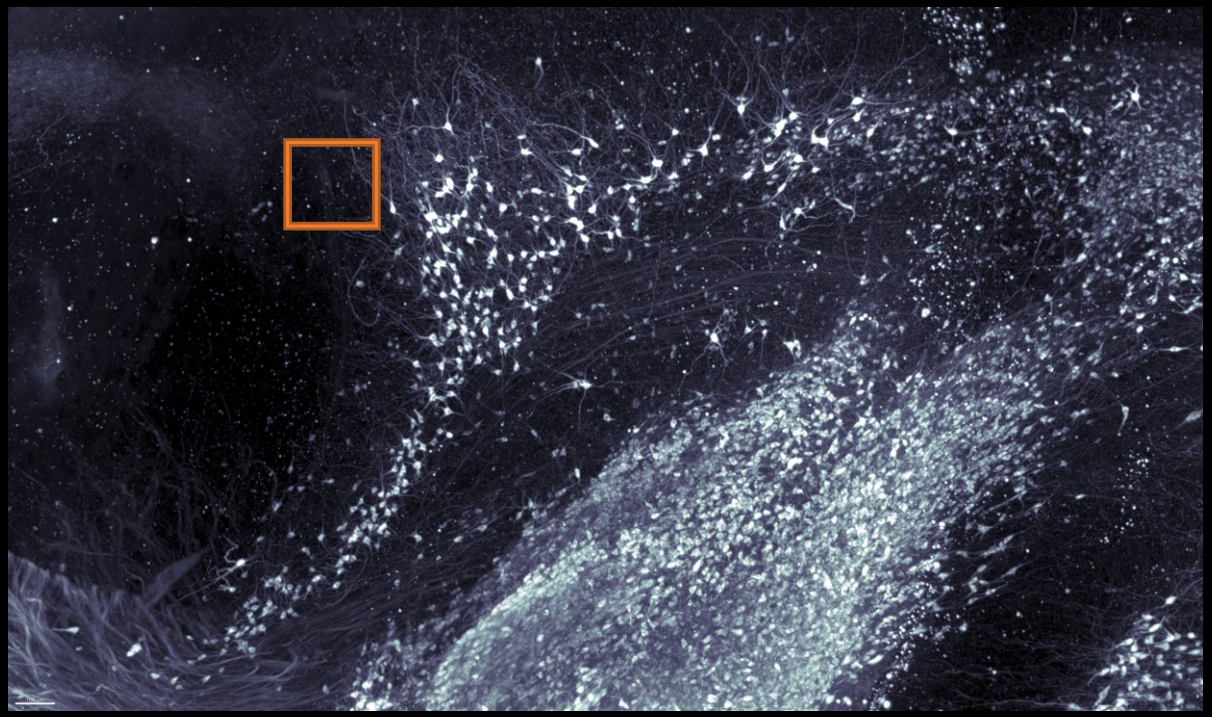
4x lens, 1 laser, NA 0,148

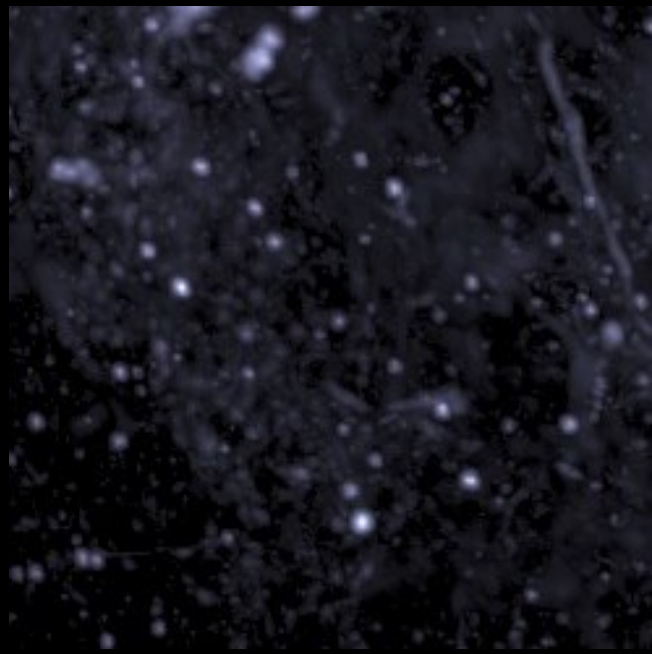


(Klingberg, J Am Soc Nephrol 2017)

Multifactorial problems: Dots artefacts - primary antibody+fixation problems

Adult mouse brain, TH staining, middle optical planes max projection

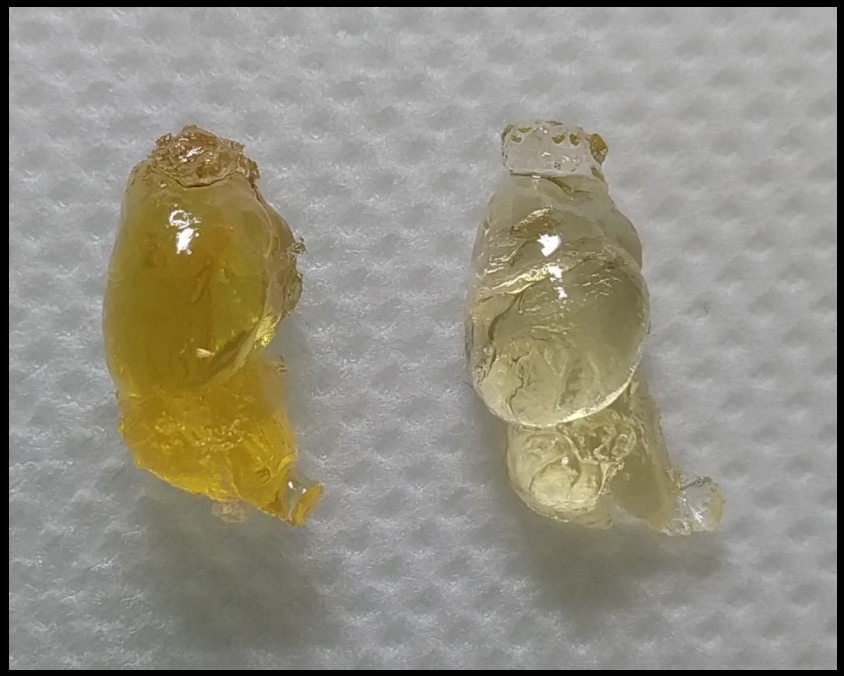




2 factors :

- Keeping the sample longer in the fridge before processing increases the dots
- Dots appear when the antibody is too concentrated compared with the epitopes
- Dots are more visible when the signal is weak (one can use PBS/Methanol)

Multifactorial problems: sample oxidation – long latencies or excess of air

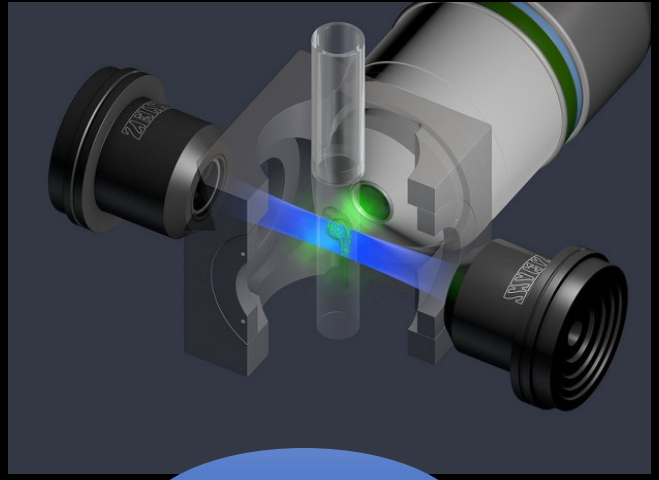


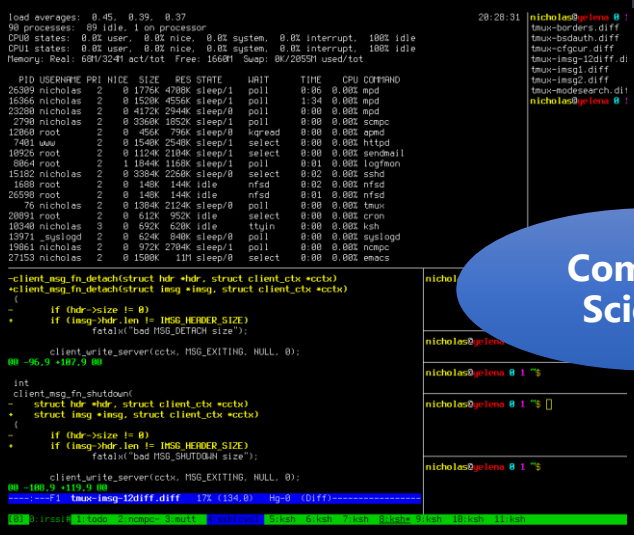
2 factors :

- Keeping the sample longer in the fridge before processing increases oxidation
- Keeping a large bubble of air in the sample's tube throughout the different steps of the protocol

Practical tips

- Supplement all aqueous buffers with 0,01% NaN_3
- Fill all tubes to the top, minimize air in the sample tube
- Find a MeOH-compatible ink labeling system for the samples
- Use a wheel shaker for all steps except bleaching
- Embed fragile samples in a 1% Agarose gel before final dehydration
- Shrink large samples with sparse labeling by doing the final dehydration in PBS/MeOH
- Choose red-shifted fluorophores



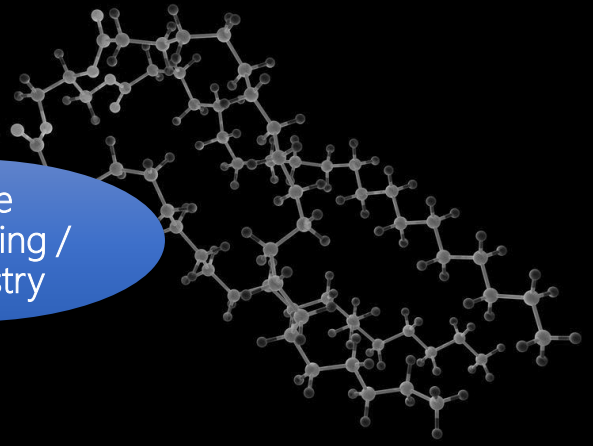


Lightsheet microscopy

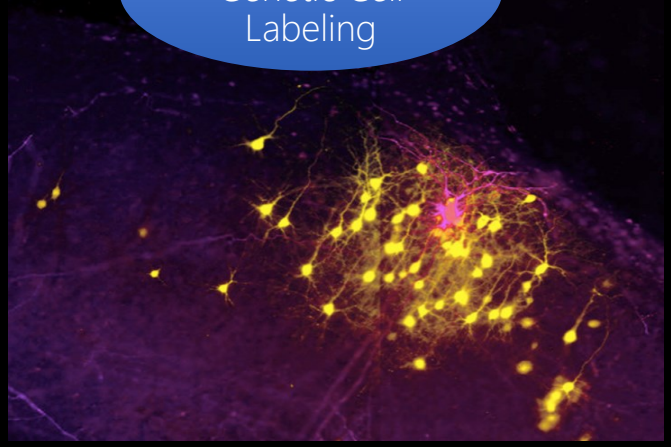
Computer Sciences

Mesoscale imaging

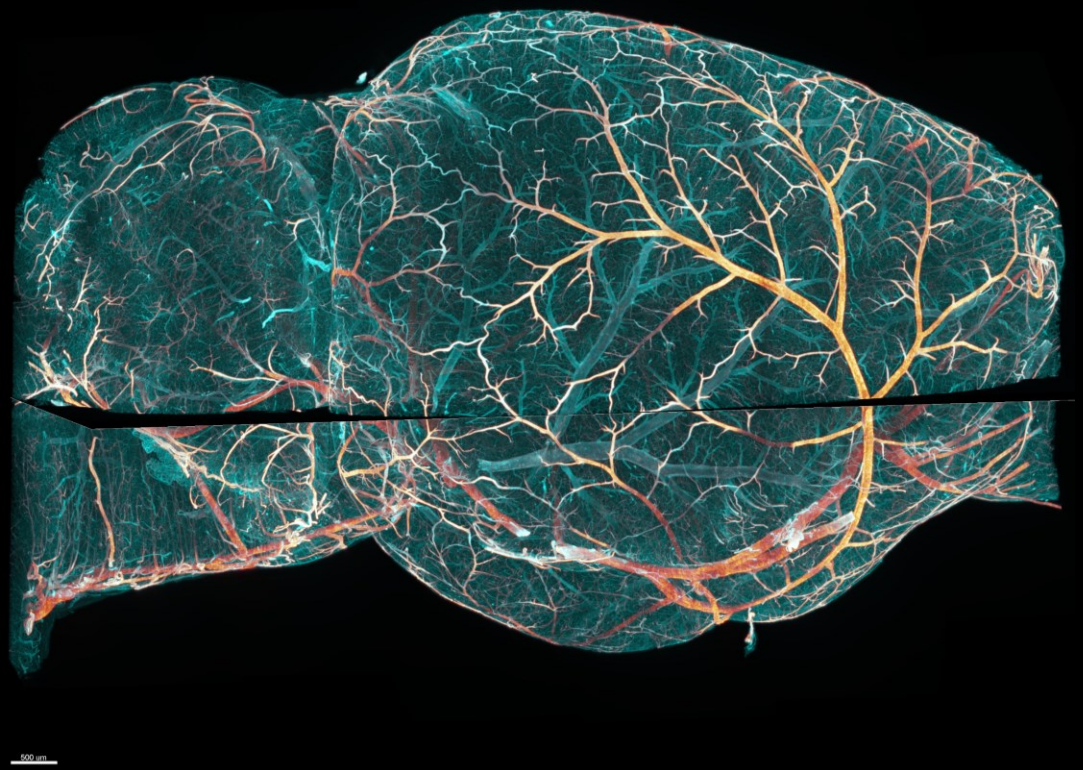
Tissue Engineering / Chemistry

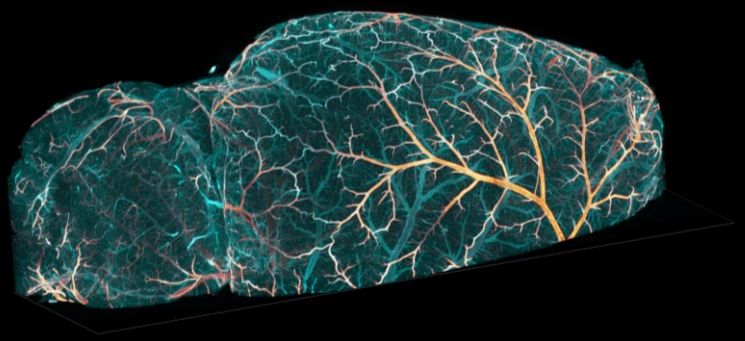


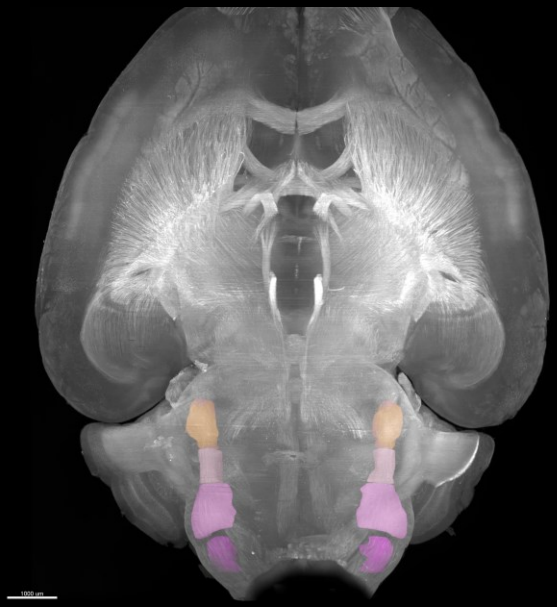
Molecular / Genetic Cell Labeling



Qualitative analysis (visualization)

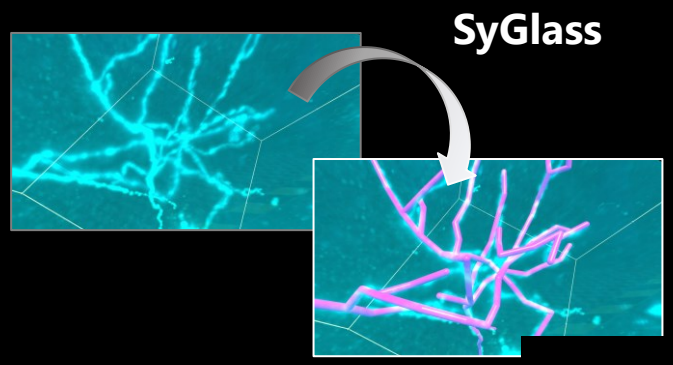


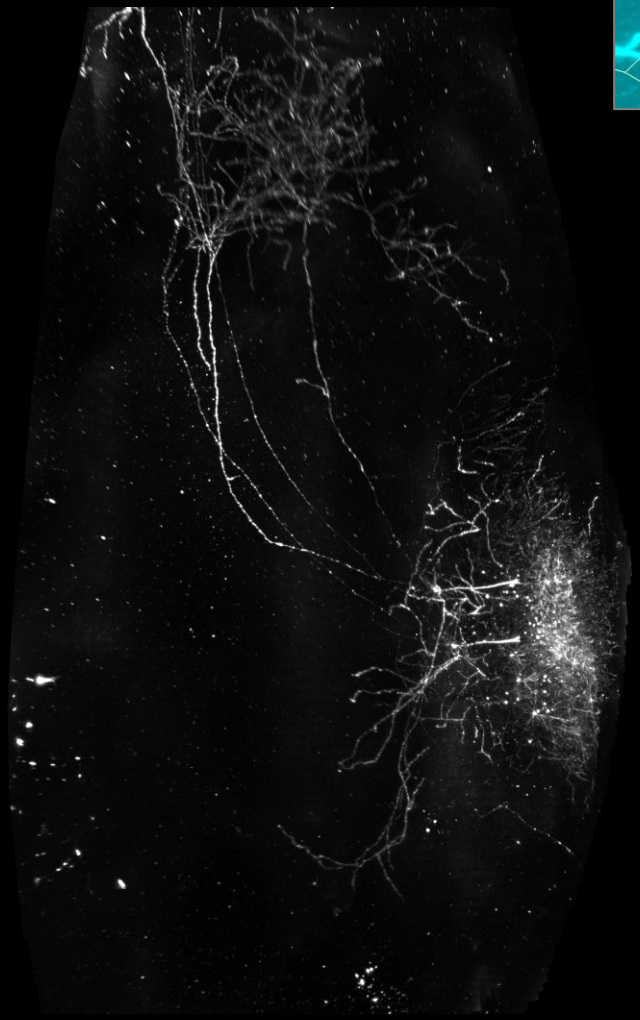


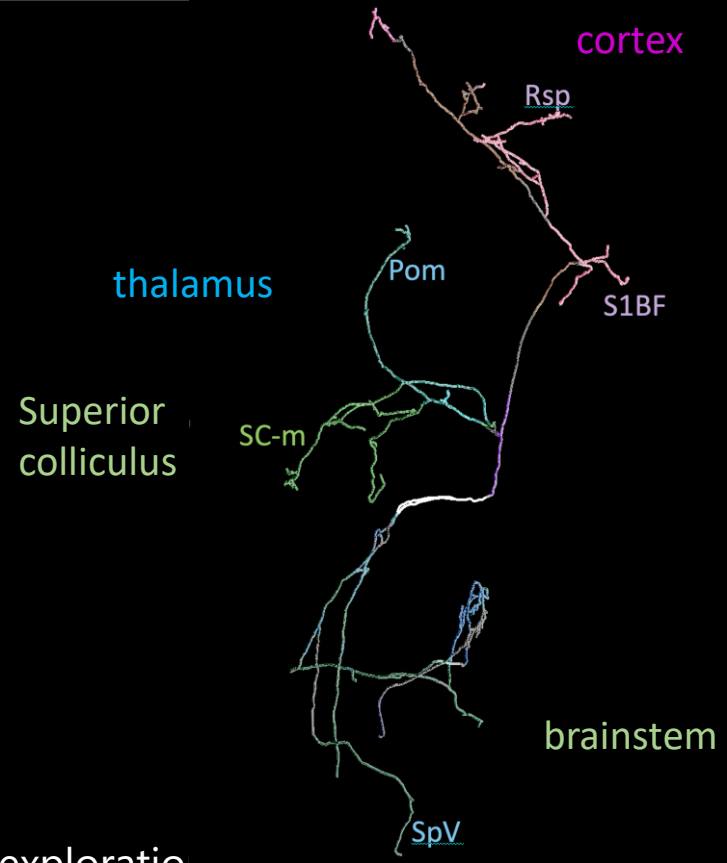


Imaris

Qualitative analysis







VR-assisted 3D image exploration

Quantitative analysis

ClearMap: CellMap, module for cell detection (Renier*, Adams*, Kist*, Wu*, et al. Cell 2016)
TubeMap, module for vasculature segmentation (Kirst*, Skriabine*, Vieites-Prado* et al. Cell 2020)