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Phenocycler Fusion BIDMC-TMC

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We use this protocol and it's working

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Abstract

The PhenoCycler Fusion protocol used by the Spatial Technologies Unit at the BIDMC for the profiling of lymphatic vessels in the BIDMC - Lymphatic Vessels TMC.

The Phenocycler Fusion (PCF, AKOYA Biosciences) consists of a liquid handling and fluidics unit connected to an integrated brightfield and fluorescent microscope with 5 channels.

Antibodies against proteins of interest are conjugated to a unique barcode, which matches via Watson-Crick base pairing to a respective sequence attached to a fluorescent reporter.

The tissue of interest is incubated with a panel of conjugated antibodies and subsequently exposed to cycles of 3 fluorophores with attached reporters plus DAPI at a time, with stripping of the reporters taking place between cycles. This allows for the imaging of three reporters per cycle.

The first and last cycles are blank and are used to examine the difference in autofluorescence occurring as part of the treatment. DAPI is imaged at every cycle and is used to set the focal plane. Imaging takes place at a constant x20 magnification with brightfield mask taken in advance to highlight the position of the section.

Troubleshooting

Necessary Materials

1

	Reagent/Antibody	Storage Temperature
	Tissuetek OCT	Room Temperature (RT)
	70% Ethanol	RT
	N Blocker	4°C
	J Blocker	4°C
	G Blocker	-20°C
	S Blocker	-20°C
	Fusion Phenocycler Antibodies	4°C
	Custom conjugated Antibodies	4°C
	Hydration Buffer	4°C
	Staining Buffer	4°C

	Reagent/Antibody	Storage Temperature
	10x Fusion Phenocycler Buffer	RT
	Fusion Phenocycler Buffer Additive	RT
	Fixative Reagent	-20°C
	Storage Buffer	4°C
	MilliQ Water	RT

	Plastic Consumables/Tools

	Humidity Chamber
	Drierite Absorbent Beads
	Fiberboard Box
	1.5mL Eppendorf Tube
	50mL Conical Tube
	Metal chuck
	Fine-Tip paintbrushes (2)
	Leica brush
	Standard Razorblade
	Kimwipes (small)
	Fisherbrand Superfrost Plus Microscope Slides

Sample Collection

2 Isolate and collect lymphatic vessels following consent as part of surgical procedures according to criteria and procedures outlined in **dx.doi.org/10.17504/protocols.io.e6nvwjeb7lmk/v1**.

Sectioning

3 The tissue sample is placed into the cryo chamber 10-60 minutes before beginning to section. This allows the tissue to equilibrate to -20°C on a Leica CM3050s cryostat.

Once equilibrated, OCT is added to the metal chuck, making sure that the entire chuck is filled to avoid issues with OCT spreading when adding the block due to viscosity. The block is taken out of the cryomold and placed it gently onto the OCT, then pushed down lightly to ensure that it's parallel to the chuck. The chuck is placedback into the cryo chamber into one of the holders on the left side.

4 Allow 5-10 minutes for the OCT to solidly freeze. While OCT is freezing, slides are labelled with the information associated with the block, the operator's initials and the date of sectioning.When OCT is sufficiently frozen, as indicated by the color switch of the block from clear to white, the chuck is placed on the objective and fastened.

5 The distance from the tissue block to the blade is gauged and calibrated until contact is made. First, the safety guard is lifted to ensure visibility of the sectioned parts and 5-10 um sections are cut after adjusting the objective to be parallel with the block.

6 Finally, the tissue is sectioned and collected under the guard or using fine-tip paintbrushes to lower the chances of the tissue curling with extremely gentle movements to avoid any kind of damage. Using the positively charged side, the sample is then laid flat onto the section. The sample should stick immediately and the OCT turn clear.

7 The chuck is unlocked and taken out of the cryostat and laid on a Kimwipe, then allowed to thaw slightly for 2 minutes. A clean razor blade is then used to very carefully dislodge the sample by prying it from the edges. The sample is placed back in the mold and stored in -80 C.

The remaining OCT can be removed with a Kimwipe and the chuck sprayed with 70% ethanol and cleaned completely. Afterwards, it can be left at room temperature until furhter use.

Sample Collection

8 After collection, samples are immediately embedded in OCT with long axis facing the plane of sectioning. Samples are then flash frozen in a mixed dry ice+isopentane bath until solidified and the orientation of the specimens is noted for registration downstream on the CCF.

9 Sample are then stored at -80C until used.

10 Samples are sectioned on Leica Superfrost plus slides to obtain serial sections starting from the top and descending, with a maximum of 5 sections/slide for a total of 5 sections/specimen.

Sample Treatment

11 Following sectioning, the slides are placed sample side up on drierite beads for 5 min, followed by dipping for 10 min in molecular biology grade acetone.

12 The slides are then allowed to air-dry for 2 min, followed by immersion in 40 mL hydration buffer for 2 min and another immersion in hydration buffer in a separate container for 2 min.

13 The samples are then fixed using 1.6% PFA in PBS for 10 min followed by re-immersion for 2 min through two rounds of hydration buffer as before and a 30 min incubation in staining buffer.

14 The slides are then treated with 190uL of antibody and blocker cocktail in staining buffer overnight at 4C.

15 The slides are subsequently washed with staining buffer and fixed with 1.6% PFA in PBS for 10 min.

16 Slides are then cleaned by immersion in PBS three times and further fixed using ice cold molecular biology grade methanol for 5 min, followed by another round of triple cleanup using PBS.

17 The slides are then treated with 200uL/slide of final fixative for 20 min at room temperature, triple washed with PBS and the flowcell applied using a specialized instrument.

18 The flowcell is allowed to harden for 20 min in staining buffer before being moved to the PCF for the initiation of the experiment. At the same time, the reporter plate was prepared.

Reporter Plate Generation

19 The reporter plate is black Costar round bottom well plate with well contents relating to the fluorophores used in each cycle of the PCF imaging.

Therefore, the barcode-tagged fluorophores were organized in groups were at most three different fluorophores/well were used at 5uL/well each and the volume adjusted to 250uL with staining buffer.

The cycle number of the experiment was then based on the number of wells that needed to be allocated based on these restrictions. Once prepared, a plate is used within 48h at most.

Imaging

20 The PhenoCycler Experiment Designer software is utilized to enter the antibody and corresponding barcode, as well as fluorophore information in accordance with the generated reporter plate.

The slide with attached flowcell is inserted into the carrier containing the fluidics. The carrier has an open area which can be further delimited by manual highlighting to aid the instrument in autolocating
the sample on the slide based on the DAPI signal using the higher-sensitivity "Algorithm B" provided by Akoya in the new Phenocycler Fusion software version 1.0.8.

Due to the small size of the lymphatic vessels, in all cases the high sensitivity algorithm for sample finding was used, with saturation protection set to DAPI and a default exposure time of 2ms for DAPI and 150 ms for all other frequencies.