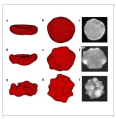

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Working

Preparation of human Red Blood Cells for confocal imaging

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ABSTRACT

This protocol is used to label red blood cells (RBC) for confocal imaging. Fluorescent beads are used to calibrate voxel size on the confocal microscope. The imaging protocol was optimised to obtain high quality data, by mounting the cells in a high refractive index and optimising acquisition parameters.

Spherical aberration results in a decrease in image intensity when the imaging plan gets further away from the coverslip. It is caused by difference in refractive indices between the different components present over the beam pathway. In order to reduce spherical aberration, the cells are fixed in glutaraldehyde after staining so they can be resuspended in a medium with a high glycerol content and maintain their shape. Glycerol increases the reflective index of the medium, bringing it closer to the reflective indices of the coverslip and the immersion oil.

Acquisition parameters are first chosen using the system optimisation function, then adjusted. The z-axis step size is chosen as close as possible to the lateral resolution to avoid under- or over-sampling. The pinhole is reduced to 50 µm to increase resolution and reduce out-of-focus light. A 2-step line averaging is selected during scanning to remove noise.

Data is analysed and quantitative volume and surface area values were extracted using a home-written matlab code (not included in this protocol).

EXTERNAL LINK

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MATERIALS

NAME	CATALOG #	VENDOR
Glutaraldehyde EM Grade 25%	G5882-50ML	Sigma Aldrich
Vybrant® DiI Cell-Labeling Solution	V22885	Thermo Fisher Scientific
PBS	17-517Q	Lonza
Glycerol	104092	Merck Millipore
TetraSpeck Fluorescent Microspheres Sampler Kit	T7284	Thermo Fisher Scientific

MATERIALS TEXT

Fresh citrated human whole blood

SAFETY WARNINGS

Concentrated glutaraldehyde should be manipulated under a fume hood. Please check SDS and local chemical risk assessment for all chemicals before starting work. Ensure you use the correct preventive safety measures and wear the right PPE.

BEFORE STARTING

Fresh human blood samples are used during this protocol: make sure you obtain the appropriate ethics clearance before starting work. Also check that the laboratory has the correct equipment and PPE for this protocol.

Imaging chamber

1 Prepare imaging chamber by using double-sided tape as a spacer between two coverslips : cut a square within the tape such as to form a

frame. Without removing the non-adhesive cover, place this pre-cut frame on a coverslip. The chamber will be closed at the end of the protocol, by removing the non-adhesive cover and placing a second coverslip on top of the double-sided tape.

2 In the center of the chamber, add **2 µl** of 4µm calibration beads, and let the slide dry in the dark

RBC isolation

3 Centrifuge fresh whole blood in a citrate tube for **00:02:00 at 1000g**

SAFETY INFORMATION

Until screening for diseases is completed, consider all blood samples as potentially contagious and use the appropriate PPE

Ethical clearance must be obtained before any exeriment using human blood samples.

4 Remove supernantant and buffy coat, keeping the RBC fraction

Staining

5 Wash **5 µl** of RBC in **500 µl** PBS, centrifuge for **00:01:00 at 1000g** , and remove the supernantant

6 Resuspend in **500 µl** PBS

7 Add **5 µl** of DiI and incubate for **00:02:00 in the dark** at **37 °C**

8 Wash 3 times in **500 µl** PBS (**00:01:00 at 1000g**) and remove supernanant

Fixation

9 Mix **10 µl** of the pellet containing the RBC with **20 µl** of **[M]3 Volume Percent** glutaraldehyde in PBS

SAFETY INFORMATION

Prepare glutaraldehyde solution under a fume hood

10 Incubate **00:15:00 in the dark**

11 Wash 3 times in **500 µl** PBS (**00:01:00 at 1000g**) and remove supernanant

Imaging

12 Resuspend in glycerol to obtain a [M]**50 Volume Percent** glycerol solution, deposit on the prepared slide containing the calibration beads, then close the imaging chamber by placing a second coverslip on top of the double-sided tape spacer

13 Image with x63 oil immersion objective