Endothelialized flow models for blood transfusion research

Monica S.Y. Ng,[1,2] Jacky Y. Suen,[1] John-Paul Tung[1,2] and John F. Fraser[1]

[1]Critical Care Research Group, Faculty of Medicine, University of Queensland, Brisbane and [2]Research and Development, Australian Red Cross Blood Service, Brisbane, Australia

©2019 Ferrata Storti Foundation. This is an open-access paper. doi:10.3324/haematol.2018.205203

Received: October 31, 2018.

Accepted: January 15, 2019.

Pre-published: February 14, 2019.

Correspondence: *MONICA S.Y. NG*

monica.ng91@gmail.com

Supplementary 1 – 3mm endothelialised *in vitro* vascular flow model (an example)
Below is an example of a 3mm endothelialised *in vitro* vascular flow model used at the Critical Care Research Group, Brisbane, Australia

Methods

Model construction

SolidWorks (Dassault Systèmes 2014, version 22) was used to draw blueprints for the top and bottom pieces of the model container, side plugs and gas trap (Figure 1). The top piece consisted of a 70 mm x 30 mm x 5 mm flat slab with inlet, outlet, injection and decompression ports. The bottom piece contained a lengthwise 60 mm x 5 mm x 3.8 mm rectangular channel for the collagen scaffold and two 3mm diameter holes to accommodate the stainless steel moulding rod. The model container components were Computer Numerical Controlled (CNC) machine manufactured out of Poly(methyl-methacrylate) (PMMA) (Figure 2). Six M3 x 5 mm full thread cap head screws were used to secure the top and bottom pieces of the PMMA container together. Inlet and outlet port plugs were formed by cutting 2.8mm diameter silver brazing rods into 10mm length pieces. The two side plugs were CNC machine manufactured out of Polytetrafluoroethylene (PTFE). The gas trap was selective laser sintering 3D printed using Nylon 12 (PA2200)/White.

Two stainless steel barbed M3 connectors (Festo: 15872), barbed 1/8-1/8 Kynar connectors (Harvard Apparatus: 721525), Tygon ST R-3603/R-3607 tubing (Harvard Apparatus: 729198), 3-stop Polyvinyl chloride (PVC) tubing (Harvard Apparatus: 720671) and P70 Harvard Apparatus Peristaltic pump (Harvard Apparatus: 70-7000INT) were used for circuit construction.

Model and circuit assembly

Equipment and model parts were sterilised with 70% (v/v) ethanol, oxygen plasma treatment or autoclave, prior to use. Sequential chemical surface treatments were used to enable collagen adherence to PMMA. First, the top and bottom PMMA pieces were submerged in 1% (w/v) Polyethylenimine (Sigma Aldrich: P3143) in sterile water for 10 mins at room temperature. The pieces were then rinsed thoroughly with sterile water and allowed to air dry for 30 mins at room temperature (RT). Then both PMMA pieces were submerged in 0.1% Glutaraldehyde (Sigma Aldrich: 49629) in sterile water for 30 mins at room temperature. Both pieces were rinsed with sterile water and allowed to air dry completely. Upon completion of treatment, the surface treated pieces was stored in a sterile container at 2-4 °C for up to 4 days if required. The two PMMA model container pieces, six M3 stainless steel screws, 3mm stainless steel rod, two silver plugs were assembled as per Figure 2A in preparation for collagen injection moulding.

High concentration Type 1 Rat tail collagen (Falcon: FAL354249) was diluted to 6mg/mL and neutralised prior to injection moulding with 1M sodium hydroxide (NaOH; Sigma Aldrich: S2770), 10X phosphate buffered saline (PBS; Gibco: 70011044) and Endothelial Growth Media 2 Bullet Kit (EGM, Lonza: CC-3162). The volumes of each reagent required were calculated using the following equations:

a. $Volume\ 10x\ PBS = \frac{Final\ volume\ collagen}{10}$
b. $Volume\ collagen = \frac{Final\ collagen\ concentration \times final\ volume\ collagen}{Stock\ collagen\ concentration}$
c. $Volume\ 1M\ NaOH = Volume\ collagen \times 0.023$
d. $Volume\ EGM = volume\ collagen - volume\ 10x\ PBS - volume\ 1M\ NaOH$

Final concentration = 6 mg/mL *Final volume collagen = 1.5-2 mL per model*

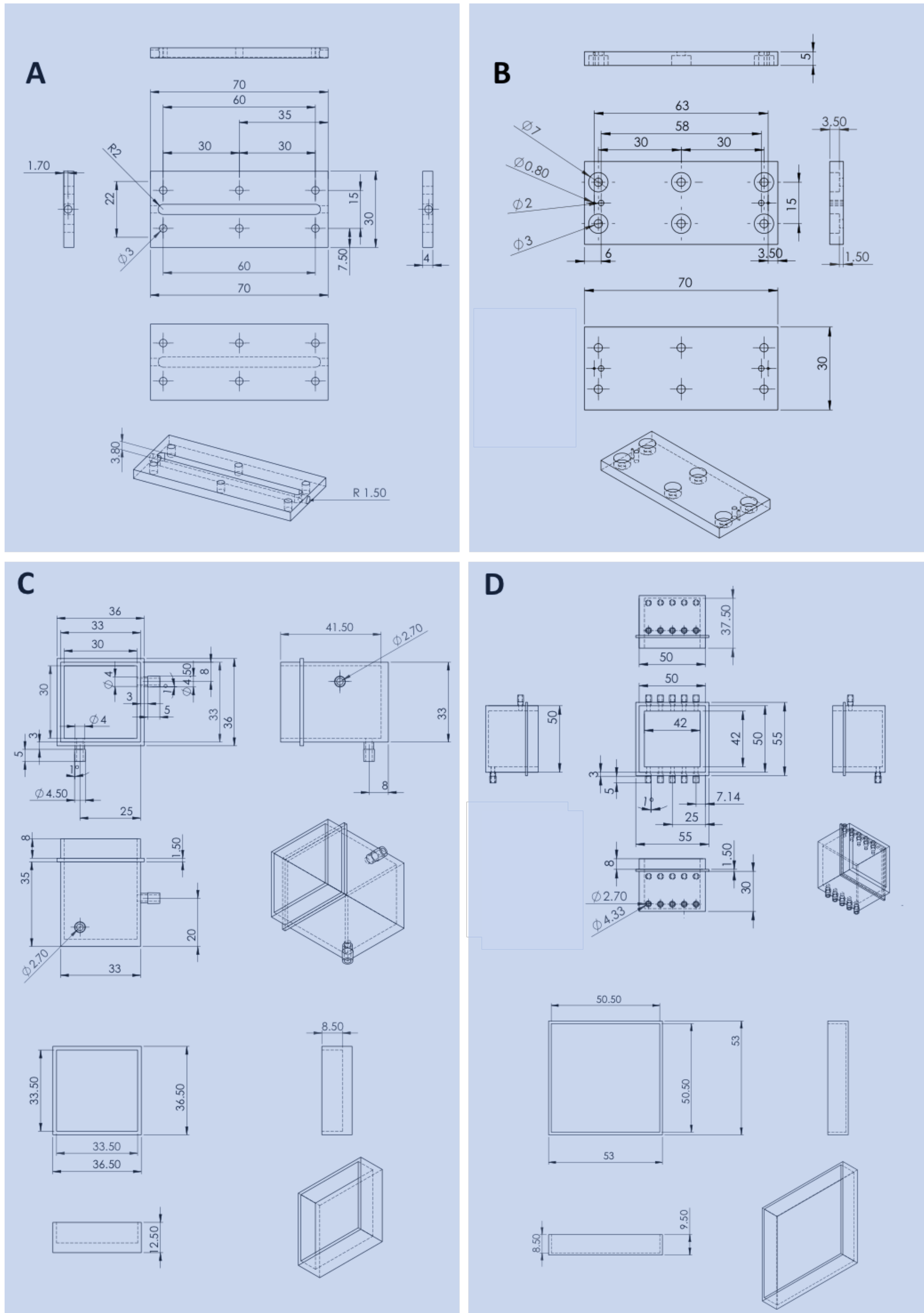

Figure 1: Drawings of model container (A=bottom piece, B=top piece), single channel gas trap (C) and 5 channel gas trap (D).

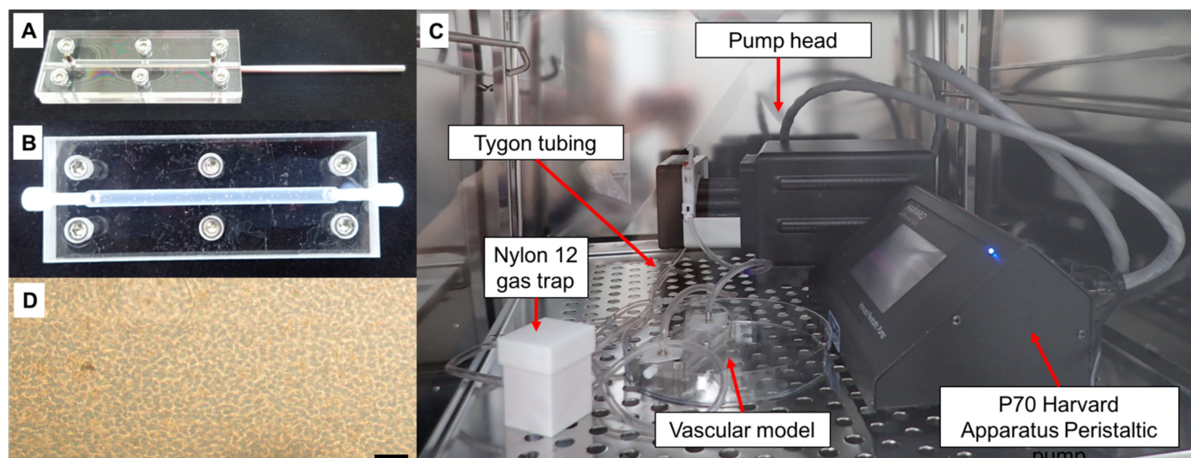

Figure 2: Assembly of model components and flow circuit. (A) Assembly prior to collagen injection, (B) Assembly after collagen injection, (C) Flow circuit development, (D) confluent endothelial cell layer after 18 hours of laminar flow at 16mL/min (10X magnification, scale bar=50μm)

The collagen suspension was prepared under sterile conditions at 4 °C. A buffered solution formed from 10X PBS, 1M NaOH and EGM, was added dropwise to high concentration Type 1 Rat tail collagen to minimise bubble formation. The resulting 6 mg/mL collagen suspension was stirred in circular movements with a stainless steel stirrer for 2 mins to homogenise mixture and minimise bubble formation. pH strips (Sigma Aldrich: P3536) were used to titrate mixture to physiological pH of 7.3-7.4. The collagen suspension was slowly aspirated into a 19G tapered needle (BD Medical: 305187) attached to a 5 mL syringe (BD Medical: 302130) to prevent bubble formation. The 6 mg/mL collagen mixture could be stored overnight at 4 °C in the syringe if required.

For injection moulding, the 19G needle (BD medical: 305187) was replaced with a 25G needle (BD Medical: 305127) and the collagen suspension was slowly injected into the injection port of the PMMA container with constant pressure. The system was then incubated at 37°C for 30 mins to allow the collagen to crosslink into a gel. The stainless steel rod and silver plugs were then removed from the assembly and PTFE plugs were inserted into the two side holes to form a U-shaped channel. The model was filled with EGM and allowed to equilibrate for at least 1 h at 37 °C prior to cell seeding. Human umbilical vein endothelial cells (HUVECs; Lonza: CC-2517A) between passage 1-5 were seeded onto the model at 300,000 cells/mL and allowed to adhere for 2 h at 37 °C. Prior to connecting the model to the circuit, the HUVEC seeding suspension was replaced with fresh EGM media and stainless steel barbed M3 connectors were attached to the inlet and outlet ports.

The circuit was connected as per Figure 2C and primed with EGM. HUVECs were cultured to confluence for 18 h under 16mL/min laminar flow at 37 °C and 5% CO_2. HUVEC confluence was confirmed prior to blood perfusion testing with an inverted light microscope (Nikon: Eclipse TS100). The gas trap was removed from the circuit prior to whole blood perfusion.

Fluorescence and confocal microscopy
HUVECs in the model were fixed with 3.7% (w/v) paraformaldehyde (Sigma Aldrich: P6148) in PBS for 30min at room temperature. HUVECs were washed 3 times for 5 mins with PBS. The inlet and outlet ports could be sealed with paraffin film and stored overnight at 4 °C. HUVEC cells were treated with 3% (w/v) bovine serum albumin (BSA; Sigma Aldrich: A2153) and 1% (v/v) Triton X-100 (Sigma Aldrich: X100) in PBS for 1 h at room temperature to prevent non-specific binding and permeabilise cell membranes. After removing the BSA/Triton X-100, primary antibodies (Table 1) in 1% BSA were added to the model and allowed to stain overnight at 4°C. The model was sealed with paraffin film (Sigma Aldrich: P7793) to prevent evaporation. The HUVECs were washed 3 times for

5 mins with PBS. The HUVECs were stained with secondary antibodies in 1% BSA and 1:1000 4',6-Diamidino-2-Phenylindole (DAPI; Life Technologies: D1306) per Table 1 for 1 h at room temperature. The inlet and outlet ports could be sealed with paraffin film and stored overnight at 4 °C. HUVECs cells were imaged *in situ* using Olympus IX73 inverted microscope or Nikon A1R Confocal. Images were analysed using Image J software (version 2.0.0).

Table 1: Antibody concentrations for fluorescence and confocal microscopy

Mode	Primary antibody		Secondary antibody	
	Antibody	Manufacturer: catalog #	Antibody	Manufacturer: catalog #
F	1:100 Rb pAb to VE-cadherin	Abcam: ab33168	1:50 Goat anti-Rb Alexa Fluor 568	Invitrogen: A11011
	1:500 Rb pAb to PECAM	Abcam: ab3245		
C	1:50 Rb pAb to VE-cadherin	Abcam: ab33168		
	1:100 Rb pAb to PECAM	Abcam: ab3245		

Abbreviations: C=confocal microscopy, F=fluorescence microscopy, pAb=polyclonal antibody, PECAM=platelet and endothelial cell adhesion molecule, Rb=rabbit, VE-cadherin=vascular endothelial cadherin

Blood perfusion testing

30 mL whole blood was collected from 18 donors into sodium citrate tubes (Greiner Bio-One: 454334) using a 21G vacutainer blood collection set (BD medical: 367286, 364815) on day of perfusion experiment. Haematocrit was determined using Beckman Coulter Ac.T Diff hematology analyzer. Whole blood was diluted 1:1 with PBS and perfused through the circuit for 4 h at 16 mL/min at 37°C with 5% CO_2. For static test conditions, the diluted whole blood remained in the circuit for 4 h with the pump set at 0mL/min. The perfusant was harvested from the circuits into polyethylene conical bottom centrifuge tubes for outcomes testing.

Microparticle measurement

Microparticles were concentrated from circuit perfusant using sequential centrifugation steps. The perfusant was initially centrifuged at 1550 xG for 20 mins at room temperature to pellet cells. The supernatant was centrifuged twice at 17000 xG for 30 mins at room temperature to pellet microparticles. The supernatant was aliquoted for cytokine analyses and replaced with 0.2 μm filtered PBS before repeat centrifugation at 17000 xG for 30 mins at room temperature. The microparticle pellet was then resuspended in 75 μL filtered PBS. Nanoparticle tracking analysis (NTA) with NanoSight NS300 (Malvern) was used to measure microparticle concentration in 50 μL of 0.45 μm filtered microparticle suspension. Samples were diluted with PBS to achieve final concentrations of 1 x 10^8 particles/mL to 1 x 10^9 particles/mL prior to analysis. The NanoSight was calibrated to record 10x 1 min videos which were analysed to calculate mean, median and mode particle size, along with particle concentration. All samples were analysed in duplicate.

Neutrophil activation assay

Aggregate microparticle suspensions were formed by combining microparticles from all donor circuits for each treatment (static vs. flow). Whole blood was collected from 9 donors on day of experiment into Ethylenediaminetetraacetic acid (EDTA, BD Medical 367525) tubes. Neutrophils were isolated using EasySep™ Direct Human Neutrophil Isolation kit (Stemcell Technologies: 19666) and EasySepTM Magnet (Stemcell Technologies: 18000). 200,000 neutrophils, 50 μL microparticle suspension and PBS to final stimulation volume of 300μl was coincubated for 1 h at 37°C in an orbital shaker set at 120 rpm. Stimulation with Phorbol myristate acetate (PMA, final concentration: 100 ng/mL; Sigma Aldrich: P1585) for 30 mins at 37 °C was used for positive control and PBS was used for negative control. 150 μL stimulation mixture was stained with 20 μL PE-CD63 (BD Biosciences: 556020), 5 μL FITC-CD11b (BD Biosciences: 562793), 5 μL PerCP Cy5.5-CD66b (BD Biosciences: 562264), 20 μL APC-CD13 (BD Biosciences: 557454) and 5 μL APC H7-CD16 (BD Biosciences: 560195) for 15 mins at room temperature. Isotype antibodies were used to assess for non-specific binding (PE-IgG1κ, BD Biosciences: 555749; FITC-IgG1κ, BD Biosciences: 554679; PerCP Cy5.5-

IgM, BD Biosciences: 560857; APC IgG1ϰ, BD Biosciences: 555751). Neutrophils were then washed with 3% (v/v) fetal calf serum (FCS, Gibco: 10437028) in PBS, centrifuged for 1000 xG for 2 mins and resuspended in 100 μL 1% (v/v) stabilising fixative (BD Biosciences: 338036) in PBS. Mean fluorescence intensity (MFI) was recorded for each fluorophore using BD FACs Canto II. Compensation beads were used to measure spectral overlap. In BD FACsDiva, neutrophils were gated based on CD16 positivity.

Results

HUVECs grown in model under laminar flow express endothelial markers

HUVECs grown under laminar flow in this model express VE-cadherin and PECAM on cell membranes (Figure 3). Due to the optical density of the model, confocal microscopy generated superior images compared to fluorescence microscopy.

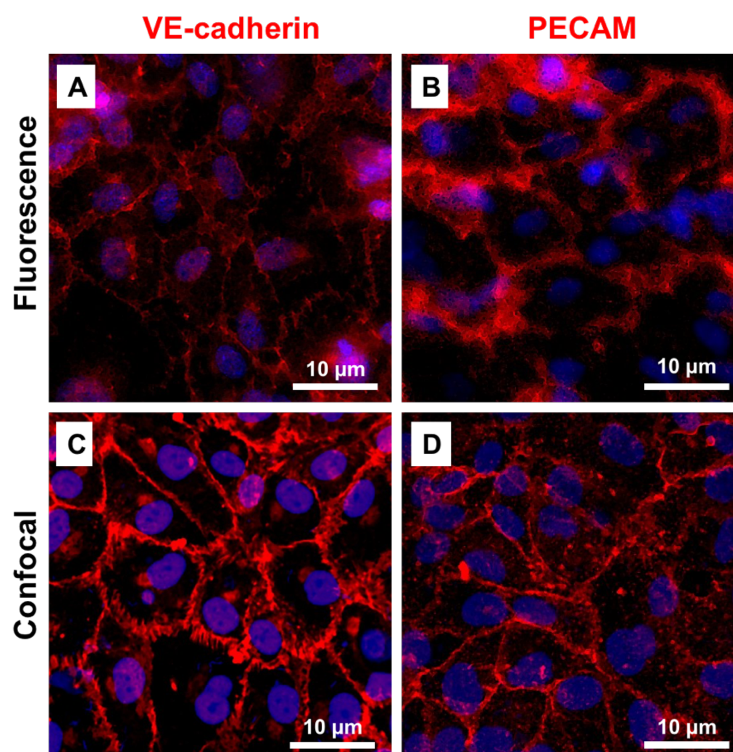

Figure 3: Fluorescence (A, B) and confocal (C, D) microscopy of HUVECs in vascular model cultured under laminar flow conditions for endothelial adhesion markers (40X magnification). Cells were counterstained with DAPI (blue).

Whole blood perfusion in model led to increased pro-inflammatory cytokine formation

Laminar flow led to increased microparticle formation compared to static condition (2.13 x 1010 vs. 6.08 x 109 microparticles/mL, p<0.0001). Microparticles generated from laminar flow led to increased neutrophil CD66b (p=0.03), CD13 (p=0.05) and CD63 (p<0.0001) expression (Figure 4) – markers of specific, secretory and azurophil granule release respectively. There was trend towards enhanced neutrophil CD11b (p=0.32) expression after flow microparticle co-incubation.

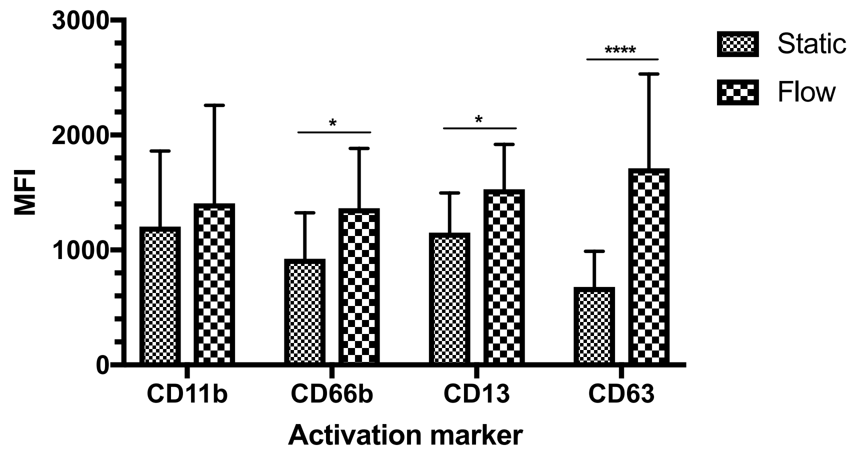

Figure 4: Neutrophil activation marker expression after 1 hour co-incubation with microparticles from flow and static condition circuits (mean ± SEM). 9 neutrophil donors were used. MFI=mean fluorescence intensity *p<0.05, ****p<0.0001