Perfusion and Sample preparation

- anesthetize the animals with a combination of midazolam, medetomidine and fentanyl (MMF) (1ml/100g of body mass for mice; i.p.)

- intracardially perfuse the animals with heparinized 0.1 M PBS (10 U/ml of Heparin, Ratiopharm; ~110 mmHg pressure using a Leica Perfusion One system) for 5-10 minutes at room temperature and with 4% paraformaldehyde (PFA) in 0.1 M PBS (pH 7.4) (Morphisto, 11762.01000) for 10-20 minutes

    - **For the collection of dissected organs** (e.g. brains)**:**
        - dissect the organs and post-fix in 4% PFA overnight at 4°C
        - wash with 0.1 M PBS for 10 minutes 3 times at room temperature
        - store in PBS with 0.05% sodium azide (Sigma, 71290) up to 3 weeks

    - **For the collection of whole bodies:**
        - remove eyes (optional), premaxilla and maxilla bones and optionally the skin, and open the palate of the animal (without damaging the tissue beneath)
        - in animals with intact skin, shave the fur off using a razor blade (Personna, 604305-001001)
        - gently wash the feces out from the intestine with 0.1 M PBS through small cuts using a syringe
        - post-fix in 4% PFA for 1 day at 4°C
        - wash with 0.1 M PBS for 10 minutes 3 times at room temperature
        - store in PBS with 0.05% sodium azide up to 6 months

Nanobooster validation

- cut post-fixed brains into 400µm slices using a vibratome

- image the slices pre-IHC with a fluorescence microscope (e.g confocal or AxioZoom) to make sure the samples are from animals which are fluorescent protein positive

IHC:

- all the steps are done with gentle shaking on a rocker or a shaker

- incubate the slices for 3 hours at 37°C with permeabilization solution containing 1.5% goat serum (Gibco, 16210072), 0.5% Triton X-100 (AppliChem, A4975,1000), 0.5 mM of Methyl-beta-cyclodextrin (Sigma, 332615), 0.2% trans-1-Acetyl-4-hydroxy-L-proline (Sigma, 441562) and 0.05% Sodium Azide (Sigma, 71290) in 0.1 M PBS

- incubate overnight at 37 °C with the same permeabilization solution adding the nanobooster of interest with dilution 1:500. Final volume=500µL . Cover with aluminum foil to keep them in dark and seal the containers well in order to prevent the slices to dry out

- note: Remember negative CTRL too: sample without adding the nanobooster!

- wash with washing solution (1.5% goat serum, 0.5% Triton X-100, 0.05% of sodium azide in 0.1 M PBS) at room temperature for 15 minutes, 4 times

- wash with PBS at room temperature for 10 minutes, 3 times

- image the slices post-IHC with a fluorescence microscope (e.g confocal or AxioZoom) to make sure the staining worked

Clearing:

- clear the slice with 3DISCO: incubate at room temperature in the following gradient of tetrahydrofuran (THF) (Sigma, 186562) in distilled water (45minutes for each step): 50 Vol% THF, 70 Vol% THF, 80 Vol% THF, 100 Vol% THF and 1hour 100 Vol% THF; after dehydration, 15 minutes in dichloromethane DCM (Sigma, 270997), and finally in BABB (benzyl alcohol + benzyl benzoate 1:2, Sigma, 24122 and W213802) until transparency. Be sure to perform the clearing in Eppendorf 5mL tubes which are resistant to clearing solutions, inside a fume hood and keeping the samples in dark

- image the slices post-IHC and clearing with a fluorescence microscope (e.g confocal or AxioZoom) to make sure the staining worked even after clearing.

- the signal over background is good, it is possible to use this batch of nanobooster for vDISCO whole organ staining or vDISCO whole body staining

vDISCO whole-mount immunolabeling of individual organs

IHC:

- all the steps are done with gentle shaking on a rocker or a shaker

- incubate the post-fixed brains or organs for 2 days at 37°C in 4.5 ml of permeabilization solution containing 1.5% goat serum , 0.5% Triton X-100, 0.5 mM of Methyl-beta-cyclodextrin , 0.2% trans-1-Acetyl-4-hydroxy-L-proline and 0.05% Sodium azide in 0.1 M PBS

- incubate the brains or organs for 12-14 days at 37°C in 4.5 ml of permeabilization solution with the nanobooster of interest with the concentration adjusted to expression of the target (for example Atto647N conjugated anti-GFP nanobooster dilution 1:600, which is ~5-8µg of nanobooster in 4.5 ml (1.1-1.8 µg/ml) (stock concentration = 0.5 – 1 mg/ml) for Thy1-GFPM brains)

- wash with washing solution (1.5% goat serum, 0.5% Triton X-100, 0.05% of sodium azide in 0.1 M PBS) at room temperature for 2 hours 3 times and once overnight

- wash with PBS at room temperature for 2 hours 4 times

Clearing:

- clear the brains with 3DISCO: incubate at room temperature in the following gradient of THF in distilled water (2 hour for each step) in final volume of 4.5ml: 50 Vol% THF, 70 Vol% THF, 80 Vol% THF, 100 Vol% THF and overnight 100 Vol% THF; after dehydration, 1hour in DCM, and finally in BABB until transparency. Be sure to perform the clearing in Eppendorf 5mL tubes which are resistant to clearing solutions, inside a fume hood and keeping the samples in dark

- During all the clearing steps, the tubes were wrapped with aluminum foil to keep them in dark

vDISCO whole-body immunostaining, PI labeling and clearing

IHC:

- The solutions for the immunolabeling pipeline were pumped inside the body of the animal by transcardial-circulatory perfusion exploiting the same entry point hole into the heart created during the PBS and PFA perfusion step (see above, perfusion and tissue preparation paragraph) and following the procedure already described in Pan et al., 2016

- place the post-fixed mouse body in a 300 ml glass chamber (Omnilab, 5163279) which will be filled with 250-300 ml of appropriate solution, which covered the body completely

- establish the transcardial-circulatory system involving a peristaltic pump (ISMATEC, REGLO Digital MS-4/8 ISM 834; reference tubing, SC0266) keeping the pressure at 160-230 mmHg (45-60 rpm)

- Set one channel from the pump, made by a single reference tube, for circulation of the solution through the heart into the vasculature: connect one ending of the tube to the tip of a syringe (cut from a 1 ml syringe-Braun, 9166017V) which holds the perfusion needle (Leica, 39471024) and immerse the other ending in the solution chamber where the animal is placed. In this way, the perfusion needle pumps the appropriate solution into the mouse body, and the other ending collects the solution exiting from the mouse body in order to recirculate the solution, pumping it back into the animal

- fix the needle tip in place to ensure extensive perfusion, putting a drop of super glue (Pattex, PSK1C) at the level of the hole where the needle is inserted inside the heart

- now start perfusing the animal: perfuse with 0.1 M PBS overnight at room temperature

- perfuse with 250 ml of decolorization solution: 25-30 Vol% dilution of CUBIC reagent 1 in 0.1 M PBS. CUBIC reagent 1 is prepared with 25 wt% urea (Carl Roth, 3941.3), 25 wt% N,N,N´,N´-tetrakis (2-hydroxypropyl)ethylenediamine (Sigma, 122262) and 15 wt% Triton X-100 (AppliChem, A4975,1000) in 0.1 M PBS. Perfuse for 2 days at room temperature,

exchanging with fresh decolorization solution every 6-12 hours until the solution turns from yellowish to clear and the spleen becomes lighter colour (indicating that the blood heme is extracted)

- perfuse with 0.1 M PBS at room temperature, washing for 3 hours 3 times
- perfuse with 250 ml of decalcification solution (10 wt/Vol% EDTA (Carl Roth, 1702922685) in 0.1 M PBS adjusting the pH to 8-9 with sodium hydroxide NaOH (Sigma, 71687)) for 2 days at room temperature
- perfuse with 0.1 M PBS at room temperature, washing for 3 hours 3 times
- perfuse with 250 ml of permeabilization solution containing 1.5% goat serum , 0.5% Triton X-100, 0.5 mM of Methyl-beta-cyclodextrin , 0.2% trans-1-Acetyl-4-hydroxy-L-proline and 0.05% Sodium azide in 0.1 M PBS for half a day at room temperature
- connect a 0.20 µm syringe filter (Sartorius, 16532) to the ending of the tube not holding the needle, in order to efficiently prevent accumulation of dye aggregates into the sample
- perfuse for 6days with 250 ml of permeabilization solution containing 35 µl of nanobooster (20-35 µg in 250 ml (0.08- 0.14 µg/ml), 1:7000 in dilution, (stock concentration 0.5 – 1 mg/ml) (the amount of nanobody was adjusted depending on the expected presence of fluorescent protein in the mouse body) and 290 µl of propidium iodide (stock concentration 1 mg/ml). At the same time, from this step use an infrared lamp (Beuer, IL21) directed to the chamber to heat up the solution to 26-28°C
- remove the animal from the chamber and with fine scissors we removed a tiny piece of skull from the back of the skull (above the cerebellum) at the level of the occipital bone
- place the body of the animal in a 50 ml tube (Falcon, 352070), filled with the same permeabilization solution, containing an extra 5 µl of nanobooster
- incubate the tubes at 37°C with gentle shaking for an additional 2-3 days of labeling
- place the animal back in the perfusion system
- perfuse with washing solution (1.5% goat serum, 0.5% Triton X-100, 0.05% of sodium azide in 0.1 M PBS) for 3 hours, 3 times at room temperature, to remove the excess of dye
- perfuse with 0.1 M PBS for 3 hours, 3 times at room temperature

Clearing:

- all the steps are done with gentle shaking on a rocker or a shaker at room temperature inside a fume hood and in dark (for example covering with aluminum foil)
- clear the animal in a glass chamber with 3DISCO protocol: incubate in the following gradient of THF in distilled water (12 hours for each step, final volume=200 ml): 50 Vol% THF, 70 Vol% THF, 80 Vol% THF, 100 Vol% THF twice; after dehydration, 3 hours in DCM and finally in BABB until transparency