cytoMASH protocol

Citation for using this protocol:

Hildebrand, S., Schueth, A., Herrler, A., Galuske, R., & Roebroeck, A. (2019). Scalable labeling for cytoarchitectonic characterization of large optically cleared human neocortex samples. Scientific reports, 9(1), 1-10.

Notes:

The protocol described here is the generalized version of the original MASH publication. This protocol was originally carried out in 6 well plates with samples of about 2-3 cm x 2-3 cm in lateral extend. Most often this method is still used today in our group. For this method an incubation volume of 5 ml is used for all steps, except the two bleaching steps and the staining step, which is done with 6 ml. In case the tissue samples are too high to be covered, the volumes must obviously be adjusted accordingly. The rinsing and washing steps in aqua dest are not exactly measured, the wells are simply filled completely over the sink. Originally all dye working solutions were prepared in PBS of respective pH, but since PBS should not be kept at pH 4 we switched to McIlvain buffer (McIlvaine, 1921) for lower pH's. The incubation in the dye solution was originally carried out at 4°C, but since then it was done regularly at RT without noticible difference. Larger pieces can be incubated in the special made clearing jar or a similar container with adjusted volumes. If samples have to be kept at neutral pH, MG and MB have been found to work most reliable. For AO and NR always check if staining at pH 7.4 lead to the desired staining pattern.

Materials:

Chemicals

- Acridine orange (AO)
- Chloroform
- Dichloromethane (DCM)
- Ethyl cinnamate **or** 2,2'-thiodiethanol and *trans*-cinnamaldehyde
- Hydrogen peroxide (H_2O_2)
- McIlvain buffer
- Methanol (MeOH)
- Methylene blue (MB)
- Methyl green/Ethyl green (MG)
- Neutral red (NR)
- Phosphate buffered saline (PBS)
- Potassium disulfite (also pyrosulfite or metabisulfite)
- Triton X-100

Preparations of stock solutions

- Methyl green (MG) stock solution
  - Dissolve 4 g MG in aqua dest (takes a long time to dissolve; stirr over weekend and keep covered as well as protected from light)
  - Extract impurities with chloroform in a separation funnel by mixing MG solution 1:1 with chloroform and discarding the lower (chloroform) phase until no violet color appears anymore in that phase
  - According to Prieto et al. (2014) resulting stock solution has concentration of about 2%; keep at 4°C and protected from light!

- Other MASH-dyes

- Either dissolve 0.1-1 g in 100 ml aqua dest or PBS pH 7.4 (methylene blue; MB) or in McIlvain buffer pH 4 (acridine orange; AO, neutral red; NR) by stirring over weekend covered and protected from light
- Store at 4°C protected from light
- Dilute 1:100 (with 0.1% stock) or 1:1000 (with 1% stock) for working solution

Or create ready-to-use solution

- Dissolve 10 mg in either PBS pH 7.4 or McIlvain pH 4 for MB depending on desired pH of staining or 10 mg in McIlvain pH 4 for AO and NR
- Add buffer to 1l
- Store at RT when used up quickly, else store at 4°C

The latter method is used more recently, because stock solutions precipitated a lot with 1% stock solutions used in the beginning and final concentration was therefore not reliable. It also proved more convenient. If stock solution is made, I recommend the 0.1% stock as dyes dissolve completely.

- 0,2% PBST
  - 10 ml of 10x PBS
  - 2 ml Triton X-100
  - Add 800 ml aqua dest
  - Check pH and adjust to 7.4 if necessary
  - Add to 1l with aqua dest

- 50% potassium disulfite (also pyrosulfite or metabisulfite) solution
  - 500 g potassium disulfite
  - Add 1 l with aqua dest
  - Dissolve under stirring and heating (over time a lot will precipitate because the solution is oversaturated)

- TDE/CA-RIMS
  - Mix 62% 2,2'-thiodiethanol (TDE) and 38% *trans*-cinnamaldehyde (CA) to required volume

If TDE/CA is used as RIMS, prepare enough solution to incubate samples (twice) and image them in same batch as differences through measuring errors between batches might lead to „Schlieren"/streaks between the two batches which might lead to refraction and artefacts in the images. Make sure solution is well mixed. Ideally measure RI with a refractometer and adjust to 1.56.

Protocol:

Day 1: Dehydration and 1st bleaching

- Place samples in 6 well plates (or larger clearing jar depending on size)
- Dehydration for 1h each in 20, 40, 60, 80, 100% methanol (MeOH) at RT
- Incubation for 1h in 100% MeOH at 4°C
- Bleaching overnight at 4°C in 5% H_2O_2 in MeOH (from 30% H_2O_2 stock; 1 part H_2O_2 : 5 parts MeOH)

Day 2: Rehydration, 2nd bleaching and staining

- Rehydrate samples for 1h each in 80, 60, 40, 20% MeOH

- 2x 1h permeabilization in 0,2% Triton x-100 in phosphate buffer saline (PBST) with pH 7.4
- 1h bleaching in 50% potassium disulfite ($K_2S_2O_5$) in water (needs to be warmed under stirring and filtered right before use; solution can be re-used multiple times, put back in bottle, don't throw away! If no precipitate is forming anymore in the stock and the colour becomes more faded, prepare new solution.)
- Rinse well plates 5x and wash 1h with aqua dest
- Stain for 5 day (for 5 mm thick samples; 1 day/1 mm of tissue thickness) in MASH-dye solution of choice: For MG dilute stock 1:5000 to 1:10000 (determine best concentration empirically!) in PBS pH 7.4; For AO, MB, and NR either dilute stock in respective buffer or use ready-to-use working solution

Day 5: Flip samples and check staining (is there still visible colour in the solution; are the samples visibly stained)

Day 7: Dehydration and delipidation

- Wash 2x 1h in same buffer used in MASH-dye solution
- Dehydrate 1h each in 20, 40, 60, 80, 2x 100% MeOH

Important: All steps from here onwards need to be performed in 50 ml tubes made of polypropylene or polyethylene, because the 6 well plates will be dissolved by DCM! If other container is used, check first if material is DCM resistant!

- Delipidate overnight in 66% dichloromethane (DCM)/33% MeOH (Note: very thick and lipid-rich samples may have to be delipidated for longer. We have had good clearing results in human spinal cord with 48h delipidation and one change of the 66% DCM/33% MeOH solution after 24h)

Day 8: Refractive index matching

- Wash 2x 1h in 100% DCM
- Incubate in ethyl cinnamate (ECi) **or** TDE/CA-RIMS

Day 9: Refractive index matching

- Depending on transparency, change RIMS once and let incubate for 1 more day
- Once samples are fully transparent, they can be imaged.

References:

McIlvaine, T. C. (1921). A buffer solution for colorimetric comparison. *Journal of Biological Chemistry*, 49(1), 183–186.