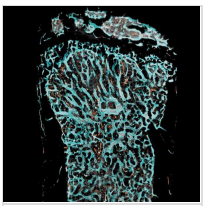

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Affordable immunofluorescence staining on bones or other tissues without cryostat

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ABSTRACT

We are happy to share the First UNICORN ★ Protocol on behalf of the UNICORN Hemato.

How to perform affordable immunofluorescence staining on bones or other tissues without cryostat

This method is an adaptation from

- Multicolor quantitative confocal imaging cytometry published by Daniel Coutu et al. in Nature Method 2017 (see supplemental methods to compose your buffers!)

- IBEX: A versatile multiplex optical imaging approach for deep phenotyping and spatial analysis of cells in complex tissues published by Andrea Radtke et al. in PNAS 2020

Not being experts in microscopy, we realized that you microscopists are the MacGyvers of the bench

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KEYWORDS

Immunofluorescence staining, vibratome, Bone section, Agarose, confocal microscopy

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MATERIALS TEXT

PFA
EDTA
Tissues Cassettes
Agarose
Plastic embedding molds
Vibratome
TBS
DMSO
FBS
Triton-X

Sample preparation

1 Collect and prepare your samples of choice (femurs, tibiae, spleen, others).

Properly remove any tissue adhering to the bones

Tissue Fixation

2 Fix your samples with 1-2% PFA

Comments: 1% for better preservation of Epitopes, 2% makes it easier to cut bones but decreases signal of some Ag compared to 1%

Incubate overnight or 16h if you start earlier than us. But no more 16h!

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3 Only if you work on bones, carry out a decalcification step!

Put your bones in cassettes and place them in 10% EDTA pH=8 for 7 days (Have to be adapted according to the age of the mouse, the size of your sample, and the % of PFA) in rotating Erlenmeyer with 100mL for 12 bones (femurs and tibias) at 4°C.

The EDTA needs to be changed 1 time a week.

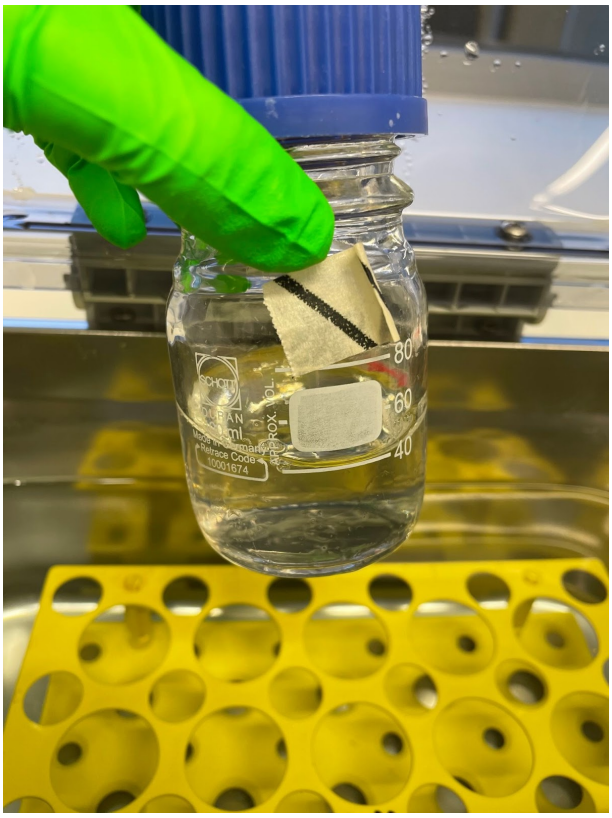
Agarose embedding

4 After 7 days, here come the tricky stages! Your bones are soft so be careful when taking them.

Comments: the twisters sometimes do damage if you pinch the bones too hard (you move the bone marrow inside the bone)

5 Weight your agarose to obtain a 4% agarose solution in water.
Boil it in a microwave to obtain translucent agarose

Comments: Low melting agarose is the easiest way but standard agarose works too, just the timing to use the agarose at optimal temperature is a little more clever.



6 Place it in a water bath to cool down your agarose to a temperature of around 45 °C (42-46 °C).

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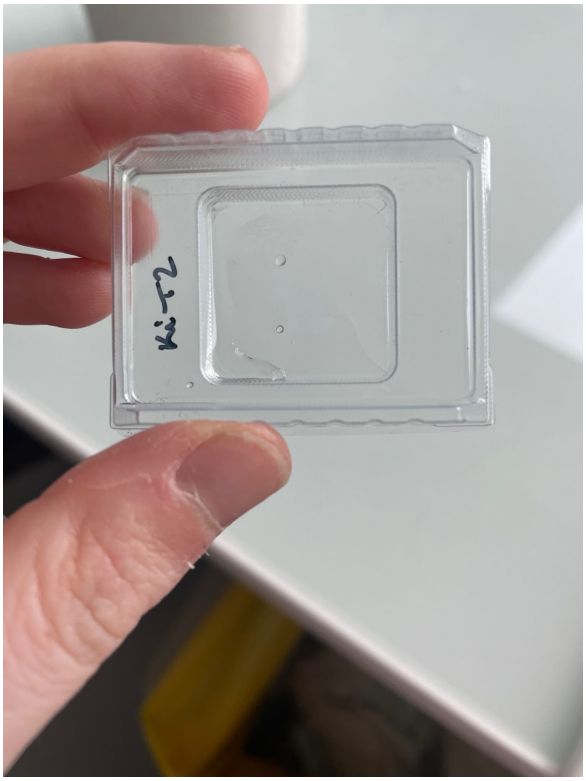
7 During the cool down, take advantage of the time to place your cassettes into the PBS bath for washing 2 times.

Then dry your bones with microscopy Kim wipes just before the embedding (this is short, do not over-dry your bones).

8 Place them into plastic embedding molds as flat as possible.



9 Pour the agarose into the molds and put them on ice for 1h.

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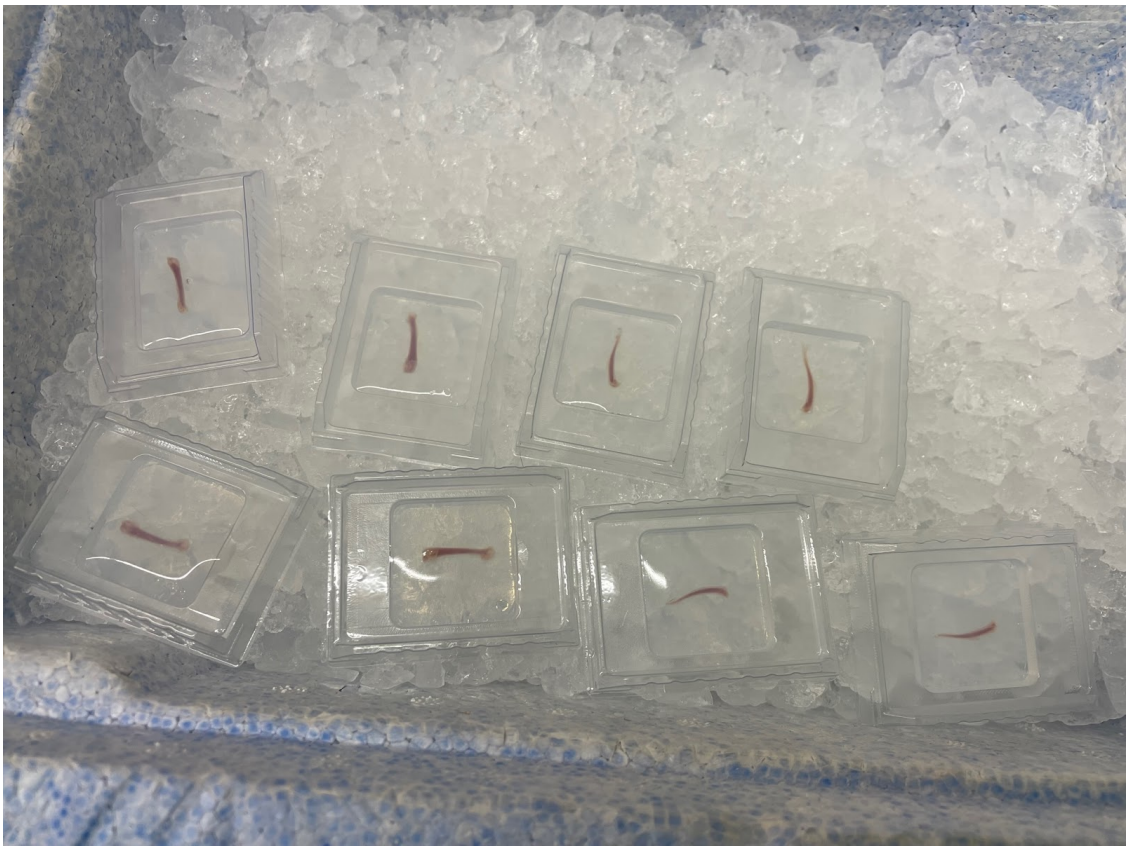

Sectioning

10 Go to your vibratome!

For this specific step, you have to take into consideration some parameters: the type of microscope your lab possesses (direct or inverted), if clearing is needed or not, and the number of antigens you want to visualize (multi-round staining or not).

The setting should be determined before you start, we cut at a thickness of 25-50µm (max if you wanted to do IBEX) to 250 um (optimal to clearing protocol).

Be aware, when your slices are thin the staining is less noisy but it is going to be more challenging to cut!

11 Select the best slices.
Keeping in mind that if you want to compare conditions between them, you have to estimate your cutting depth.

For example, we have observed that the closer you are to the bone in the metaphysis, the more adipocytes you will have. Conversely, in the center of the metaphysis, you will have

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fewer adipocytes. Indeed, the biggest part of the heterogeneous organization of your BM depends on the proximity to the bone and different areas (metaphysis, diaphysis, or epiphysis). To avoid this problem, we can cut transversely to keep the same proportion of endosteal and non-endosteal niches between your different conditions. Obviously for transverse cuts, you need to compare the same bone area: metaphysis, diaphysis, or epiphysis.

12 Place the slices on a square microscopy slide.
You have to prepare the slides before using them. To do that you can follow this simple protocol (https://www.rndsystems.com/resources/protocols/protocol-preparation-gelatin-coated-slides-histological-tissue-sections) or you can buy directly coated slides but it's very (very) expensive!!!
The cut section will stick to the slide after around 15-20min.

13 Then place the slides in the embedding molds and put TBS until staining at 4-8 °C.

Critical step: we encourage you to stain your slice as soon as possible your slice, because the more you wait, the less your antigen will be strongly recognized (believe in our experience).

Staining

14 The next day, block and permeabilize (1.25X TBS, 1% DMSO, 10% FBS, 0.1% Triton-X) during 1h.

Critical step: take into consideration that in thick sections (100-250µm) less the wavelength is large, less is it able to penetrate efficiently in the tissues. So for 405 and 488 nm, only the first layers of cells would be qualitative! You can improve this using a tissue-clearing approach (incompatible with the IBEX pipeline).

However, be aware that depending on your fluorochromes and your clearing methods, you are going to lose some part of your signal. For example, the signal loss is approximately around 80% for a TDE concentration >90%! It's why it should be used only if your staining targets are highly represented antigens.

15 Add primary antibodies diluted in the blocking buffer (1.25X TBS, 1% DMSO, 10% FBS, 0.1% Triton-X).

Comments: Incubation time depends on your section thickness. We are successful with 2-3h for 25-50 µm and overnight for thicker sections (100-250 µm). Moreover, if you have an inverted microscope, your antibodies have to penetrate all the depth of your section to stain what you imagine, so take it into consideration!

Place the embedding molds in a humidified chamber (300 µL per sample in the mold containing your slide) at 4°C in smooth rotation.

16 Wash 2-3X with washing buffer (TBS 1X, 0.1% Triton-X) for 10min

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17 Add secondary antibodies diluted in the blocking buffer. Pay attention to the order and selection of the secondaries so that it does not cross between them!

You may add a DAPI (1/5000) staining step at the final round.

Comments: Dapi staining is necessary if you want to use IBEX protocol because it will be used as a reference between your different rounds!

18 Wash 2-3X 10min with a washing buffer (TBS 1X, 0.1% Triton-X)

19 Prepare the mounting: put it in a semi-solid mounting medium like Dako and then a large slide on your previous square slide.

We don’t use spacers because of the need to easily remove the slide with the sample for potential multi-round staining, but also because of their price.

20 After waiting little time for the polymerization of the mounting medium, you are ready to see beautiful pictures!!!

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