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Clear+ Passive Protocol Rat Hemisphere V.1

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Protocol status: In development
We are still developing and optimizing this protocol

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Abstract

This protocol describes the implementation of the Clear+ Passive brain-clearing method for the George Lab, with additional notes on reagent sources and minor workflow adaptations. The protocol steps are directly transcribed from the LifeCanvas Technologies documentation (***LifeCanvas Wiki***). Original authorship and credit belong entirely to LifeCanvas Technologies.

This protocol outlines the Clear+ Passive procedure for clearing whole rat brain hemispheres to achieve optical transparency compatible with light-sheet imaging and immunolabeling. Following SHIELD stabilization, delipidation is performed, followed by immunolabeling and final refractive index matching in EasyIndex (RI ≈ 1.52). The Clear+ Passive method provides a gentle, detergent-based approach designed to preserve protein integrity and tissue morphology in large samples. This implementation remains a work in progress and has not yet been imaged to verify clearing and labeling outcomes in rat brain hemispheres.

Materials

Locations in the George wetlab listed below each material:

BioRocker
- Located in fume hood 008921 and the bench across it (row X).

Incubator:
- Located directly to the right of fume hood 008921.

Fridge:
- Located on the left wall of the wetlab entrance.

50 mL Conical Tubes
- located under bench in row W.

Scooper/spatula spoon (optional, can also scoop brains gently with hands)
- Located in fume hood 008921

Reagents:

Milli-Q H20
- Located on the left wall of the wetlab entrance, close to the boundary of the adjacent lab.

PBS 20x
- Located in row W on the highest shelf.

NaN3
- Located in row W and top shelf labeled "chemicals".

4% PFA
- Located in freezer

Clear+ Passive kit (includes everything except blocking & immunolabeling solutions)
- SHIELD Epoxy located in freezer, uper right wall of freezer
- SHIELD Buffer located in row X in "whole brain clearing shelf"
- SHIELD ON located in fridge, upper right wall of fridge
- Delipidation Buffer located in row X in "whole brain clearing shelf"
- EasyIndex located in row X in "whole brain clearing shelf"

Antibody Blocking Solution
- located in fridge, upper right wall of fridge

Primary: **Anti-c-Fos antibody [EPR20769]**
- located in the freezer. Box titled "Brain Clearing Antibodies"

Secondary: **SeTau-647**
- Located in fridge. Box titled "Brain Clearing Secondary Antibodies"

Before start

This protocol is the Clear+ Passive Tissue Clearing method provided by LifeCanvas Technologies (2022, v4.07; https://sites.google.com/lifecanvastech.com/protocol/outline)

Buffers:

1 PBS 1x + NaN3 (0.02%) (1 L)
PBS 20× 50 mL
Milli-Q H2O 950mL
0.2g NaN3

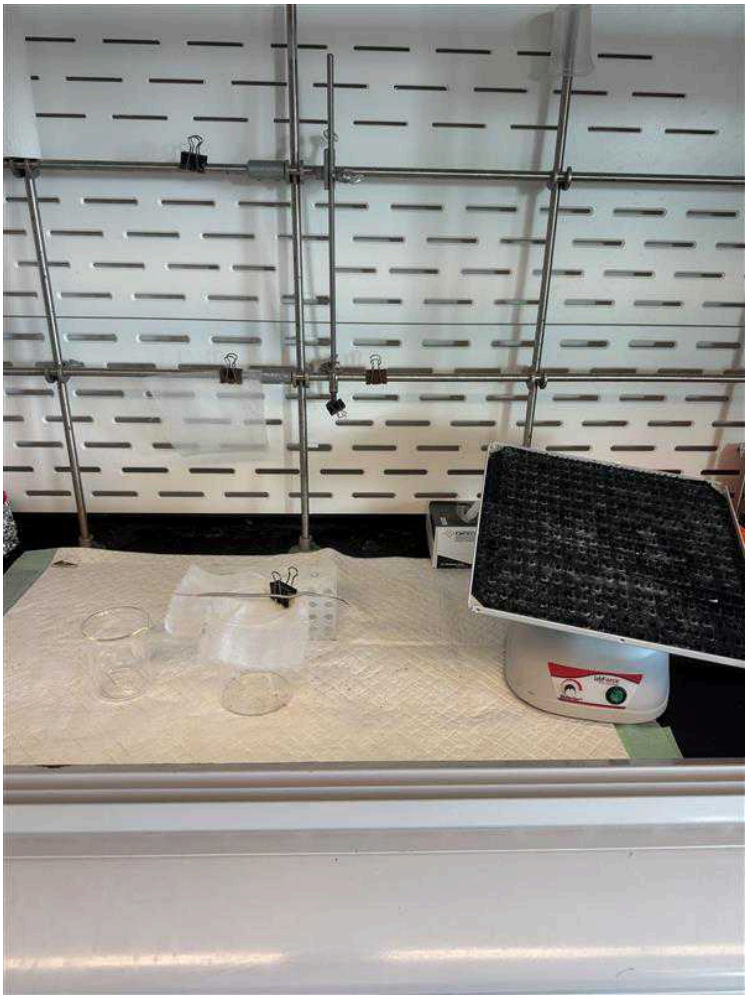
Mix solution at room temperature (RT) until fully dissolved. Store at room temperature. Adding NaN_3 0.02% to buffer stock solutions is highly encouraged to prevent microbial growth.

Sample Preparation

2
1. Anesthetize the rat.
2. Perfuse with ~72 mL saline.
3. Perfuse with ~360 mL 4%PFA/PBS.
4. Dissect the brain.
6. Fix in 1xPBS/4%PFA at 4°c, overnight with shaking.
7. Wash in 1xPBS with shaking: room temperature 30 min x 3 times.
8. Store brain in 1xPBS + NaN3 (0.02%) until ready for clearing

General Tips

3
1. Each step is performed on a rotator from 6-10RPM. Longer incubation times are ok, shorter is not.
2. Brains can be optionally put in PBS + NaN3 (0.02%) after SHIELD OFF/ON step, the delipidation step, and the immunolabeling step for several months without significant loss of signal
3. Brains cannot over delipidate during the delipidation step, but they can under delipidate. There is no need to refresh this solution.
4. Refer to the LifeCanvas website for **tested antibodies**.



Brain clearing Setup

SHIELD OFF/ON

4 Unless otherwise stated, the brain should be fully submerged in solution for every step at all times so ~20-30 mL per sample (in 50 mL tube) is sufficient per wash.

Day 1:
1. SHIELD OFF Solution in fridge for 6 days at 4°C (40 mL per tube)
- 40 mL SHIELD OFF solution: 20 mL SHIELD-EPOXY, 10 mL SHIELD Buffer, 10 mL Milli-Q

Day 7:
1. Give brain ~10 min to adjust to room temperature. Then, place brain with SHIELD ON solution for 24 hrs in incubator at 37°C (40 mL per tube)

Delipidation

5 Day 8:
1. Delipidation buffer: 21+ days passive incubation at 37°C

Immunolabeling

6 Day 37:
1. LifeCanvas Antibody blocking Solution (add 5 mL of 5% Donkey serum per sample): 2 days 37°C

Day 39:
1. Primary antibody (Anti-c-Fos abcam): 28 days at 37°C

Day 57:
1. Take brain out of incubator and give brain ~10min to adjust to room temperature. Then, do 4-6 washes of 1X PBS + NaN3 (0.02%) for 2 hr per wash RT
2. 4% PFA in 1X PBS overnight at RT

Day 58:
1. Place brain in incubator with secondary antibody (Donkey Anti-Rabbit – SeTau-647): 28 days at 37°C

Day 76:
1. Take brain out of incubator and give brain ~10min to adjust to room temperature. Then give 1X PBS + NaN3 (0.02%) 4–6 washes for 2 hr per wash RT
2. 4% PFA in 1X PBS overnight at RT

Clearing

7 Day 77:
1. EasyIndex: 30 mL overnight RT

Day 78:
1. EasyIndex in sealed tube, RT or 4°C

Further Improvements

8
- To minimize damage during skull opening and preserve olfactory bulbs and cortical surfaces, consider decalcifying the head before brain extraction. After transcardial perfusion, remove the head, then remove skin and fur, and immerse the intact skull in 10% (w/v) disodium ethylenediaminetetraacetic acid (EDTA, pH 7.4–7.6) at 4 °C with gentle agitation. Add 0.02% sodium azide to prevent microbial growth and replace the solution every 24–48 hours. Incubate until the skull and sutures are soft and flexible

when gently pinched (typically ~6 days for a mouse head and ≥12 days for a rat hemisphere, depending on specimen size and age). After decalcification, rinse thoroughly in 1× PBS (3 × 30 min) to remove residual, then open the skull and extract the brain. This step reduces mechanical damage and bone debris but adds time to the protocol (1–2 weeks for rat tissue), can slightly soften or swell the brain if prolonged, and requires frequent solution changes. Following extraction, postfix the brain in 4% paraformaldehyde in PBS for 24 hours at 4°C before proceeding with AdipoClear+ labeling and clearing steps.

- A bleaching step of hydrogen peroxide could be beneficial to help with removing pigment and clearing blood residues. Too much time however can affect fluorescent signal, so times would need to be tested and optimized should this be incorporated into the protocol.
- We tested: Primary: **Anti-c-Fos abcam** (1:1000) and Secondary: **Donkey Anti-Rabbit – SeTau-647** (1:1000). Consider testing different concentrations (250, 500, 1,250, etc.) if current signaling is not good.

Protocol references

LifeCanvas Technologies. (2022). *Clear+ Passive Tissue Clearing Protocol (v4.07)*. Retrieved from https://sites.google.com/lifecanvastech.com/protocol/outline