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**Fluorescent measurement of dense granules and intracellular calcium in individual platelets in microfluidic flow chambers**

1. Preparation of flow chambers

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| **For quantity and location see** Reagent\_and\_location\_list.xlsx  **Equipment:**   1. Harrick Plasma Cleaner 2. Centrifuges 3. Thermostat 4. Syringe pump 5. Puncher (*69031-01 Integra Miltex*) 6. Coverslips (*48366-089 VWR Micro Cover Glasses, Square, No.1 thickness is 0.13 to 0.17mm, 25 x 25 mm*) 7. PEI tubes (outer and inner diameters are 0.9 and 0.6 mm, respectively) 8. Microfluidic master mold # 1. On microfluidic master mold there are 4 parallel channels. One channel dimensions: 0.05, 1, 13 mm for height, width and length, respectively. 9. Plastic flow chamber holder. Width and length 25 and 75 mm, respectively. “Window” for PDMS flow chamber is 28\*28 mm. “Window” for flow chamber coverslip and oil objective is 22\*22 mm. Holder also has two grooves for plastic tubes and a holder for two 1.5 ml Eppendorf tubes. Cover of the holder is attached by two screws. 10. For blood collection - (*02.1067.001 SARSTEDT, S-Monovette® 5ml 9NC)*   **Reagents:**   1. Silicone Elastomer Base and Silicone Elastomer Curing Agent *(24236-10 Electron Microscopy Sciences, Sylgard™ 184)* 2. Fibrinogen aliquots 3. Isopropanol (34863 Sigma Aldrich) 4. Buffer A with BSA 5. Calbryte-590AM 6. Mepacrine 7. CaCl2 |
| **Preparation of fibrinogen solution**   1. Fibrinogen aliquot (20 mg/ml of Fibrinogen in PBS solution) is defrosted at RT and diluted to concentration 1 mg/ml by PBS buffer. 2. Fibrinogen solution is centrifuged 30 min at RT at 16800 g. 3. After centrifugation 90% of solution is taken. |
| **Preparation of PDMS slabs**   1. Silicone Elastomer Base is added to Silicone Elastomer Curing Agent (ratio is 10:1). 2. PDMS mixture is mixed by dry and clean (!) metal spatula until all is mixed and there are a lot of air bubbles. 3. To remove air bubbles mixture is centrifuged during 15 min at 200g 4. PDMS mixture is poured on the clean master mold\* # 1. The height of PDMS mixture should be 6-8 mm. 5. It is left covered for 20-25 minutes at RT to remove small bubbles. 6. PDMS mixture on master mold is placed to thermostat for 2 hours at +75 °C. 7. PDMS slab is cut out carefully by scalpel (pay attention and cut all sides, otherwise PDMS will be teared) and carefully unstick from the microfluidic master fabrication. Avoid master mold breakage! 8. PDMS slab is placed on a new micro slide and is kept closed in a clean Petry dish (or clean plastic box). Write the data on it. Keep at room temperature. It might be kept for 1-2 weeks.   \*microfluidic master mold should not be cleaned by mQ or ethanol. Before pouring the new PDMS mixture remove the old one from the mold. Inspect that there are no pieces of PDMS left at the corners of the mold. |
| **Preparation of flow chamber**   1. PDMS slab is cut by scalpel to 27\*27 mm size (because holder is 28\*28 mm). 2. In PDMS slab holes are made in the channels of the relief by puncher of 1 mm diameter. Holes should be made vertical to the relief plane on PDMS slab and close to the ends of a channels of the relief. This helps to prevent bubbles in the channels and stagnant zones. 3. PDMS slabs and coverslips are placed for 15 min in a Petri dish filled with isopropanol. 4. PDMS slabs are wiped by Kimwipes and washed by mQ flow. Coverslips are just washed by mQ. 5. Both PDMS slabs and coverslips are dried by N2 flow from N2 cylinder. 6. Cleaned coverslips and PDMS slabs are treated by oxygen plasma using Harrick Plasma Cleaner at 400 mTorr, HI RF level during 30 seconds. 7. Attach PDMS slabs to coverslips. Coverslip is pressed by fingers very carefully to prevent glass crack, but it helps to remove air bubbles. 8. Leave prepared flow chambers for 5-7 min for attachment of PDMS slabs to coverslips. 9. Insert slowly and carefully blunt needles in flow chamber holes. Blunt needles are connected to PEI tubes. One “inward” tube (6 cm) of the channel is going to Eppendorf tube with solutions, second “outward” tube (about 75 cm) is going to withdrawal syringe. |
| **Fibrinogen attachment to coverslip surface**  Do the following steps right after the preparation of flow chamber. Do not wait otherwise the effect of plasma cleaning and attachment of fibrinogen will be reduced.   1. 1ml syringe is attached to outward tube. Inward tube is placed in 0.6 ml Eppendorf tube filled with 500 µl of PBS solution. Channel is washed with PBS solution by withdrawal mode by syringe. 2. 0.6 ml Eppendorf tube filled with PBS solution is replaced by 0.6 ml Eppendorf tube filled with 200 µl of fibrinogen solution (1 mg/ml). 3. Leave flow chambers and their channels filled by fibrinogen solution for 1 hour at RT. 4. 0.6 ml Eppendorf tube filled with fibrinogen solution is replaced by 0.6 ml Eppendorf tube filled with 500 µl of buffer A with 4% of BSA.   Prepared flow chambers (filled with buffer A with 4% BSA) coverslips with covered fibrinogen could be kept not more than overnight at +4 °C. |

1. Blood preparation

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| **Platelet loading with dyes**   1. 12.5 µl of 40 µM of Calbryte-590AM is placed on the bottom of 1.5 ml Eppendorf tube. 0.5 µl of 10 mM Mepacrine is placed on the wall of the same 1.5 ml Eppendorf tube (final concentrations of Calbryte-590AM and Mepacrine are 1 and 10 uM, respectively). 2. 500 µl of citrated blood is added to 1.5 ml Eppendorf tube. Blood is pipetted slowly and carefully to prevent bubbles appearance. 3. Blood supplemented with dyes is incubated for 20 minutes at RT in a dark place.   Citrated blood is used during 2 hours after collection. Load blood with dyes directly prior to microscope experiment. |

1. Measurement on big Nikon microscope

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| **Microscope settings**  **To work on microscope, see the following protocol:**  ×100 oil objective is used. For transmitted light air condenser is used.  **Imaging scheme:**   1. First field\*: DIC photo -> Measurement of Mepacrine and Calbryte-590AM fluorescence, fps=2 during 4-5 min before activation -> DIC photo 2. Second field: DIC photo -> Measurement of Mepacrine and Calbryte-590AM fluorescence, fps=2 during 10 min -> DIC photo   **Epifluorescent settings:**   1. Angle – , some experiments could be made in a TIRF regime. In that case angle is 2. Mepacrine: excitation - 488 nm laser, emission empty filter – 1st position, 2% of intensity, 50 ms exposure, 1000 EM Gain. 3. Calbryte-590AM: excitation – 561 nm laser, emission empty filter – 1st position, 1% of intensity, 50 ms exposure, 1000 EM Gain. 4. Both Calbryte-590AM and Mepacrine fluorescence are measured using Triggered Acquisition Regime. 5. AnnexinV-Alexa-647: excitation – 640 nm laser, emission 670+ nm, 50% intensity, 50 ms exposure, 1000 EM Gain.   \*Fields are chosen in a center of the width and length of the channel. Length distance between two fields should be at least 1 mm. |
| **Solution preparation**  Solutions are prepared in advance and are kept in a dark place (i.e. plastic box). Note that inhibitors and activators are kept on ice and are added just before using the solution.   1. **Stock of Buffer А with 1 % of BSA with Mepacrine\***   In 15 ml Falcon tube 10 ml of Buffer A with 1 % of BSA and 1 µl of 10 mM mepacrine are mixed.   1. **Washing Buffer**   Content: Buffer А with 1 % of BSA with Mepacrine (1 uM)  Eppendorf tube #1 volume – 800 µl  Eppendorf tube #2 volume – 425 µl (for 25 µl/min velocity) \*\*   1. **Activation buffer**   Content\*\*\*: Buffer А with 1 % of BSA + 1 µM of Mepacrine + 2 mM CaCl2 +activator (see protocol of activator preparation)  Activation buffer volume – 800 µl  In 1.5 ml Eppendorf tube mix Buffer А with 1 % of BSA with Mepacrine and 8 µl of 250 mM CaCl2 and activator (ADP or thrombin).  Note that this activation buffer is added to Buffer #2 during experiment. Because platelets are washed during 9 min, 225 µl of Buffer #2 is used up and 200 µl of Buffer #2 is left in a tube. So, when mixed with Activation buffer, final volume is 1000 µl.\*\*  \*Mepacrine addition help to prevent mepacrine leakage from platelets.  \*\*If you change the flow rates of the solution, then buffer volumes also should be adjusted. |
| **1 trial = 1 chamber experiment scheme**   1. Flow chamber is installed in the plastic flow chamber holder. Tubes are placed in the grooves. Holder cap is placed and is fixed by small screws. 2. Holder is placed on Nikon microscope table and fixed by clamps. Objective with oil is moved up until oil drop reaches the coverslip glass. Then autofocus is switched on. By looking in a Transmitted light mode focus on the internal coverslip surface. 3. Outward tube is connected to 10 ml syringe on a syringe pump (diameter is set as 14.6 mm). 10 ml syringe is filled with 500 µl of mQ (control no bubbles in it). Also control that in inward tube there is no bubble. 4. Inward tube is placed in a 1.5 ml Eppendorf tube with labelled blood (see Blood preparation). 5. For 30 seconds syringe pump (45 µl/min) is switched on in a pumping mode to prevent any bubbles in an inward tube. 6. Then syringe pump is switched to a withdrawal mode. 7. By looking in oculars in transmitted light mode and on a tube (to see if there are any air bubbles) note the moment when blood entry into the channel. Turn on timer. 8. Blood is perfused through the channel for 20 seconds. 9. After that syringe pump is switched to a pump mode. 10. When you see in oculars that blood flow is almost stopped transfer tube to 1.5 mL Eppendorf tube #1 with 800 µl of Washing buffer. At this step look at this 1.5 mL Eppendorf tube and note if there are any bubbles in an inward tube\*. 11. If there are no bubbles in an inward tube, then syringe pump is switched to a withdrawal mode (45 µl/min). 12. Wash platelets with Washing buffer for 3 minutes. 13. After that syringe pump is switched to a pump mode. 14. When you see in oculars that blood flow is almost stopped transfer tube to 1.5 mL Eppendorf tube #2 with 425 µl of Washing buffer. At this step look at this 1.5 mL Eppendorf tube and note if there are any bubbles in an inward tube\*. 15. If there are no bubbles in an inward tube, then syringe pump is switched to a withdrawal mode (25 µl/min). 16. Wash platelets with Washing Buffer for 9 minutes. Image First field\*\* during washing. 17. Start imaging of Second field\*\*. Add Activation buffer to 1.5 ml Eppendorf tube #2 with Washing buffer after 30 seconds of imaging. Do not interrupt imaging, just add Activation buffer by 1 ml pipette immersing the tip into the solution of Washing buffer.   \*If there is a bubble, wait until it will come out or use Kimwipe to wipe the end of inward tube.  \*\* Platelets in the field should be located as far as possible from each other. Do not image platelet aggregates, you need single platelets. Try to find the field where there are at least 10-20 platelets. |