11-13 SEPT. 2024
LILLE GRAND PALAIS
CONGRÈS FRANÇAIS D'HÉMOSTASE
SFTH SOCIÉTÉ FRANÇAISE DE THROMBOSE ET D'HÉMOSTASE

Masterclass #3

Modèles d'étude de la thromboinflammation veineuse en flux

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INSERM U1144 Faculté de Pharmacie de Paris

Optimisation Thérapeutique en Neuropsychopharmacologie
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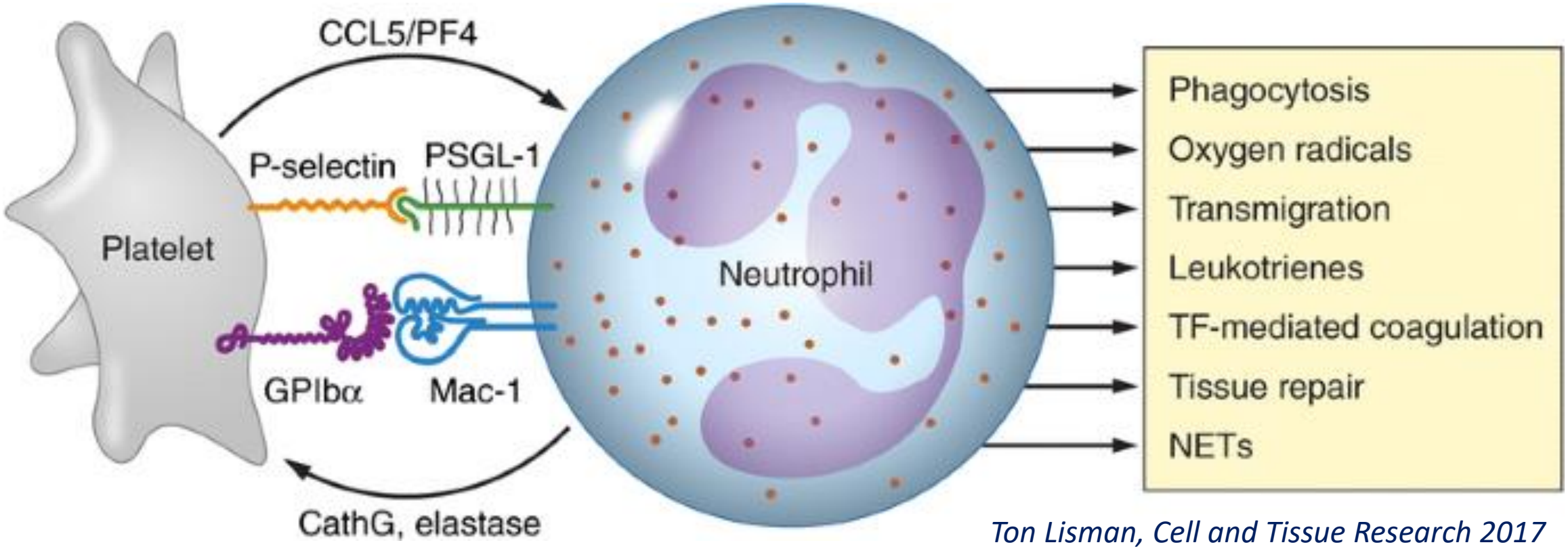
Université Paris Cité

The concept of thromboinflammation



Ton Lisman, Cell and Tissue Research 2017

Mutual interactions and reciprocal activation between the actors of thrombosis (platelets/coagulation) and immune effectors

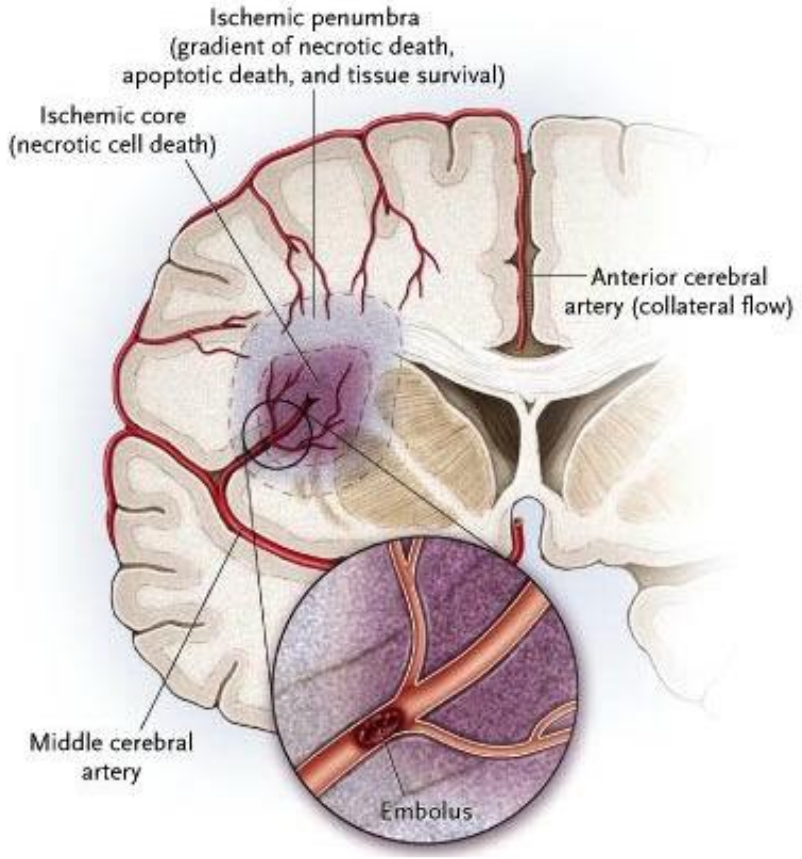
activated endothelial cells

Microvascular thromboinflammation at the (hyper)acute phase of ischemic stroke

The thromboinflammatory response to cerebral ischemia-reperfusion

Microvascular thromboinflammation at the (hyper)acute phase of ischemic stroke

> 80% of strokes are ischemic =
caused by the occlusion of an intracranial artery resulting in a profound **reduction in brain perfusion**



Microvascular inflammation at the acute phase of ischemic stroke

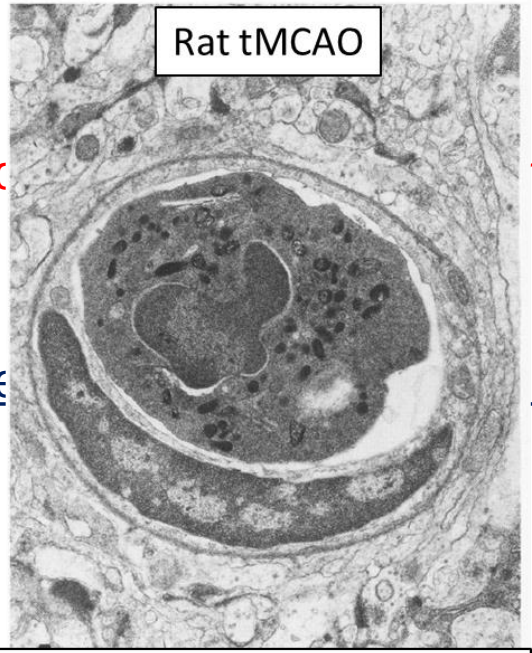
Gregory J. Del Zoppo (early 9O's – now):

- Focal ischemia is associated with obstruction of downstream microvessels after recanalization of occluded proximal arteries

- Neutrophils are present in the cerebral microvasculature during reperfusion after transient middle cerebral artery occlusion (tMCAO) in baboons: neutrophils can obstruct capillaries and post-capillary venules at sites where microvascular patency was impaired

- Administration of blocking antibodies to neutr n molecules reduce the extent of the injury after ischemia reperfusion

- Downstream microvascular obstructions by ne contribute to incomplete reperfusion and reperfusion injury in acute ischemic stroke



Garcia JH *et al., Am J Pathol 1994*

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Microvascular thrombosis at the acute phase of ischemic stroke

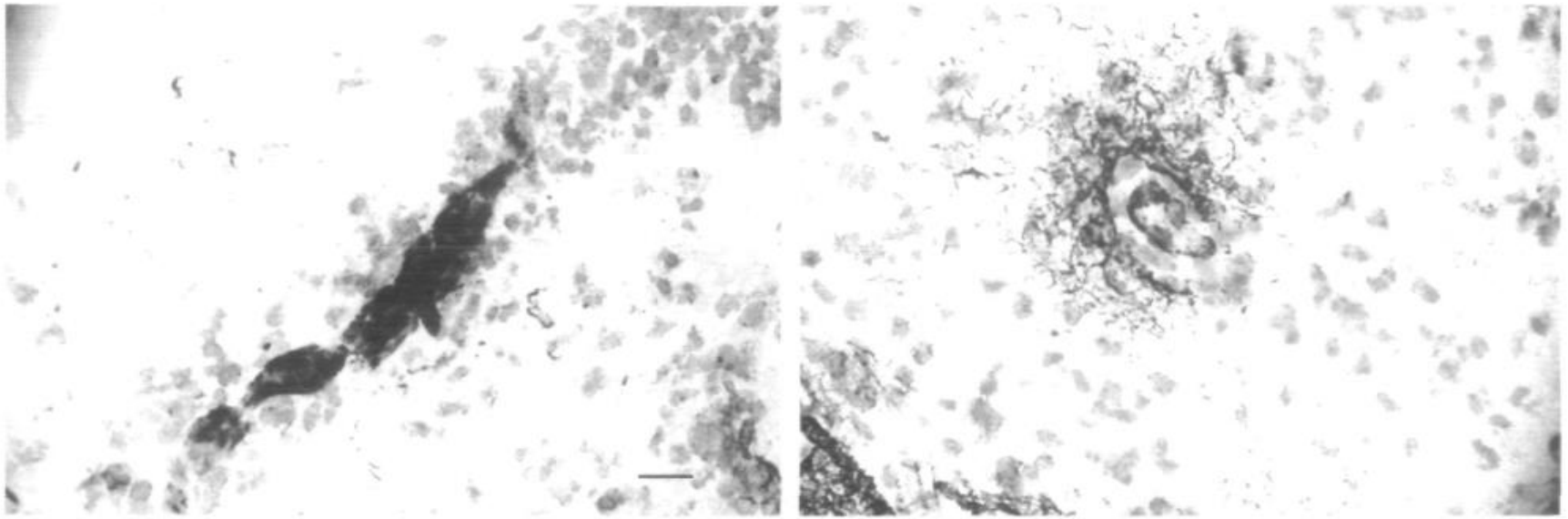
Gregory J. Del Zoppo (early 9O's – now):

- Focal ischemia is associated with obstruction of downstream microvessels after recanalization of occluded proximal arteries
- Focal ischemia is associated with fibrin(ogen) deposits in downstream microvessels beginning prior to arterial recanalization
- Administration of a blocking antibody to tissue factor reduces the extent of fibrin deposits
- Downstream microvascular occlusions by fibrin contribute to incomplete reperfusion and reperfusion injury in acute ischemic stroke

Microvascular thrombosis at the acute phase of ischemic stroke

Fibrin Contributes to Microvascular Obstructions and Parenchymal Changes During Early Focal Cerebral Ischemia and Reperfusion

Yasushi Okada, MD; Brian R. Copeland, MD; Robert Fitridge, MD;
James A. Koziol, PhD; Gregory J. del Zoppo, MD



Stroke 1994

Like neutrophils, fibrin contributes to microvascular obstructions in response to cerebral ischemia-reperfusion induced by transient occlusion of the MCA

Microvascular thrombosis at the acute phase of ischemic stroke

TABLE 1. Fraction of CD31-Positive Microvessels Associated With Fibrin (Fibrin/CD31)

			Control (−MoAb)		Fibrin	
Cohort	**Duration, h**	**n**	**Non-I/R**	**Post-I/R**	**Non-I/R**	**Post-I/R***
Control		3	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
MCA:O	2	3	0.000±0.000	0.000±0.000	0.000±0.000	0.048±0.041
MCA:O/R	1	3	0.000±0.000	0.000±0.000	0.000±0.000	0.064±0.064
	4	3	0.000±0.000	0.000±0.000	0.000±0.000	0.101±0.118
	24	4	0.000±0.000	0.000±0.000	0.000±0.000	0.143±0.072†
+Anti-TF	1	3	0.000±0.000	0.000±0.000	0.000±0.000	0.020±0.088

MoAb indicates monoclonal antibody; Non-I/R, nonischemic zone; Post-I/R, ischemia/reperfusion zone; MCA:O, middle cerebral artery occlusion only; MCA:O/R, 3-hour middle cerebral artery occlusion with indicated periods of reperfusion; Anti-TF, anti–tissue factor antibody, TF9-6B4; and Control (−MoAb), deletion of primary antibody (identical data were obtained with the irrelevant antibody TIB-115).

Values are mean±SD (per 1000 fields).

*Difference among the cohorts with post-I/R, $F_{4,26}=3.80$, $P<.05$.

†$P<.05$ vs control, using Tukey's multiple comparison method.

Microvascular fibrin deposits starts **before** arterial recanalization of the MCA

Like neutrophils, fibrin contributes to microvascular obstructions in response to cerebral **ischemia**-reperfusion induced by transient occlusion of the MCA

Microvascular thrombosis at the acute phase of ischemic stroke

TABLE 1. Fraction of CD31-Positive Microvessels Associated With Fibrin (Fibrin/CD31)

			Control (−MoAb)		Fibrin	
Cohort	Duration, h	n	Non-I/R	Post-I/R	Non-I/R	Post-I/R*

Both inflammation and thrombosis are triggered by arterial occlusion in microvessels *possibly* in relation with blood perfusion reduction in the absence of local stenosis (laminar flow) or injury

antibody, TF9-6B4; and Control (−MoAb), deletion of primary antibody (identical data were obtained with the irrelevant antibody TIB-115).

Values are mean±SD (per 1000 fields).

*Difference among the cohorts with post-I/R, $F_{4,26}=3.80$, $P<.05$.

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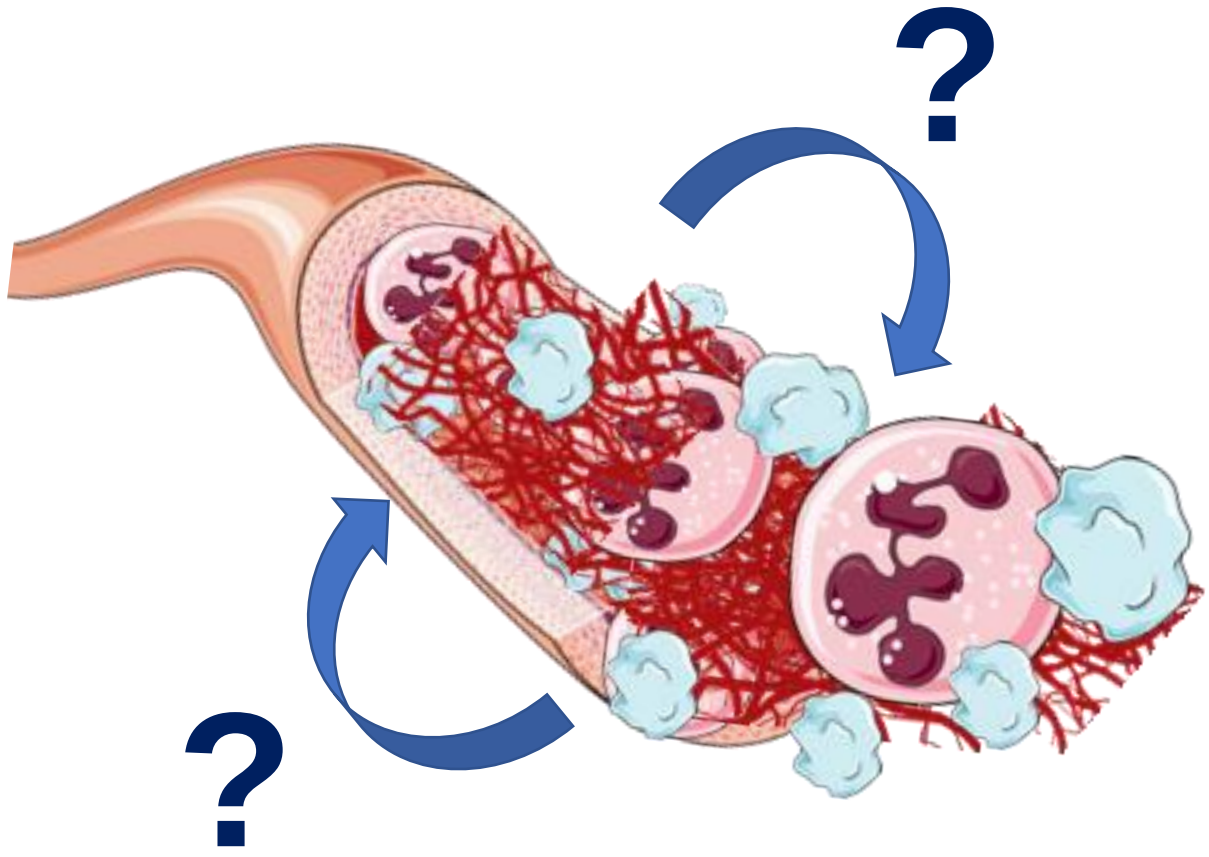
Microvascular fibrin deposits starts **before** arterial recanalization of the MCA

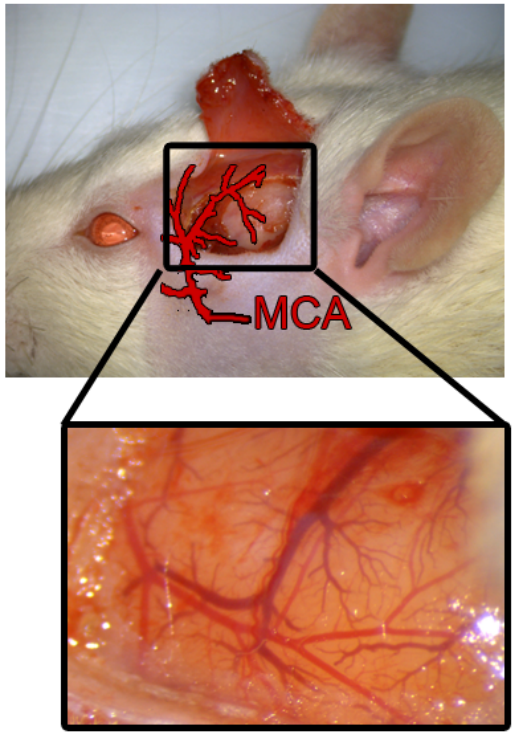
Like neutrophils, fibrin contributes to microvascular obstructions in response to cerebral **ischemia-**reperfusion induced by transient occlusion of the MCA

Interplay between microvascular inflammation and thrombosis at the acute phase of ischemic stroke? Relation to blood flow reduction?



Dynamic intravital imaging of the microvascular response to cerebral ischemia-reperfusion

Dura mera-sparing craniotomy in the fronto-parietal lobe region of rats and mice before induction of tMCAO

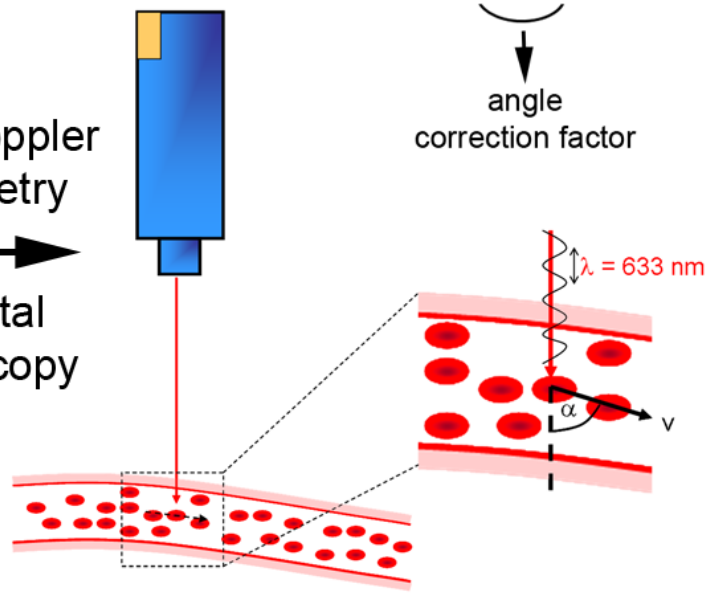


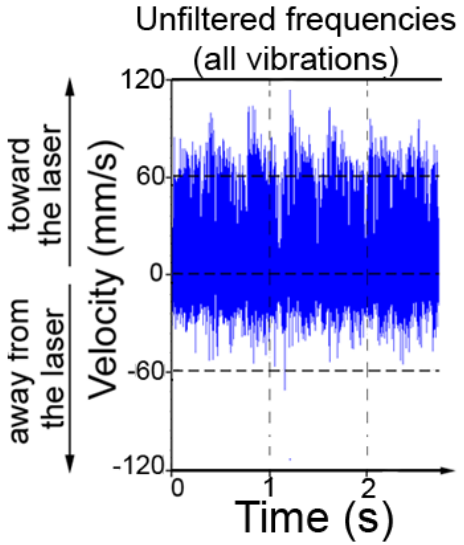
Laser doppler vibrometry

Intravital microscopy

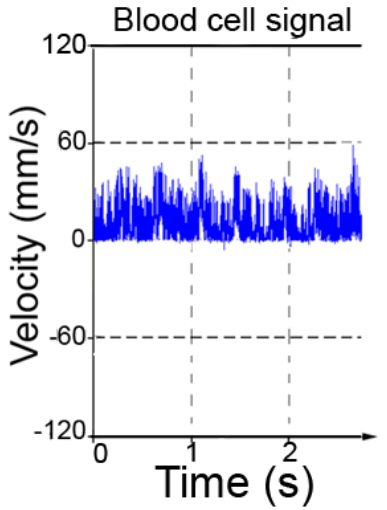
$$v = \frac{(\Delta f \times \lambda)}{2} \times \frac{1}{\cos\alpha}$$

angle correction factor



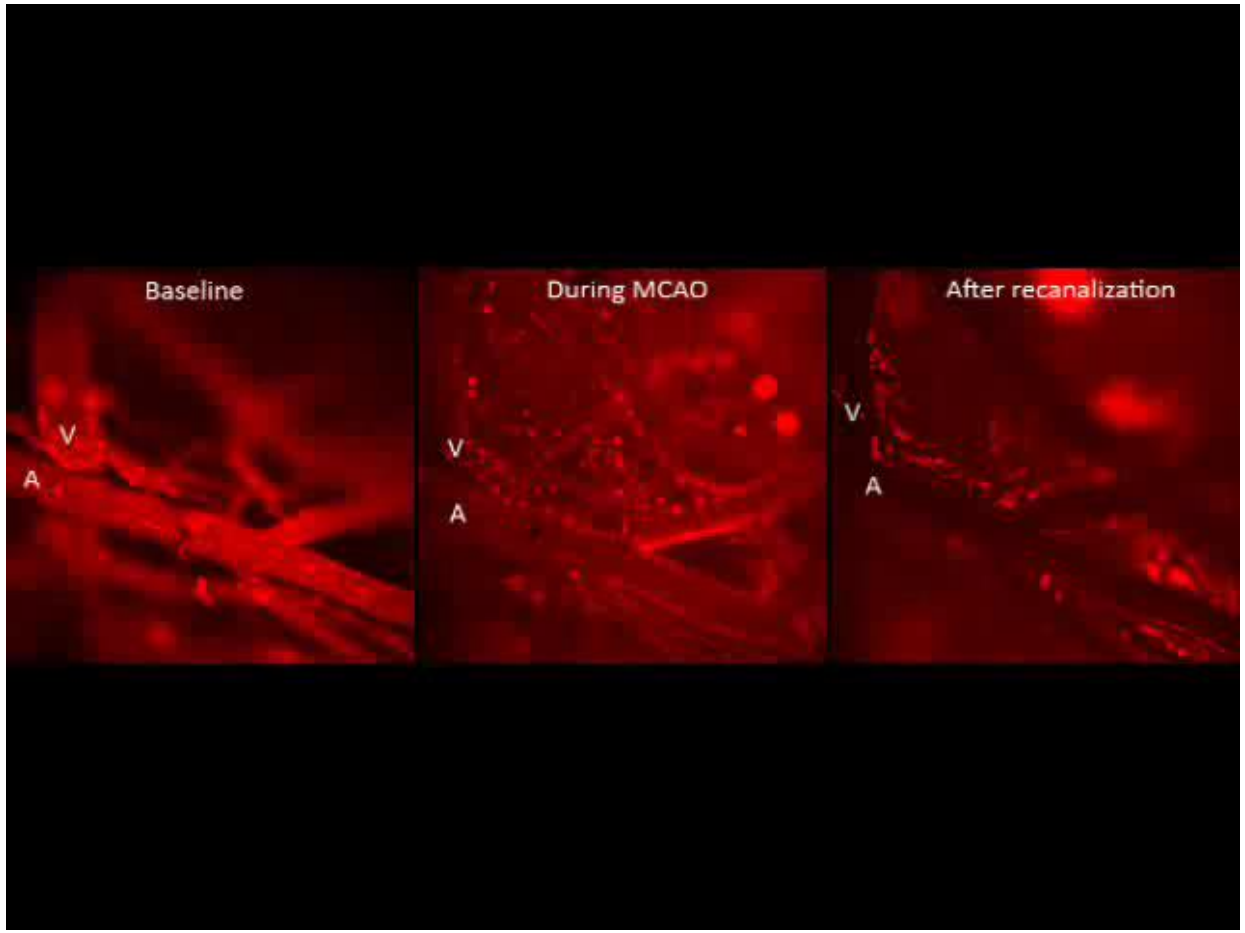


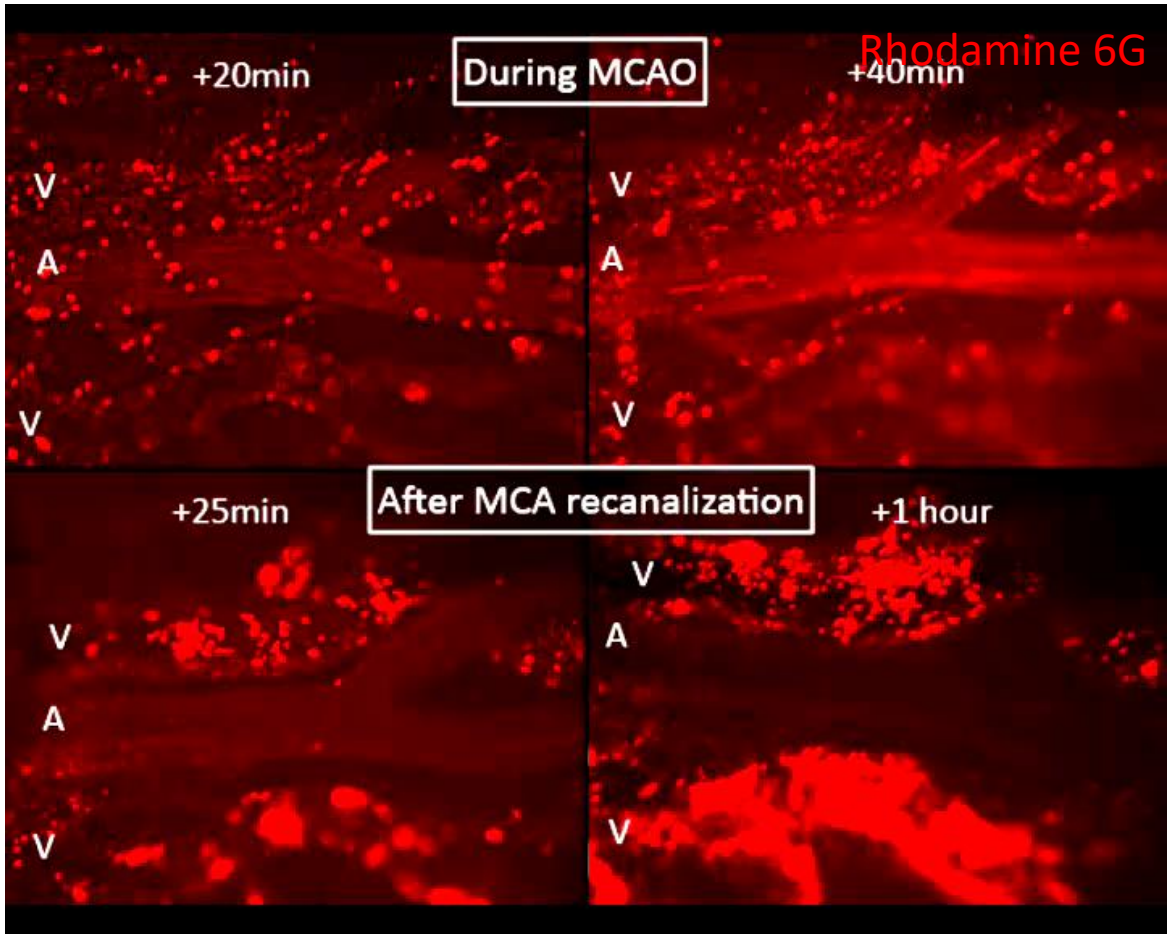
2 kHz lowpass filter



Desilles JP et al, JAHA 2018.

MCA occlusion triggers leukocyte margination in downstream microvessels where blood perfusion has dropped



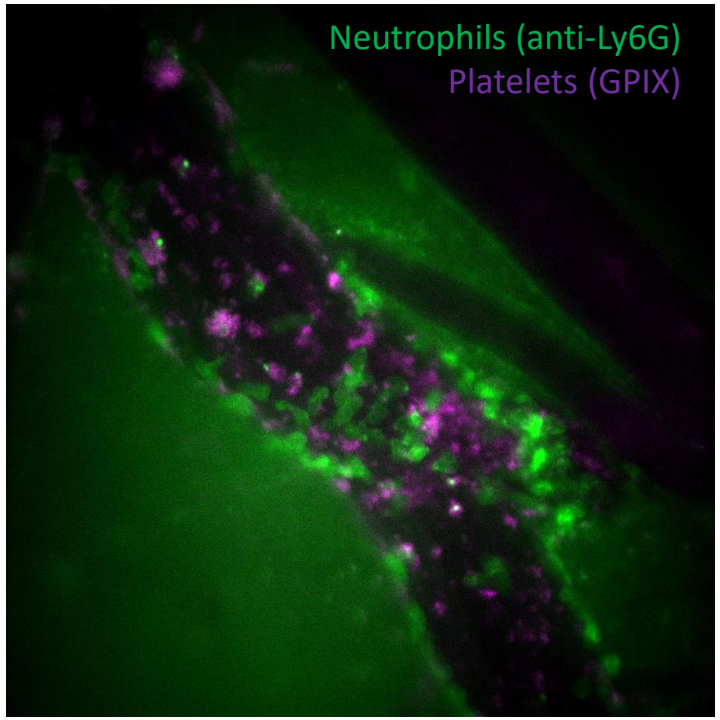


Desilles JP et al, JAHA 2018.

Occlusion of the MCA induces an immediate downstream microvascular response that continues evolving despite MCA recanalization: leukocyte margination and transmigration **mostly from post-capillary venules**

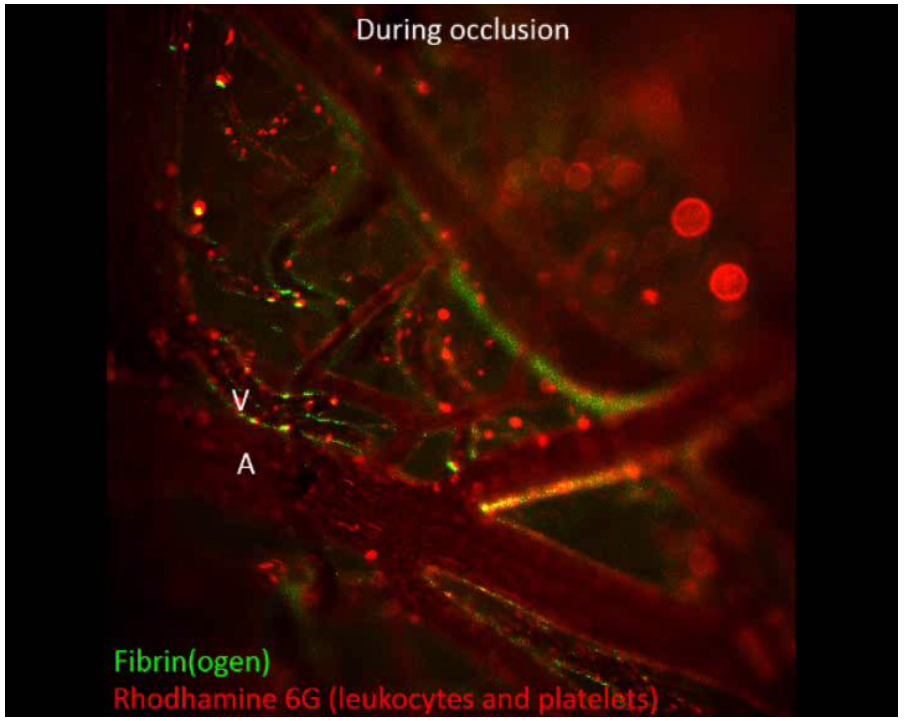
Marginating neutrophils in venules interact closely with platelets and fibrin(ogen)

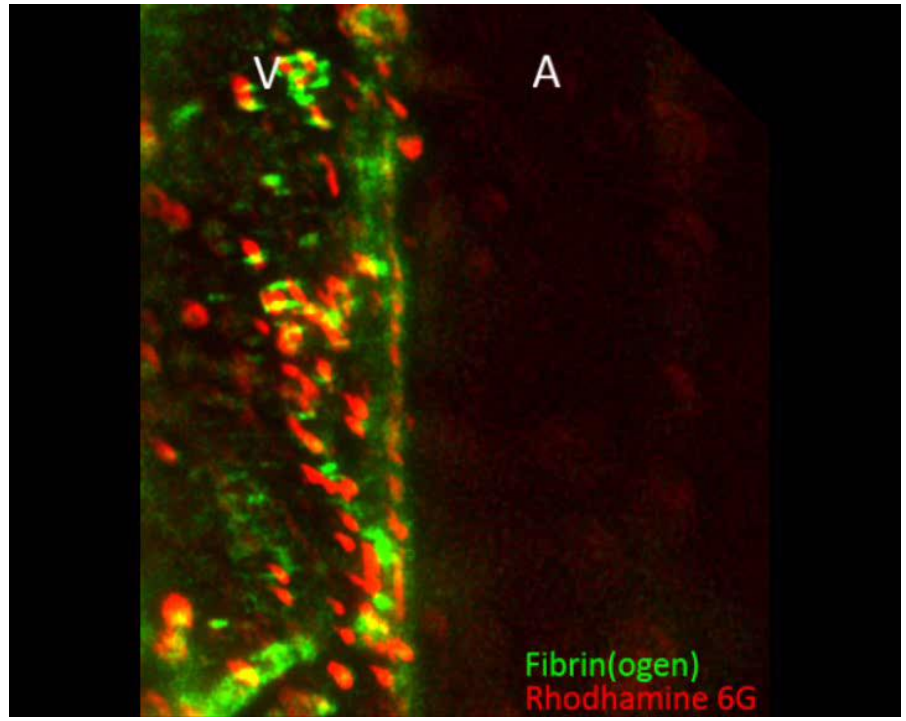
Mouse MCAO



Unpublished data

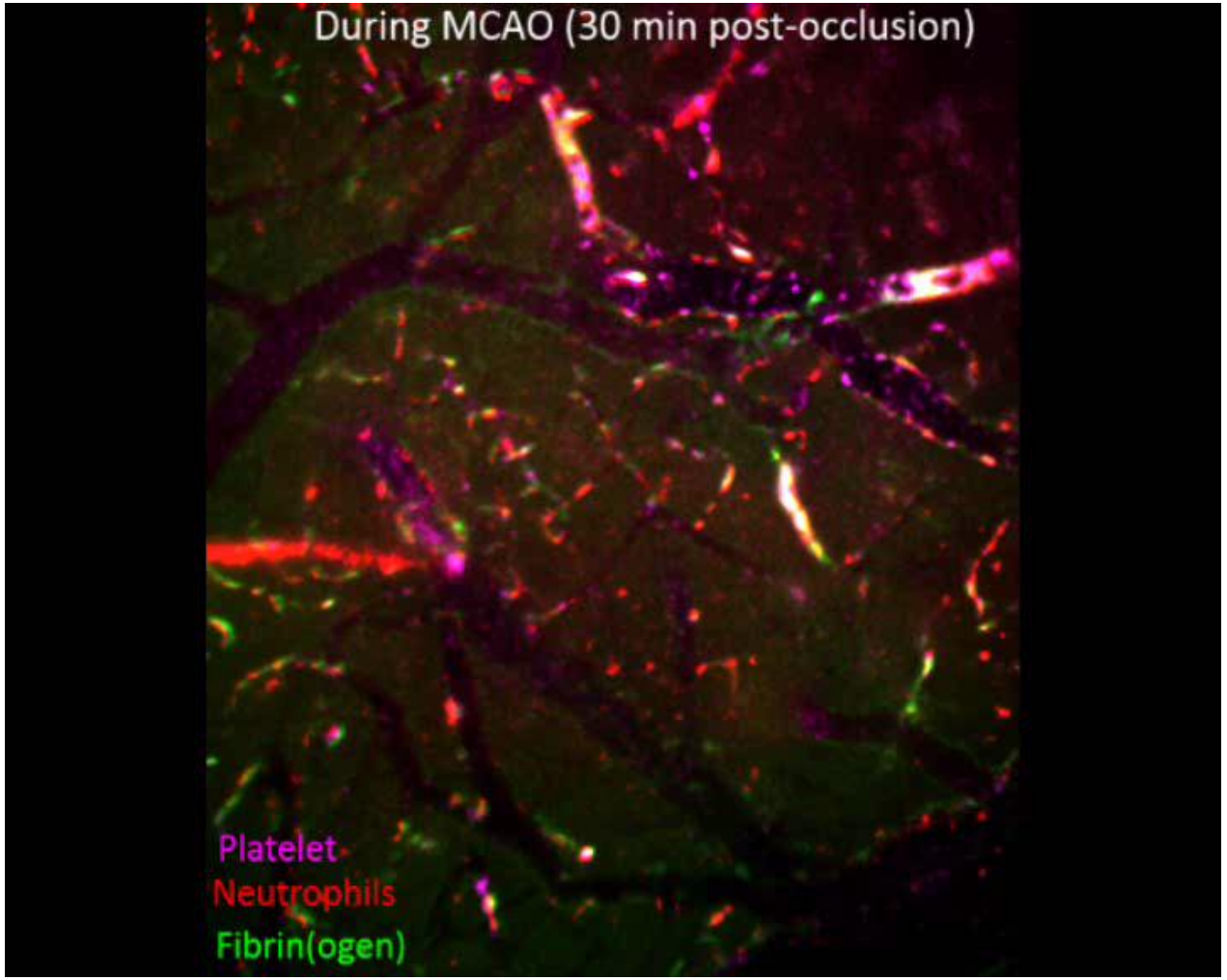
Rat MCAO





Desilles JP et al, JAHA 2018.

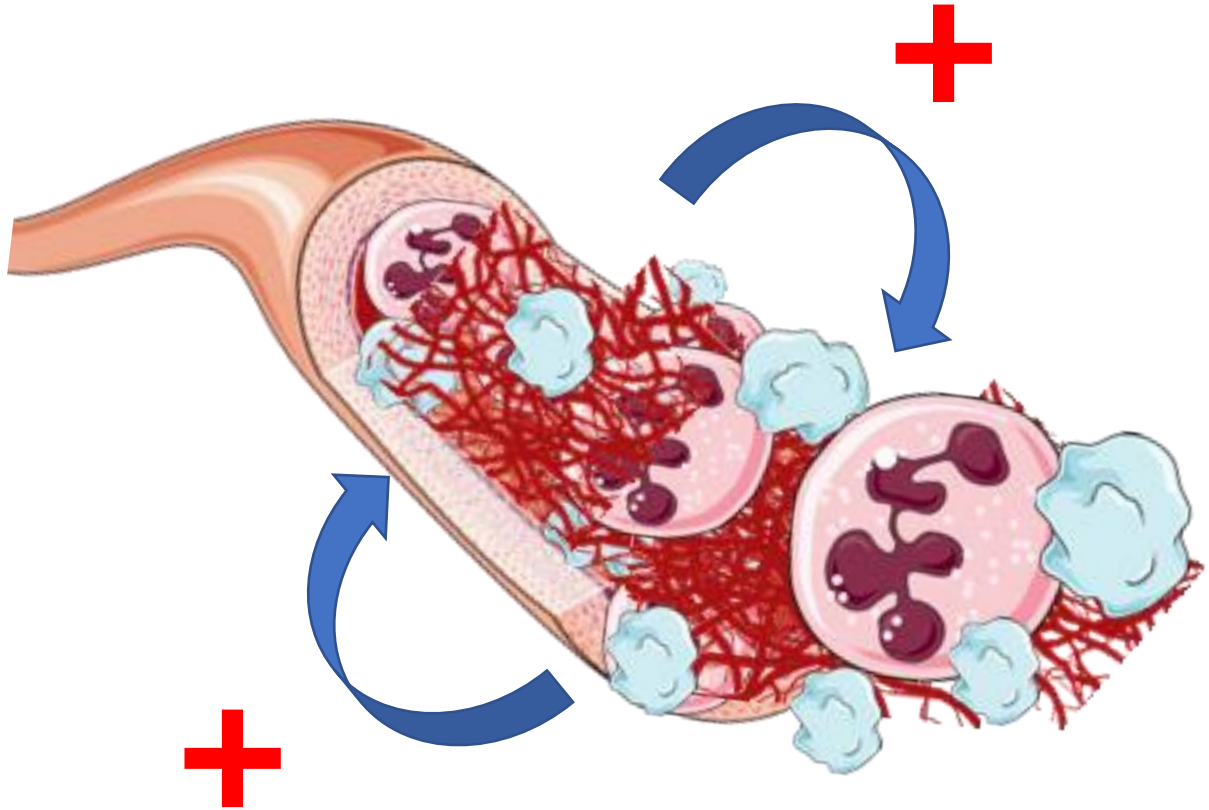
Neutrophil margination and interactions with platelets and coagulation in venules lead to microthrombis



Desilles JP et al, JAHA 2018.

Platelet and neutrophil accumulation leads to obstruction of capillaries and post-capillary venules

The interplay between microvascular inflammation and thrombosis at the acute phase of ischemic stroke leads to secondary microthrombosis and impairs reperfusion



In vitro modelization of venous thromboinflammation

4 main parameters to define

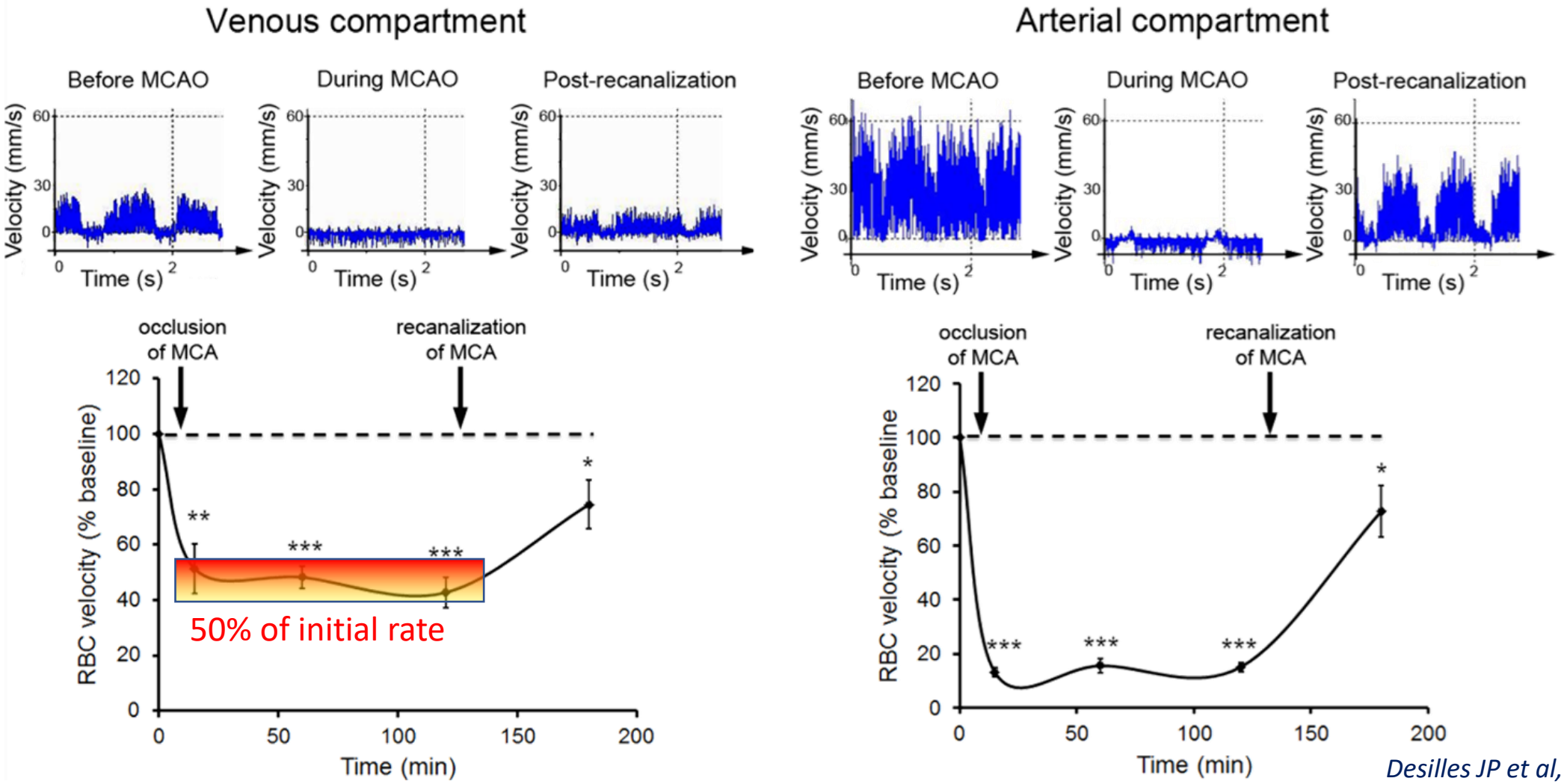
Type of flow

Adhesion substrates

Perfusate

Perfusion duration

Impact of MCA occlusion on downstream microvessel blood flow



Desilles JP et al, JAHA 2018.

In vitro modelization of venous thromboinflammation

4 main parameters to define

Type of flow: Laminar, venular, absence of stenosis but drop in RBC velocity

Adhesion substrates

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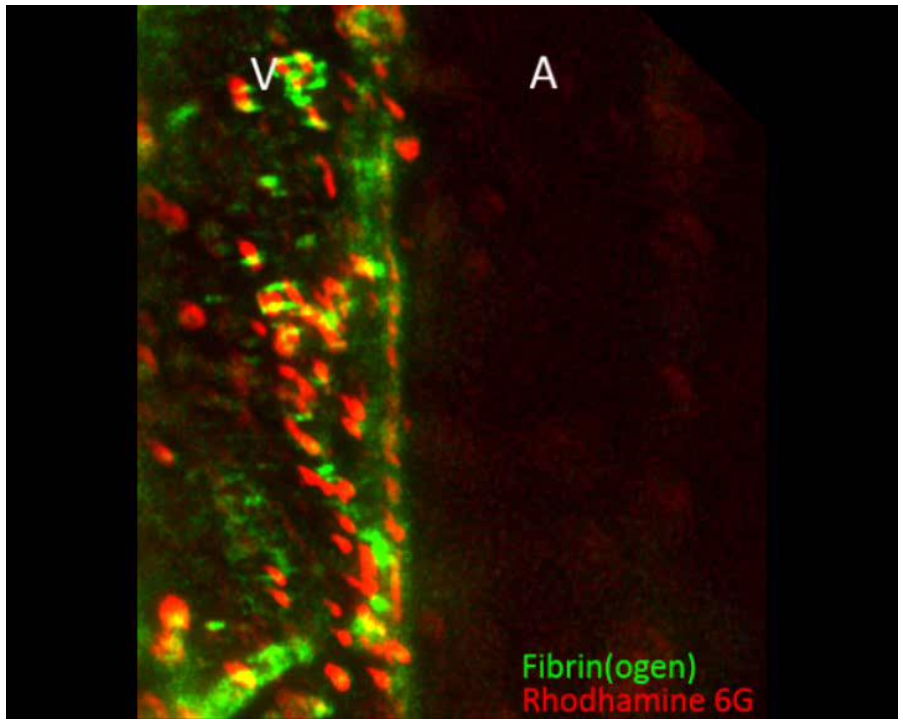

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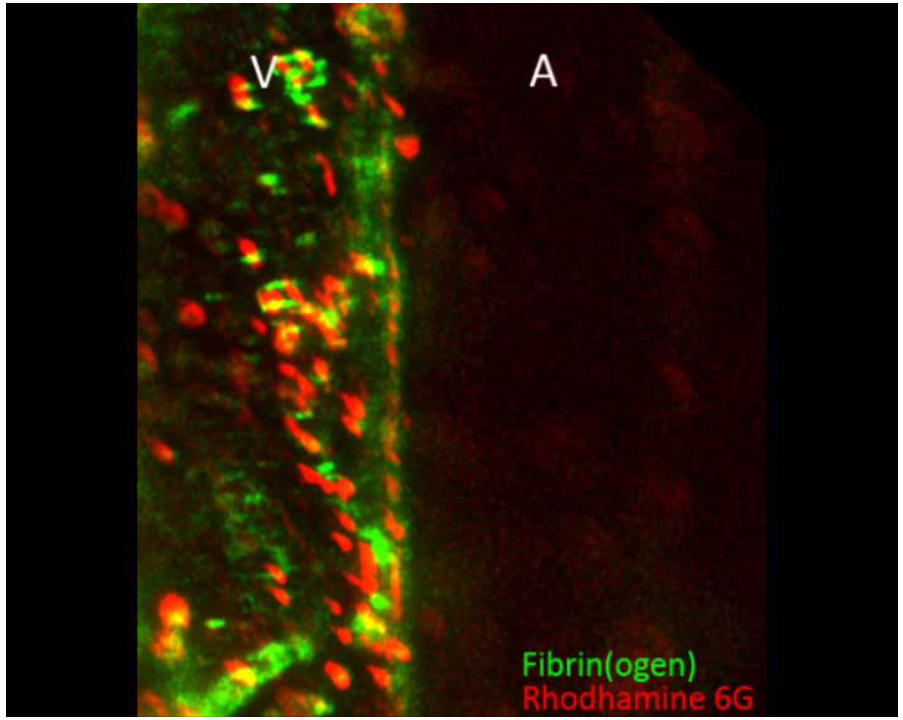

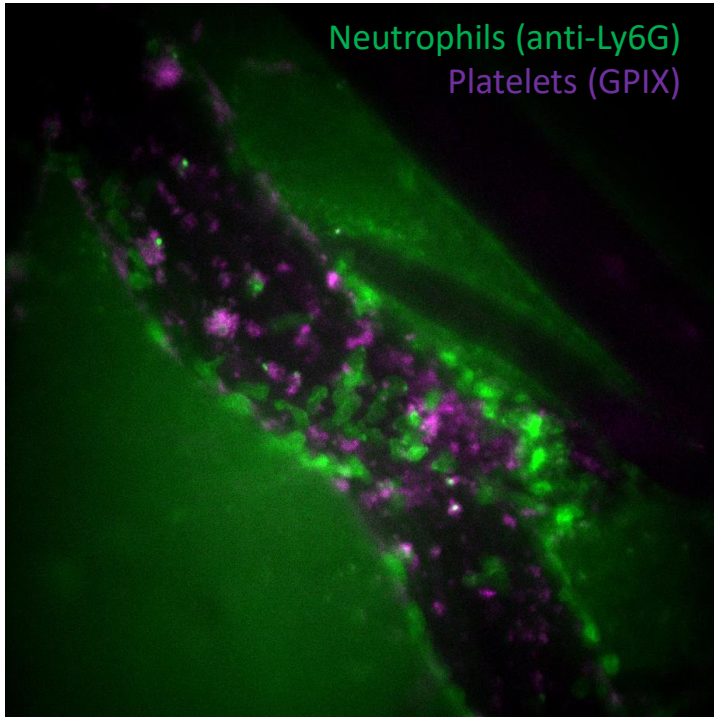

In vitro modelization of venous thromboinflammation

4 main parameters to define

Type of flow: Laminar, venular, absence of stenosis but drop in RBC velocity

Adhesion substrates: Fibrin(ogen) deposits + activated endothelial cells

Perfusate: Whole blood to have all actors i.e. leukocytes, platelets and coagulation

Perfusion duration

In vitro modelization of venous thromboinflammation

4 main parameters to define

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Perfusate: Whole blood to have all actors i.e. leukocytes, platelets and coagulation

Perfusion duration: thromboinflammation is a progressive process

In vitro modelization of venous thromboinflammation

4 main parameters to define

Type of flow: Laminar, venular, absence of stenosis but drop in RBC velocity

Adhesion substrates: Fibrin(ogen) deposits + activated endothelial cells

Perfusate: Whole blood to have all actors i.e. leukocytes, platelets and coagulation

Perfusion duration: thromboinflammation is a progressive process

Other parameters to consider

Type of blood: healthy donor vs patients

Introduction of risk factors (e.g. hyperglycemia)

Perfusion chambers for laminar flow

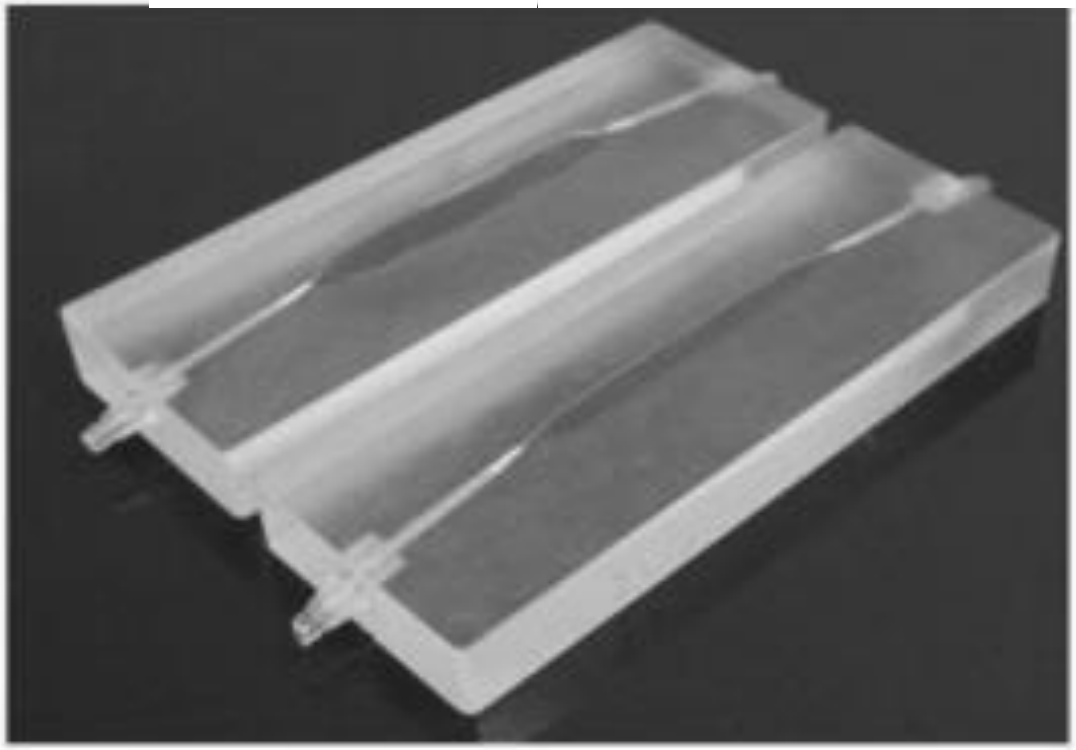
MAASTRICHT INSTRUMENTS

METHODS PAPER

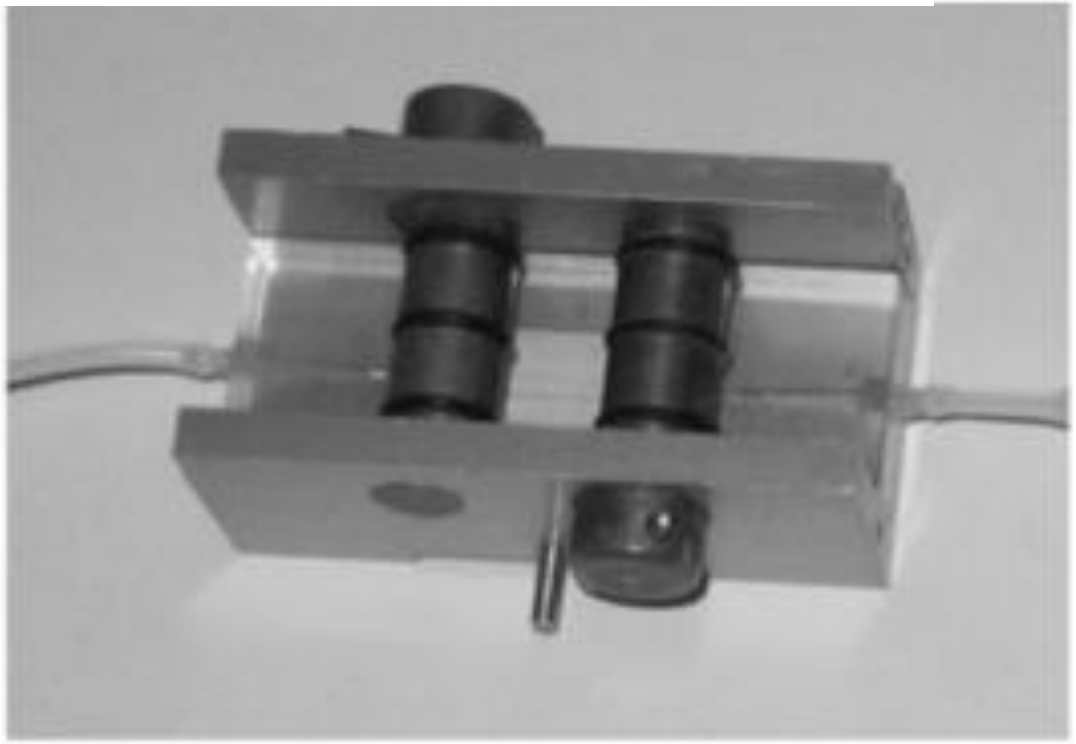
Measurement of whole blood thrombus formation using parallel-plate flow chambers – a practical guide

ROGER VAN KRUCHTEN, JUDITH M. E. M. COSEMANS, &
JOHAN W. M. HEEMSKERK

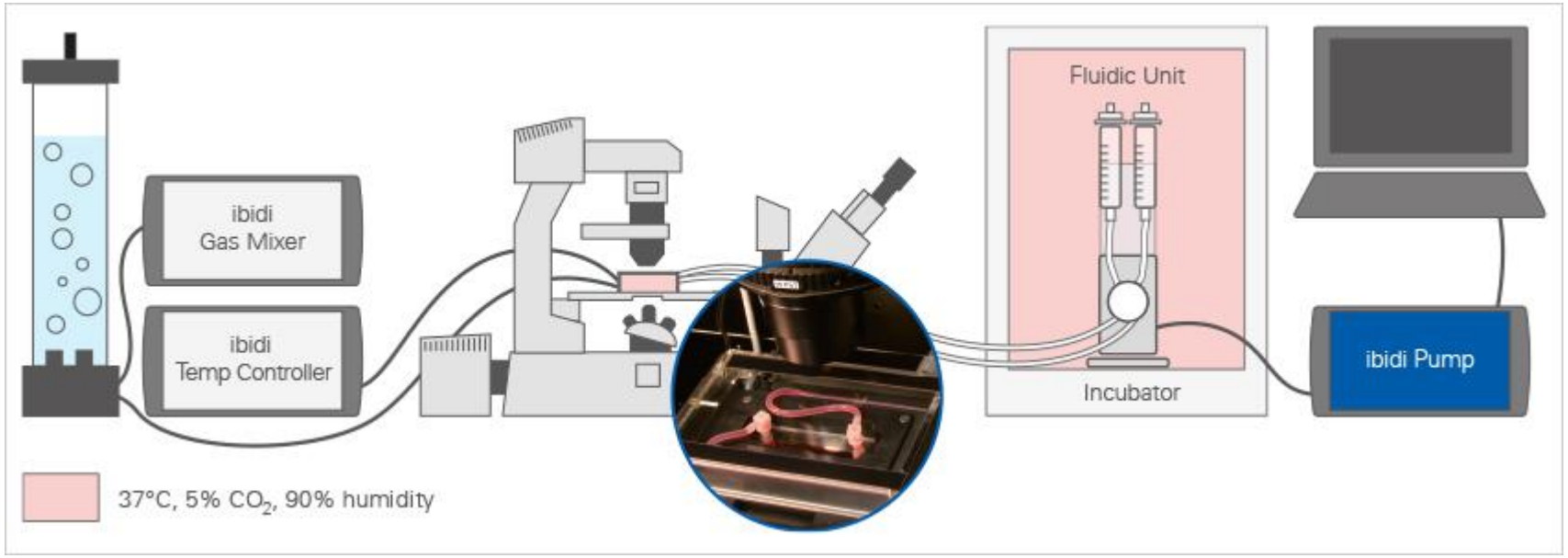
(a)

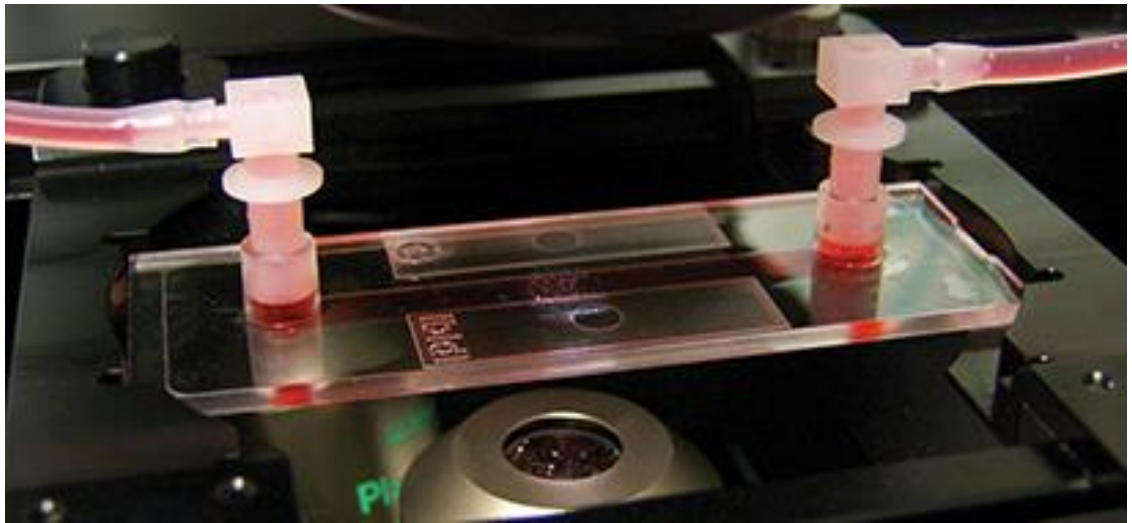


(b)

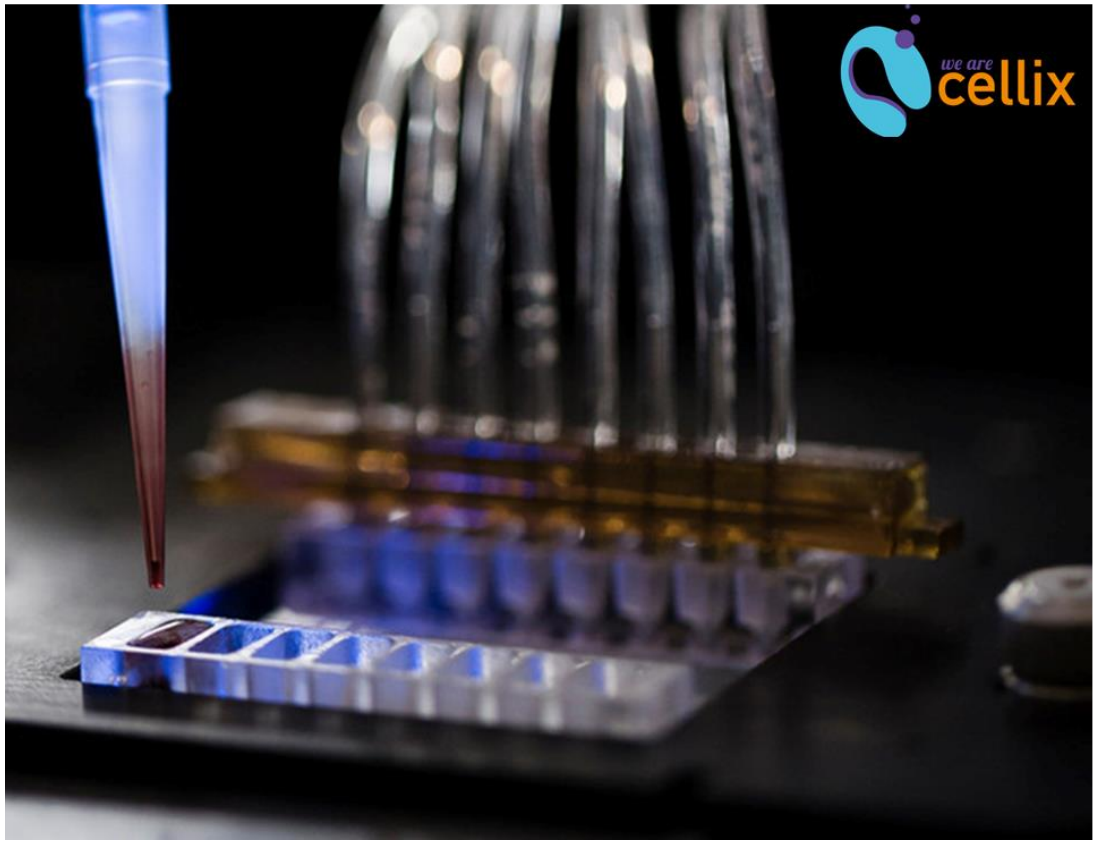


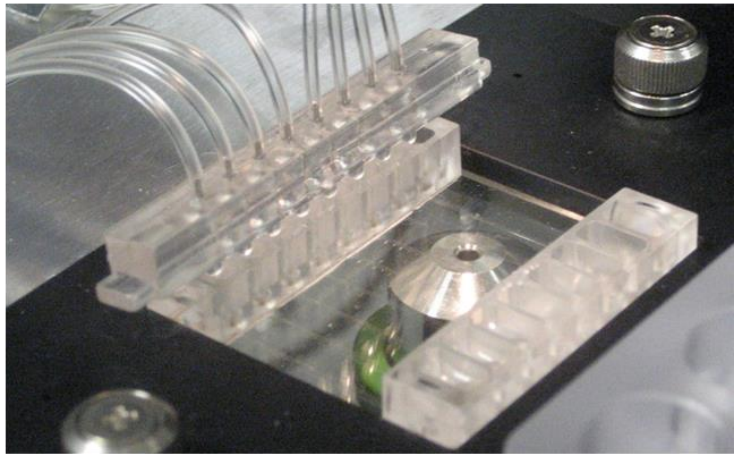
Perfusion chambers for laminar flow





Perfusion chambers for laminar flow

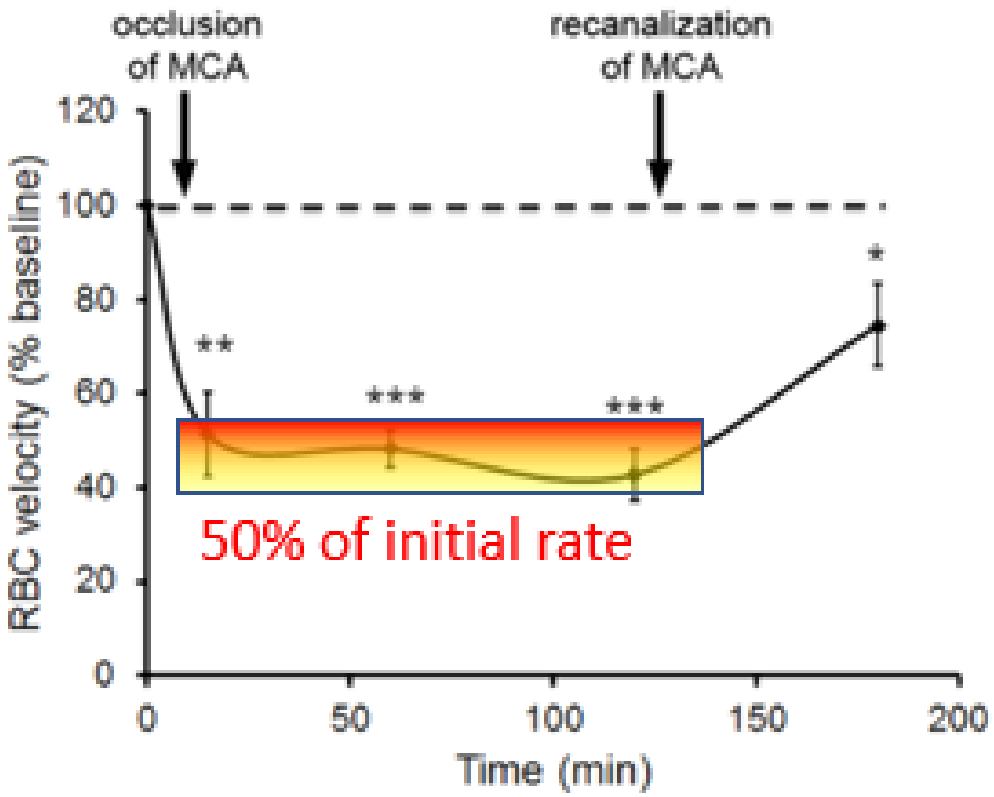


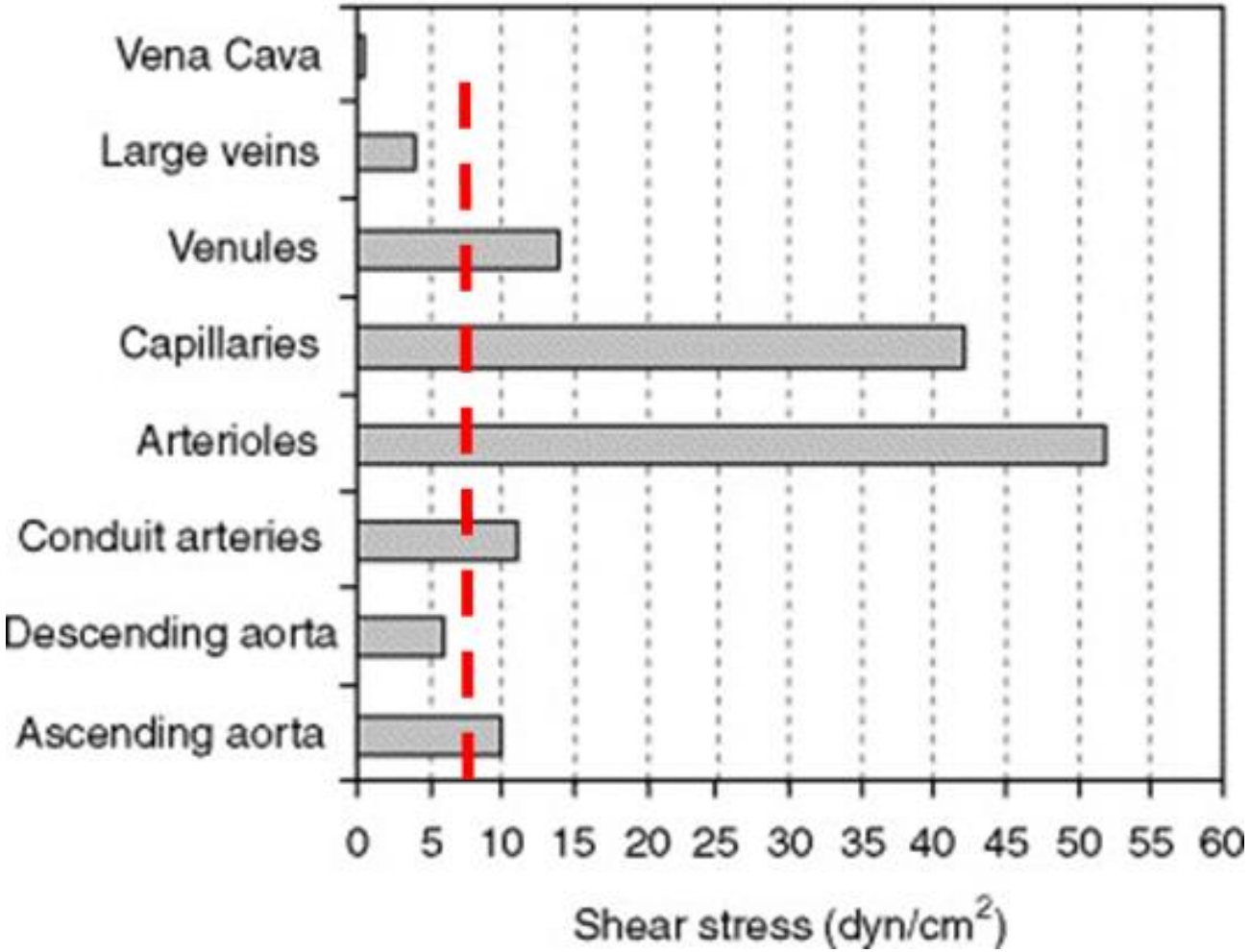


Vena 8 chambers

Exigo pump + Kima Pump for culture of endothelial cells

Defining flow rate and shear stress





International Journal of Cardiology Vol113, Issue 1, October 2006

Defining flow rate and shear stress

Target Shear Stress: 8 dyne/cm2

Shear Stress: $\tau = \frac{6Q\mu}{bh^2}$

Flow Rate: $Q = \frac{\tau bh^2}{6\mu}$

Equivalent to: $cm^3/s = 0.001 L/S = 0.06 L/min = 60mL/min = 60000\mu L/min$

Viscosity of cell culture suspension, $\mu = 0.01$ dynes/cm^2 · s

Viscosity of whole blood, $\mu = 0.045$ dynes/cm^2 · s

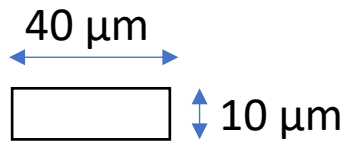
cellix

Specifications of Vena8 Fluoro+ and Vena8 Endothelial+ Biochips:

	Vena8/Vena8 Fluoro+	Vena8 Endothelial+
Channel width, b (cm):	0.04	0.08
Channel height, h (cm):	0.01	0.012
Channel length, L (cm):	2.8	2.8
Microcapillary/channel volume (cm^3)	0.00113	.00267
Microcapillary/channel volume (µL)	1.13	2.67

Shear rate: 200 sec-1

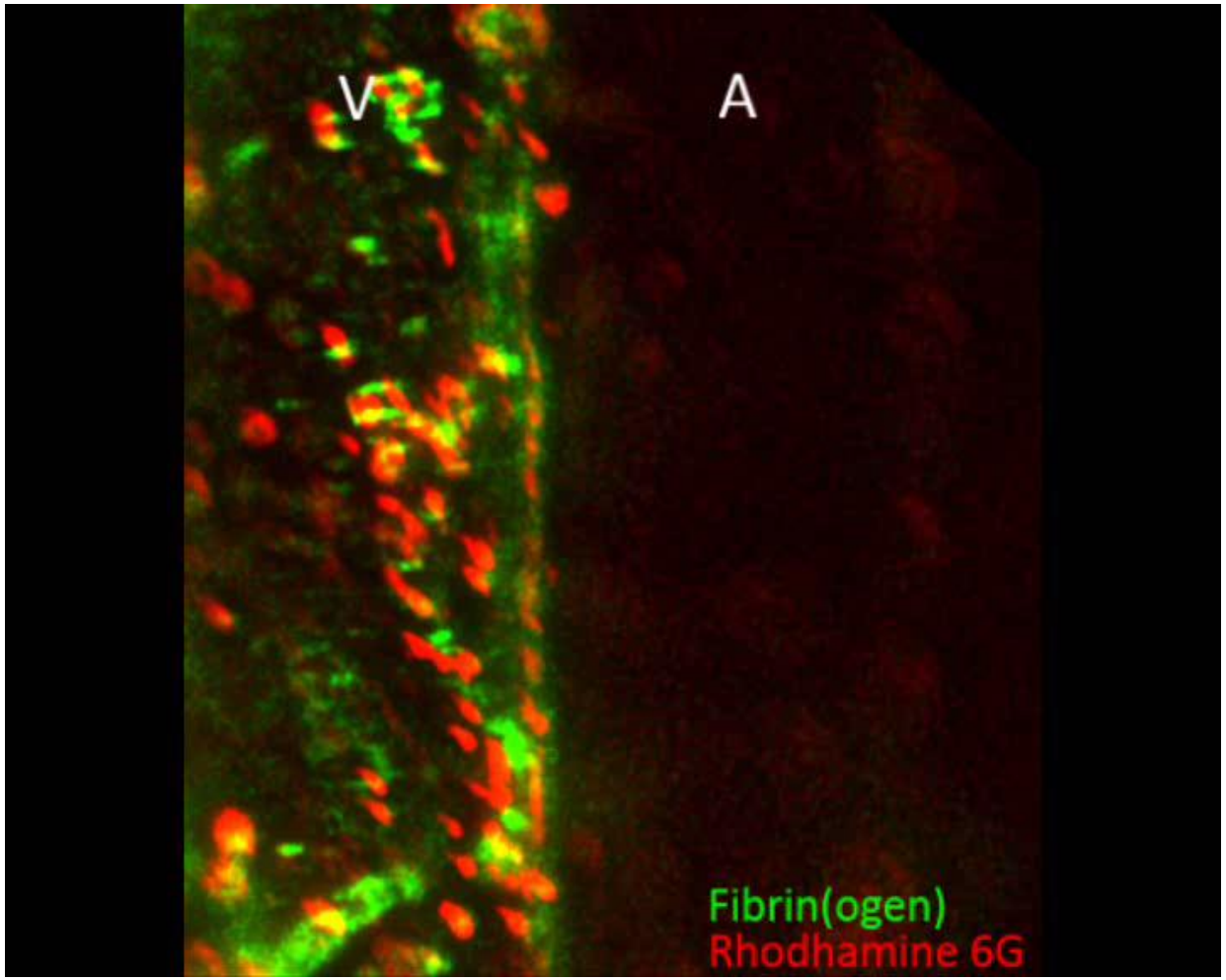
Flow rate: 8 µL/min



Defining flow rate and shear stress

Biochip			Vena8 Fluoro+ Biochip			Vena8 Endothelial+ Biochip		
Sample	Shear stress (dyne/cm^2)	Shear rate (s^{-1})	Flow rate (µL/min)	Flow rate (µL/hr)	Volume (µL) for 3 min experiment	Flow rate (µL/min)	Flow rate (µL/hr)	Volume (µL) for 3 min experiment
Cell suspension	0.5	50	2	120	6	6	259	17
Cell suspension	1	100	4	240	12	12	518	35
Cell suspension	5	500	20	1,200	60	58	2,592	173
Cell suspension	10	1,000	40	2,400	120	115	5,184	346
Cell suspension	15	1,500	60	3,600	180	173	7,776	518
Cell suspension	18	1,800	72	4,320	216	207	9,331	622
Cell suspension	20	2,000	80	4,800	240	130	10,368	691
Whole Blood	2.25	50	2	120	6	6	346	17
Whole Blood	4.5	100	4	240	12	12	691	35
Whole Blood	22.5	500	20	1,200	60	58	3,456	173
Whole Blood	50	1,111	44	2,667	133	128	7,680	384
Whole Blood	60	1,333	53	3,200	160	153	9,200	460
Whole Blood	67.5	1,500	60	3,600	180	173	10,368	518
Whole Blood	81	1,800	72	4,320	216	207	12,442	622
Whole Blood	90	2,000	80	4,800	240	230	13,824	691

Defining perfusion substrate



Fibrin or venous endothelial cells

Defining Perfusate

Thrombin and fibrin are major actors of thrombo-inflammatory reactions: Coagulation must be enabled

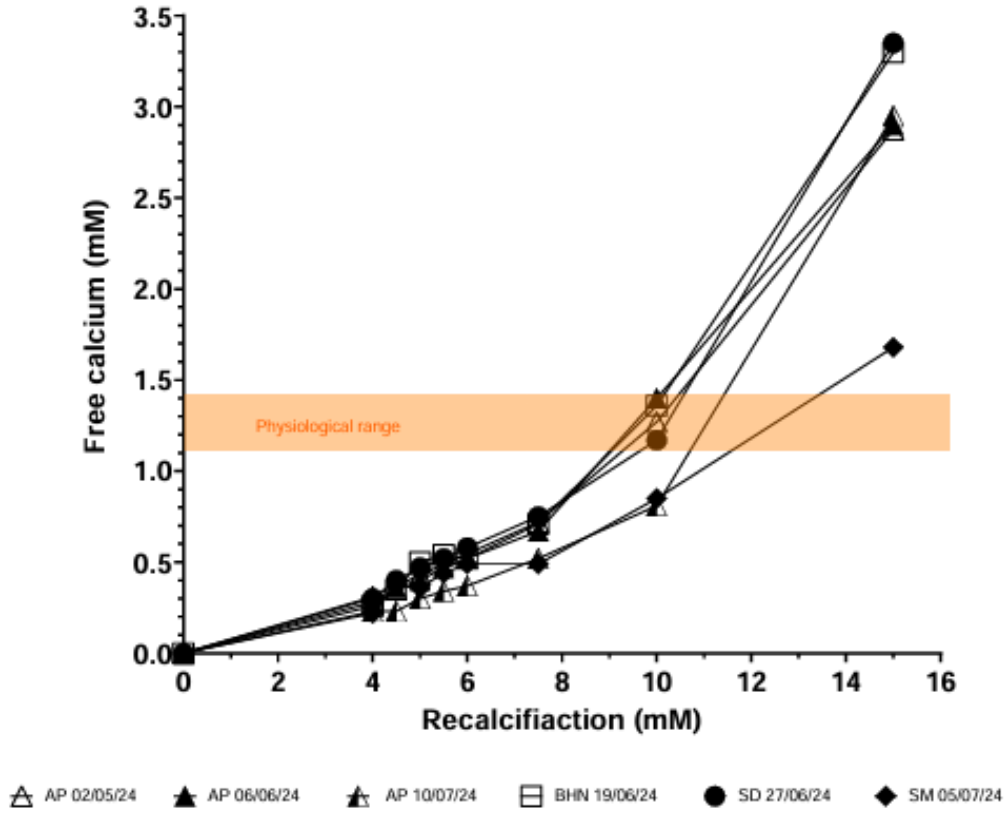
Platelets, neutrophils, and the coagulation cascade:

Ionized calcium is required: recalcification of citrated whole blood

What is ionized calcium?

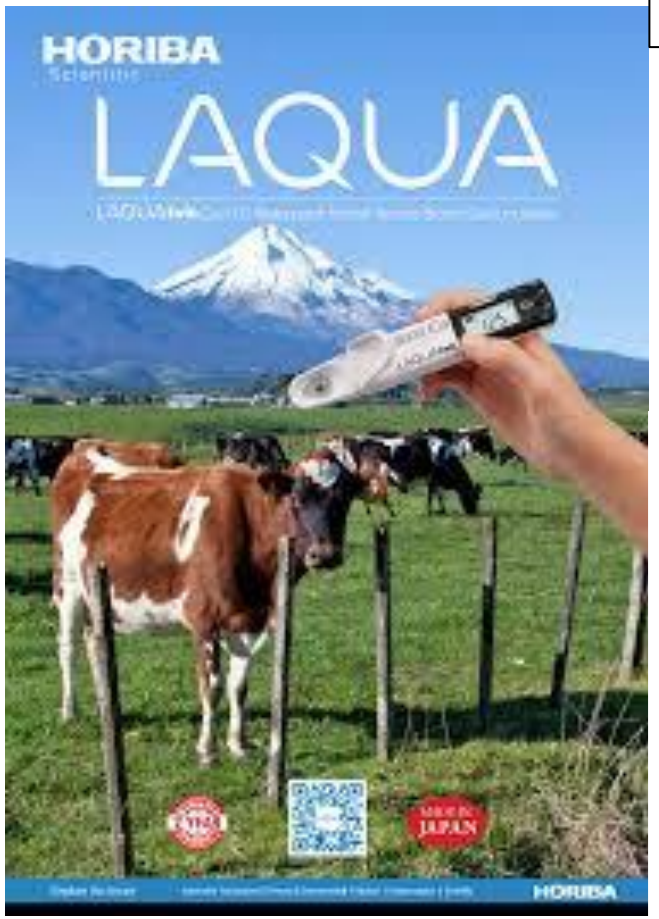
Ionized calcium or free calcium refers to blood calcium that isn't attached to proteins (mostly albumin): ~ half of total blood calcium. Unlike bound calcium, free calcium is "active"= available for use.

Normal ranges in adults:
ionized calcium ~ 4.5 to 5.2 mg/dL (or 1.15 to 1.30 mM)
total calcium ~ 2.10 to 2.55 mM

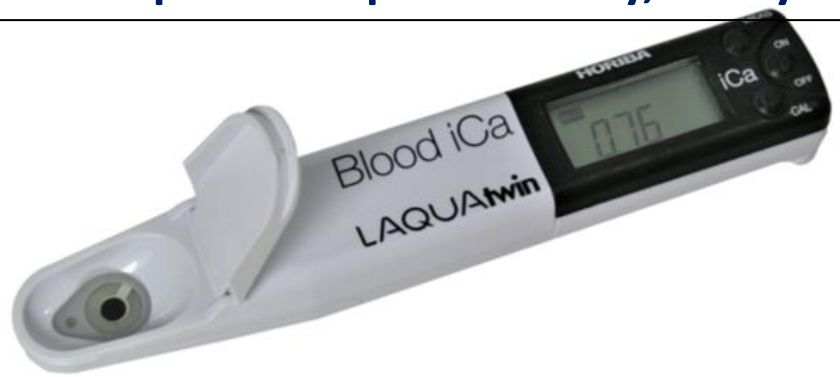


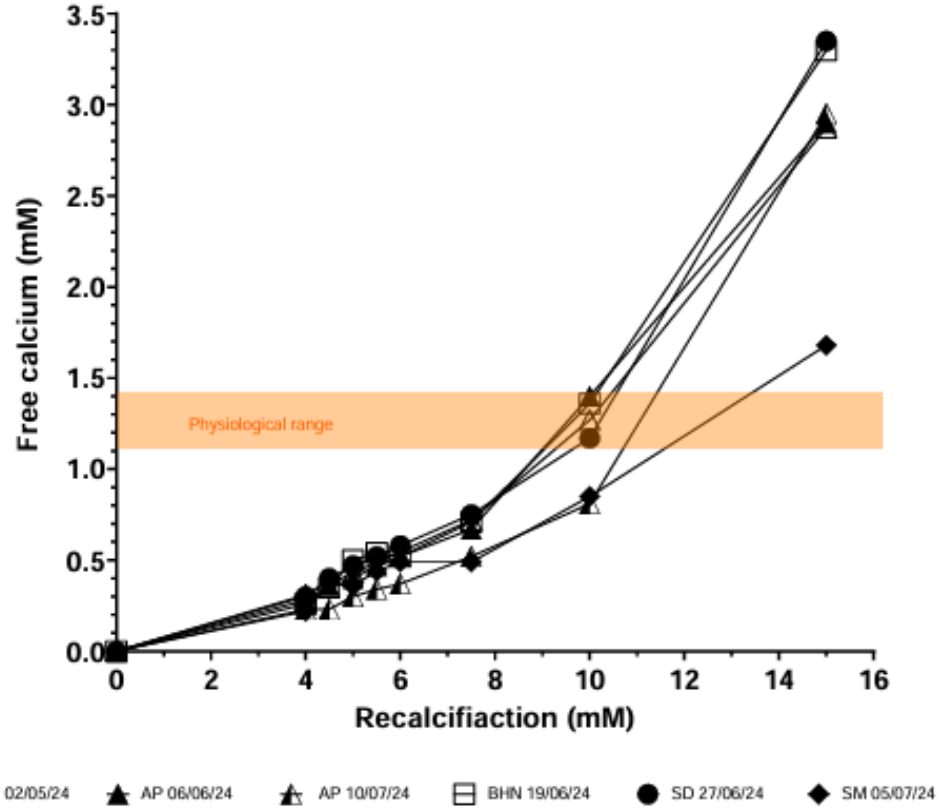
Defining Perfusate

Thrombin and fibrin are major actors of thrombo-inflammatory reactions: Coagulation must be enabled



For improved reproducibility, always check free calcium levels

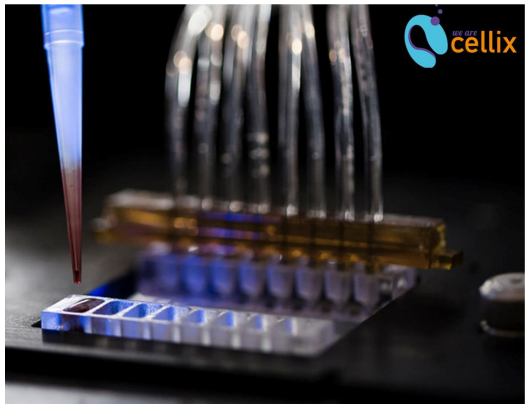


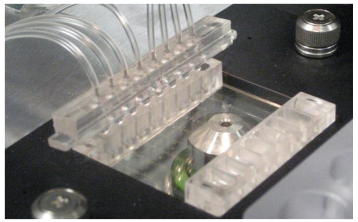


Experimental setting based on intravital observations

Type of chamber	Coating material	Perfusate	Shear rate	Coagulation status	Perfusion duration
we are cellix	Fibrin(ogen) HUVECs	Recalcified Citrated Whole blood	***200 sec-1 Venous blood flow***	Allowed by controlled recalcification	***> 10 min***

Experimental setting based on intravital observations





Surface coating → Fibrinogen 100 µg/mL → O/N 4 °C → Thrombin 1U/mL + $CaCl_2$ 2 mM 37 °C , 1h → Fibrin → Wash → Hirudin 10U/mL RT , 15 mins → Wash 3x

Differences with most common settings for the study of thrombosis and platelet aggregation

- Absence of thrombin generation (anticoagulated blood, washed platelets)
- Or artefactual activation of the contact coagulation pathway: harsh recalcification
- Short times of perfusion
- Nature of coating (i.e. collagen)
- When coagulation is allowed, TF is often added and channel occlusion occurs within a few minutes
- Not necessarily in whole blood (absence of neutrophils in PRP or washed platelets)

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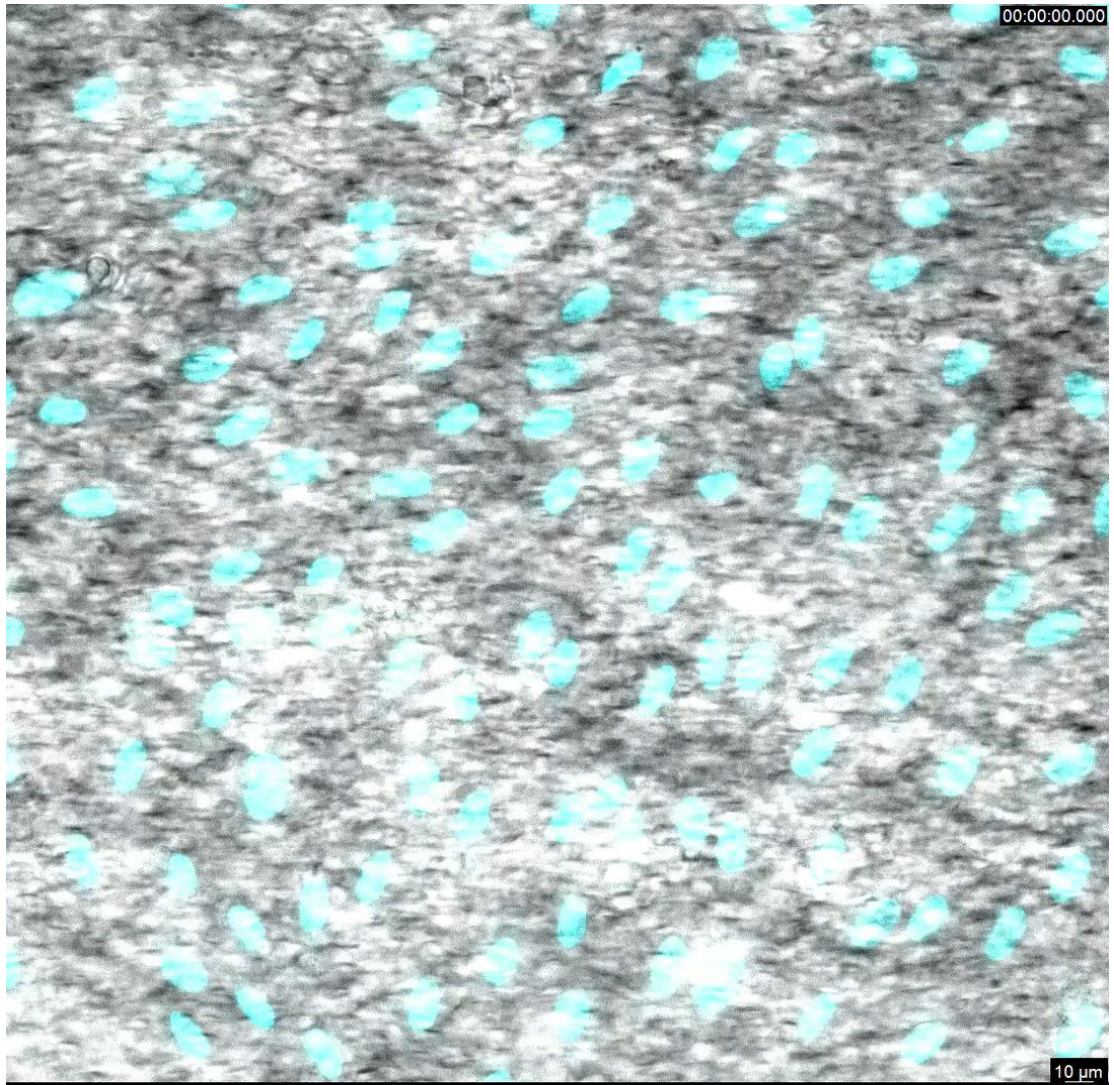
Perfusion (200 sec-1) of recalcified human whole blood onto albumin or quiescent HUVECs

Albumin

Unstimulated HUVECs



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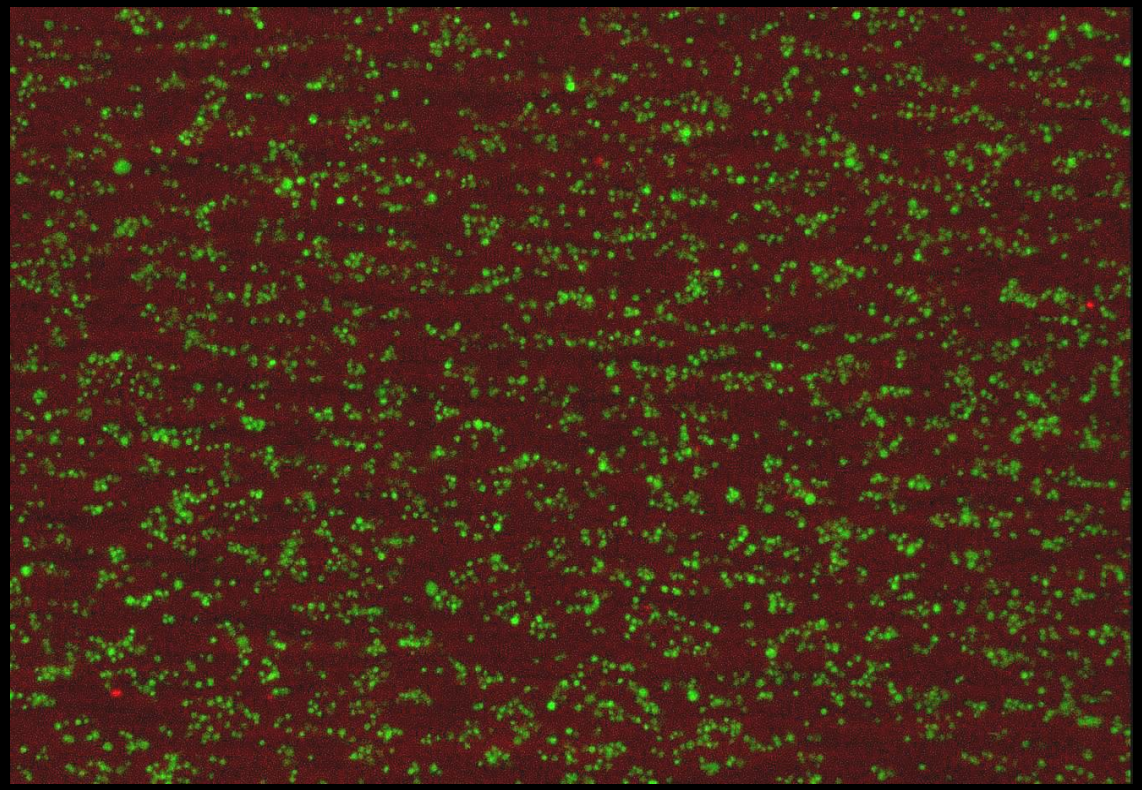
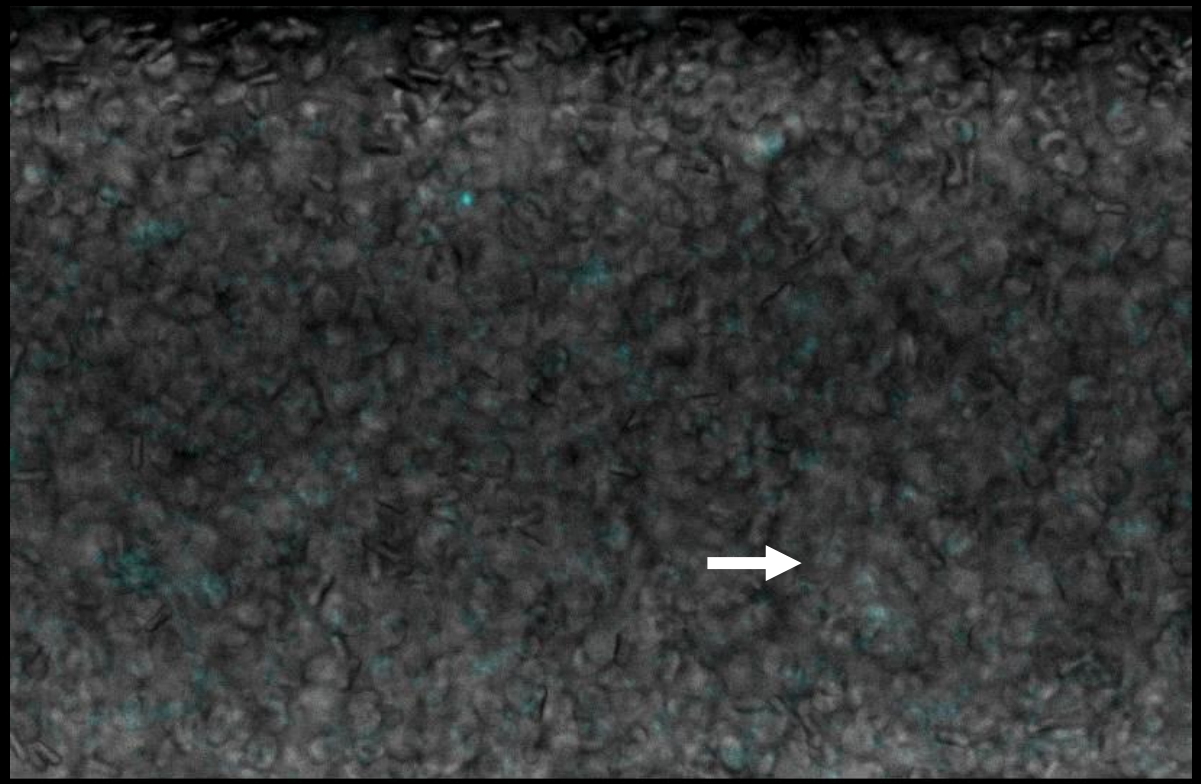
Perfusion (200 sec-1) of recalcified human whole blood onto fibrin

DIOC 6 Platelets/leukocytes
Fibrin



Brightfield and Hoechst (DNA and Polyphosphates)



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Perfusion (200 sec-1) of recalcified human whole blood onto fibrin

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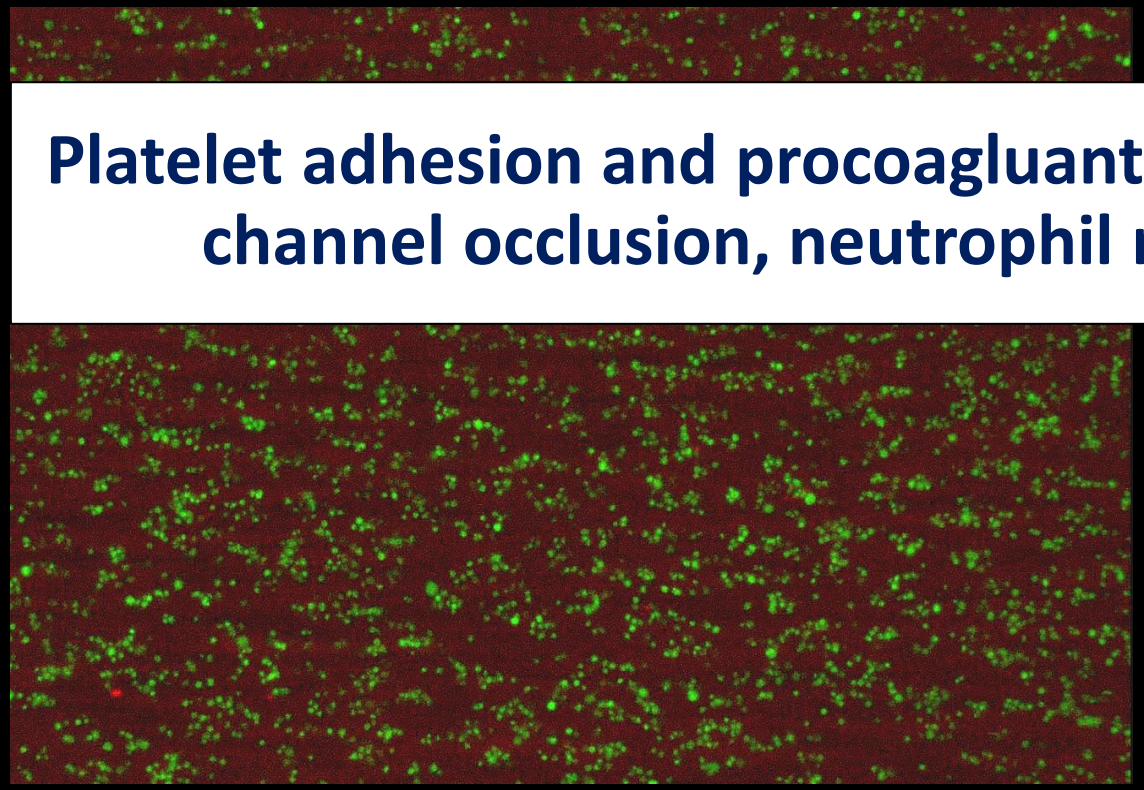

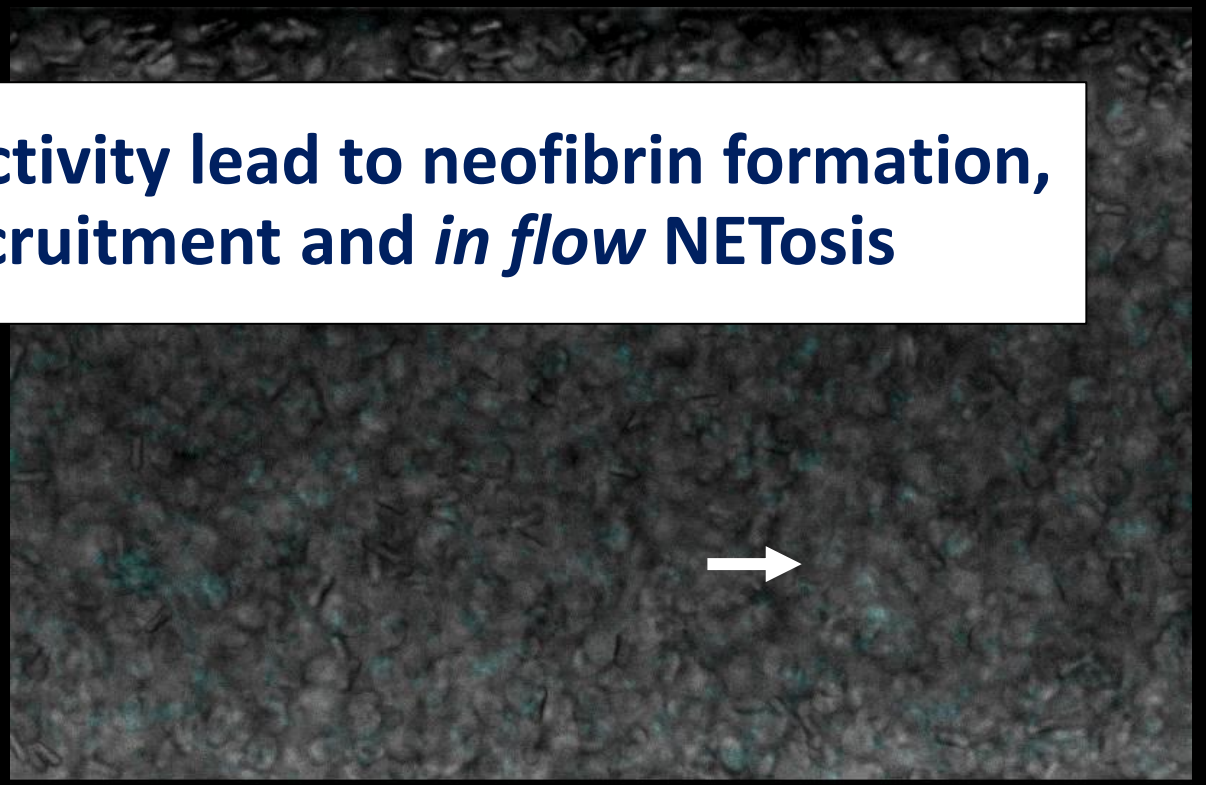

Platelet adhesion and procoagluant activity lead to neofibrin formation, channel occlusion, neutrophil recruitment and *in flow* NETosis

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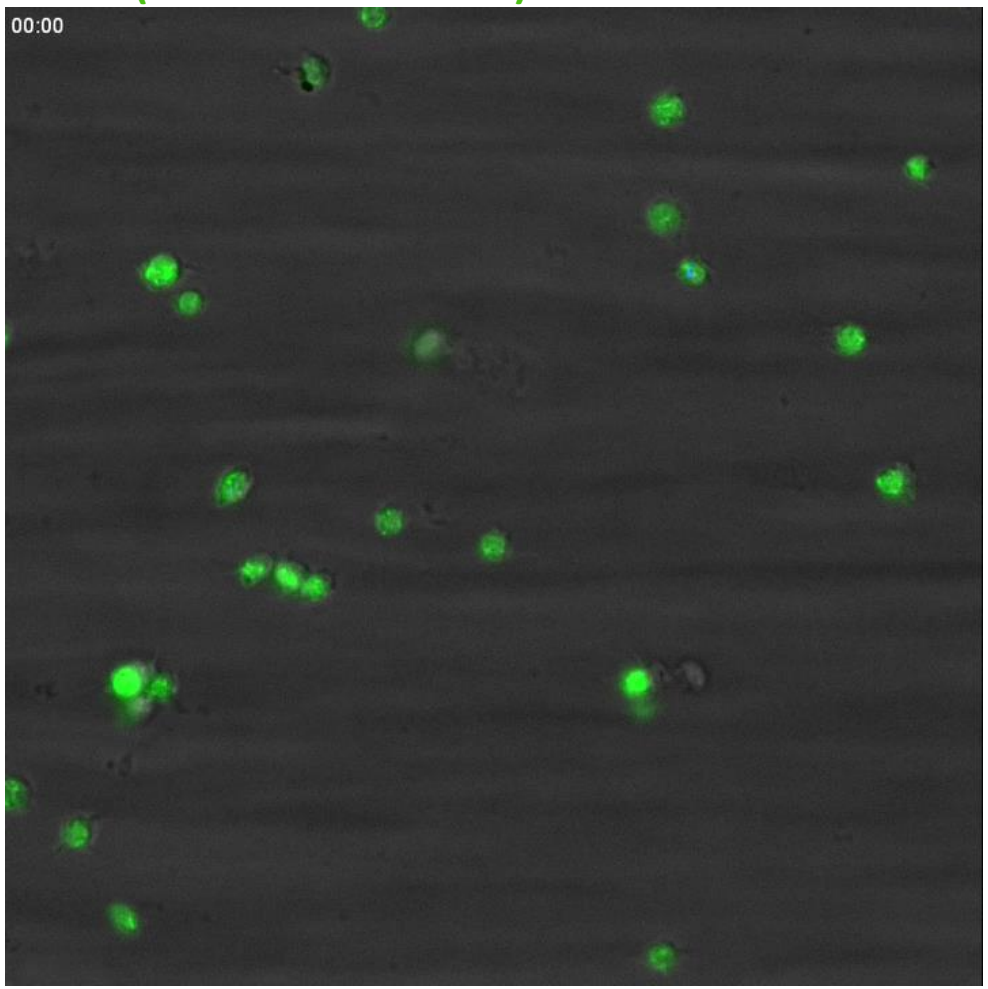
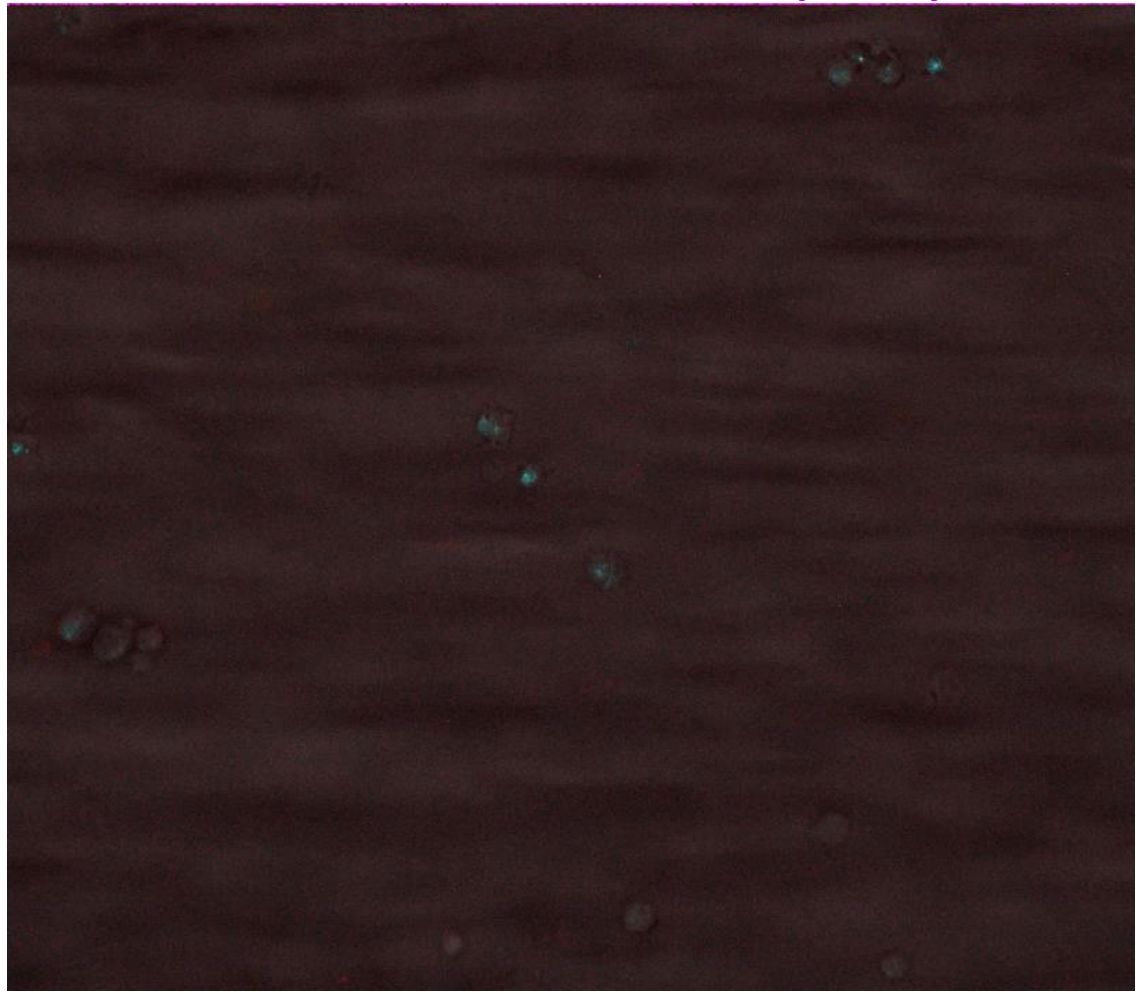
Perfusion (200 sec-1) of recalcified human whole blood onto fibrin

Hoescht (PolyPh. and DNA)
Dioc6 (Platelets&Neutros)
Fibrin
Phosphatidyl-Serine



Hoescht (PolyPh. and DNA)
P-Selectin (Activated platelets)
Fibrin
Phosphatidyl-Serine



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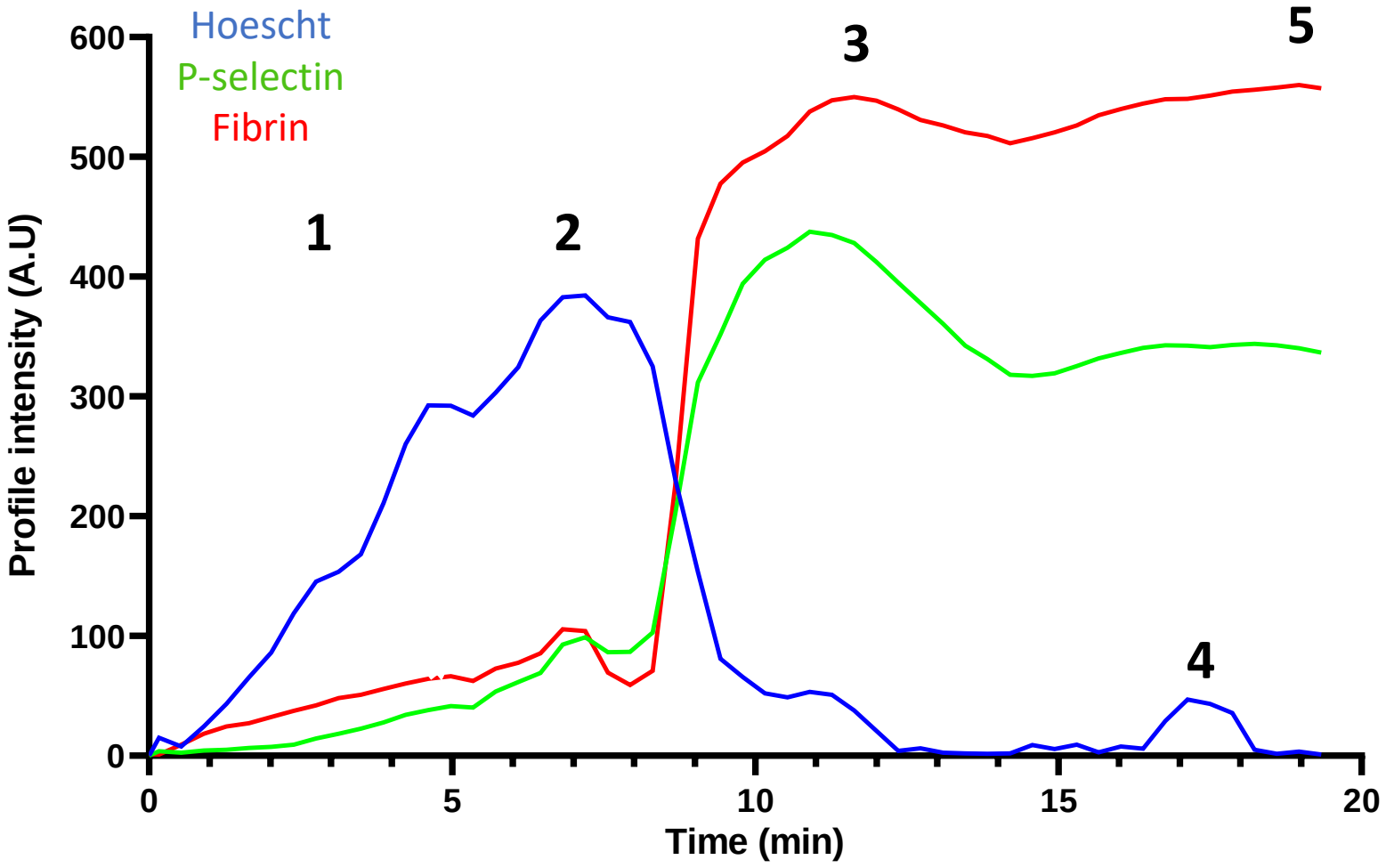
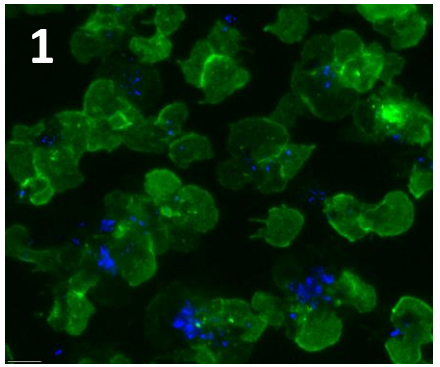
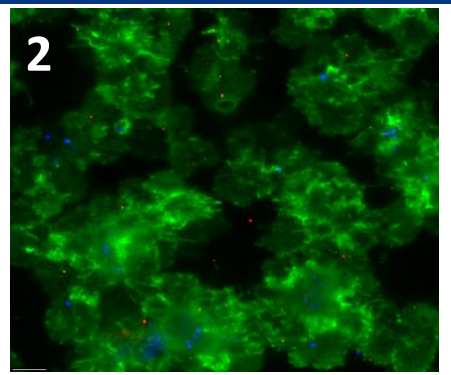
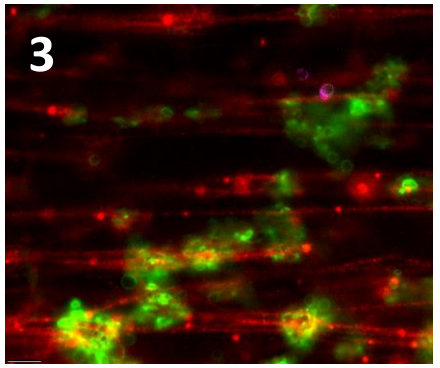
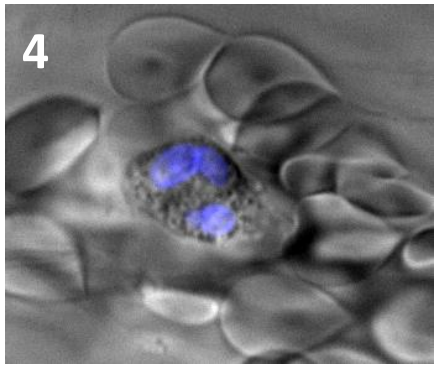
Sequence of thrombosis induced by perfusion (200 sec-1) of recalcified human whole blood onto fibrin

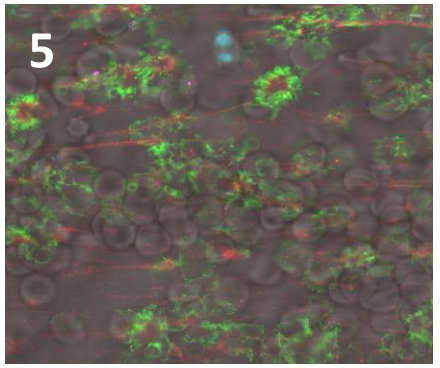




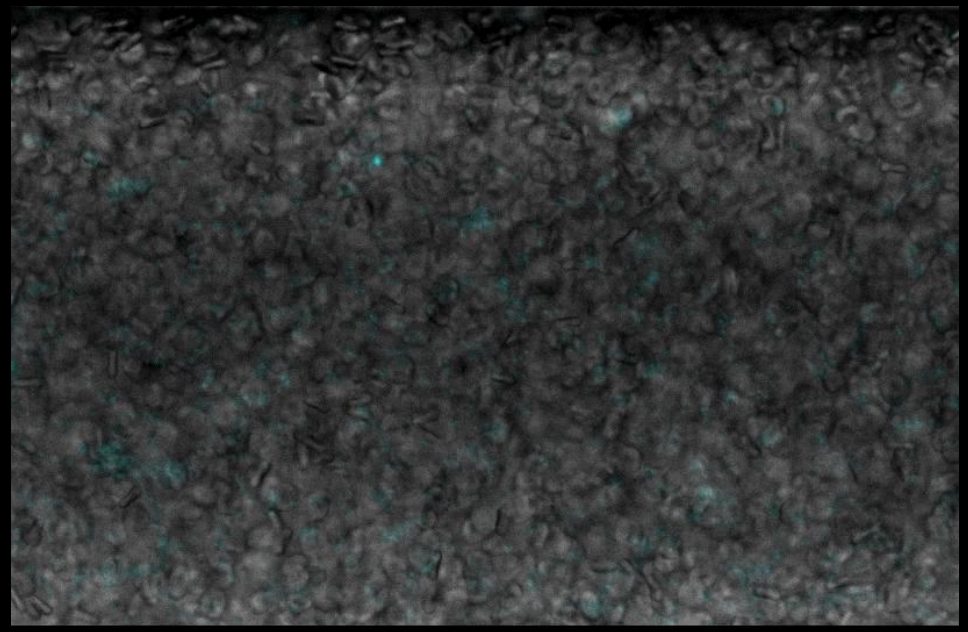




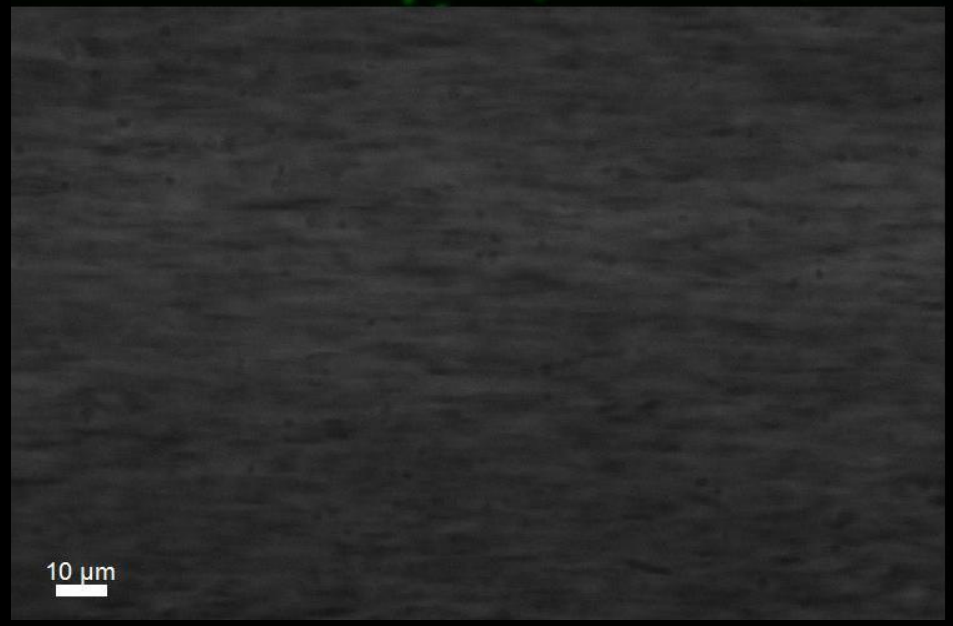




Platelets activation is required



+ glenzocimab (antiplatelet)



Targeting platelet adhesion or activation prevent thrombosis, neutrophil recruitment, and activation

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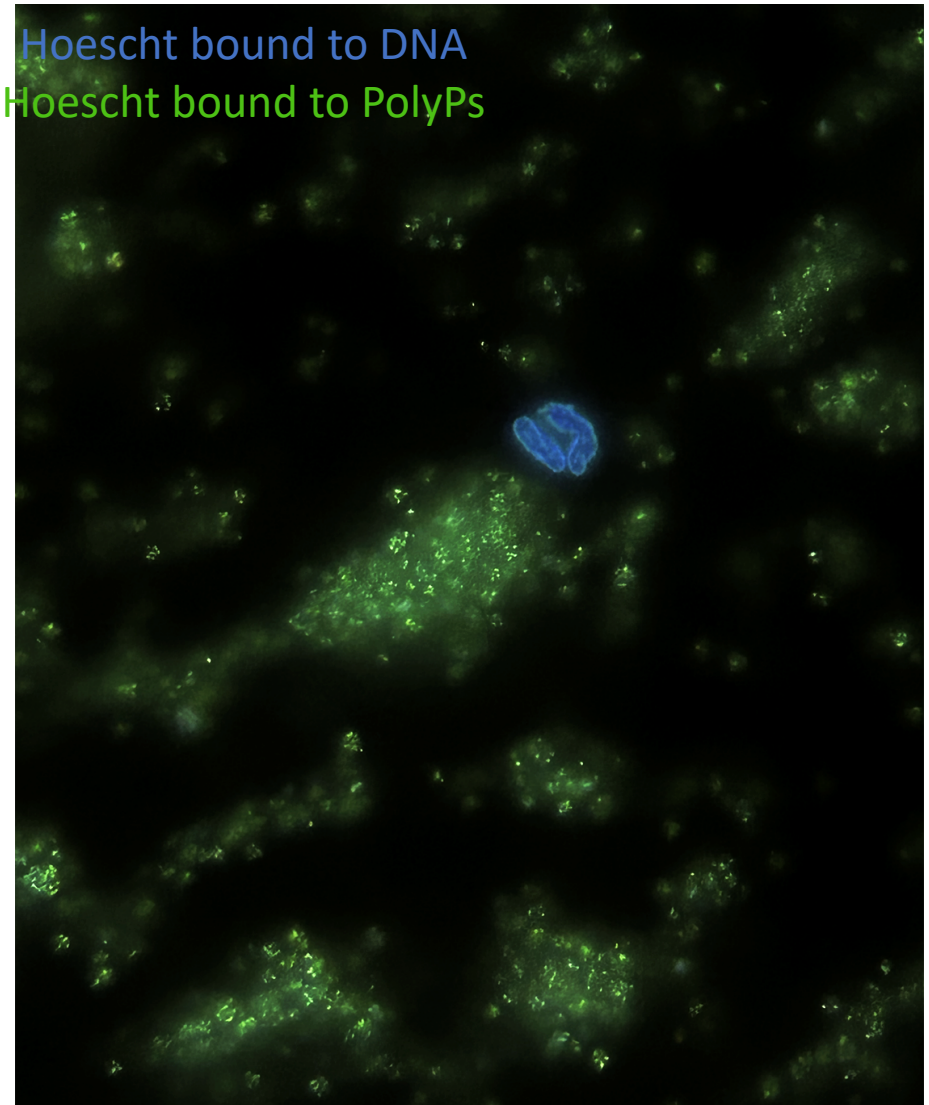
Platelets procoagulant activity is required



PSP 50 U

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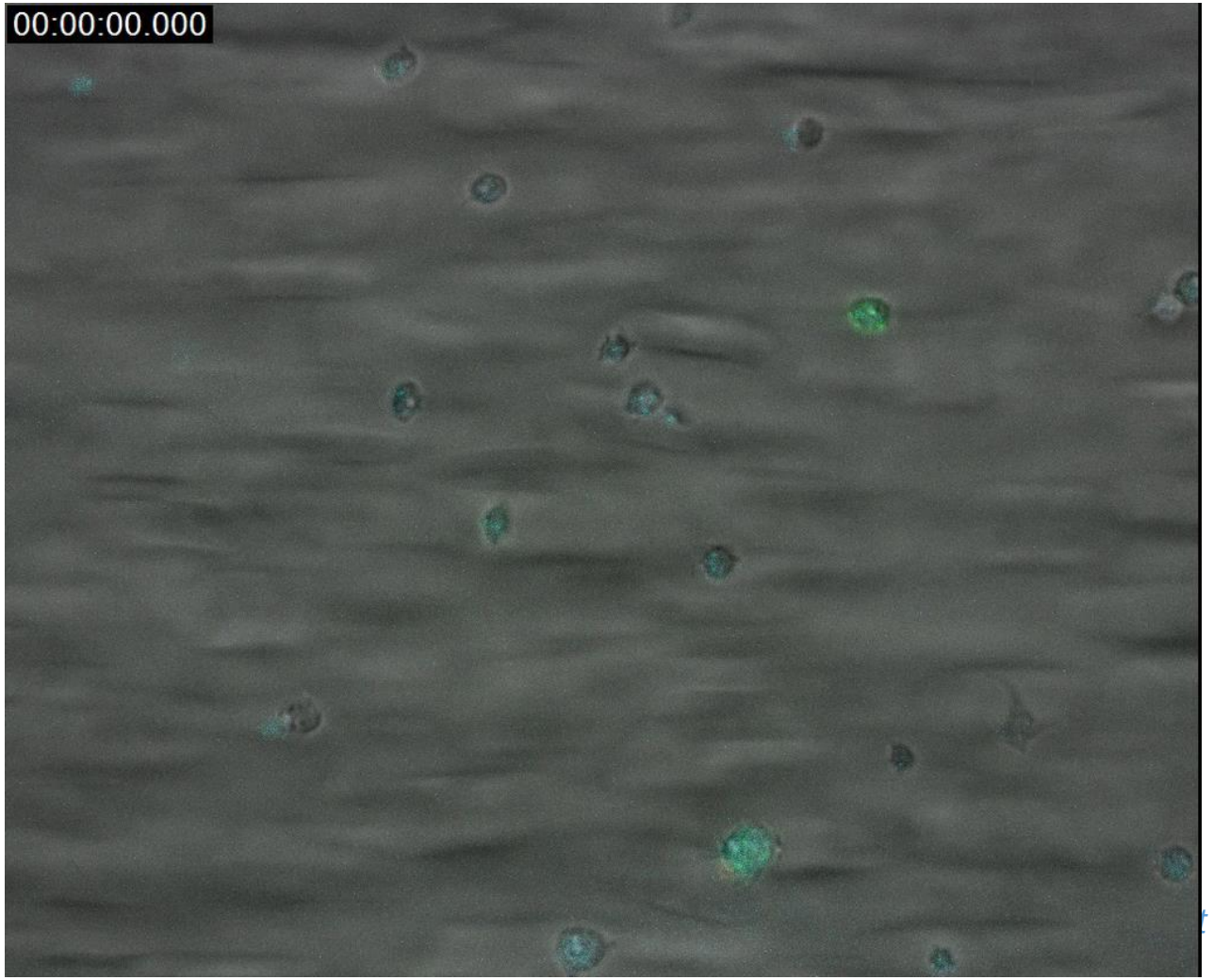

Hoescht P-selectin Fibrin PS

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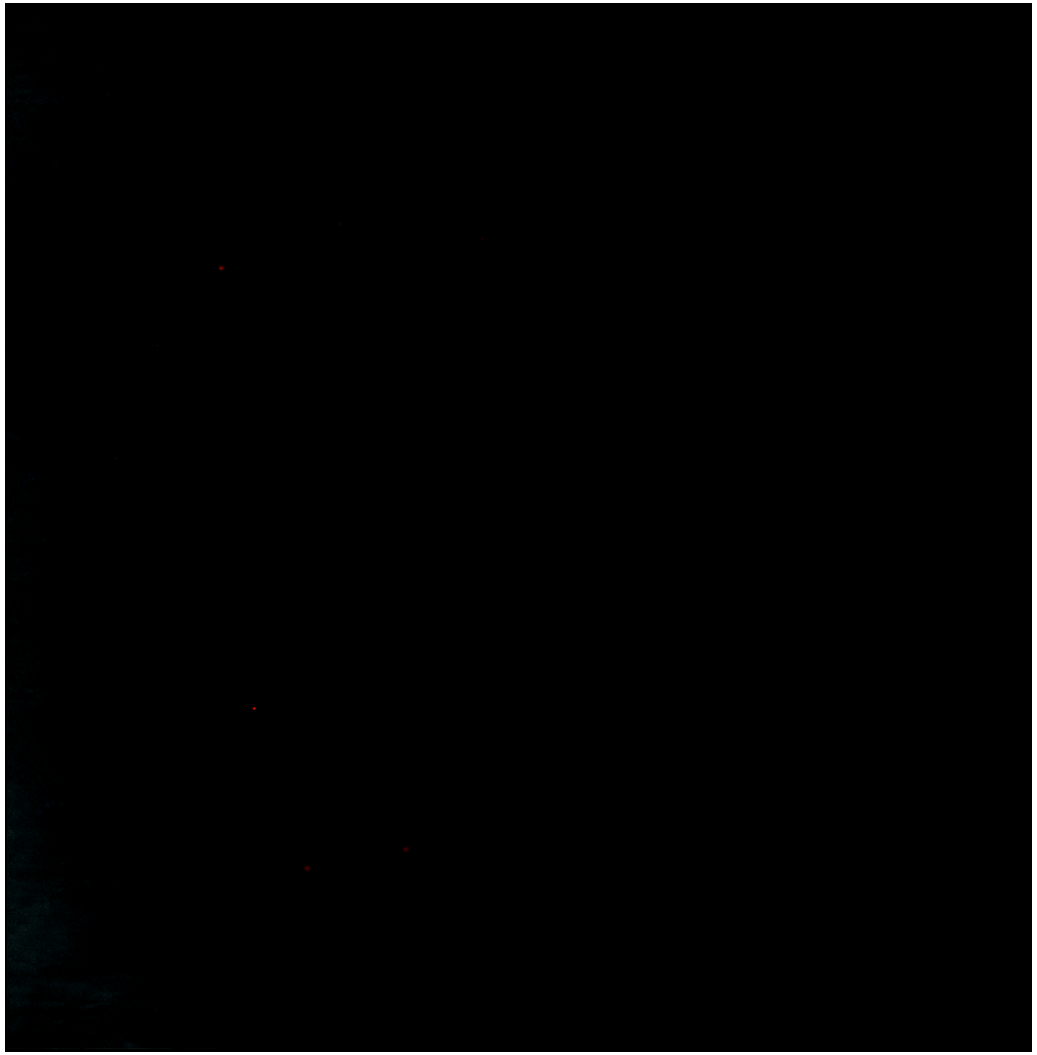
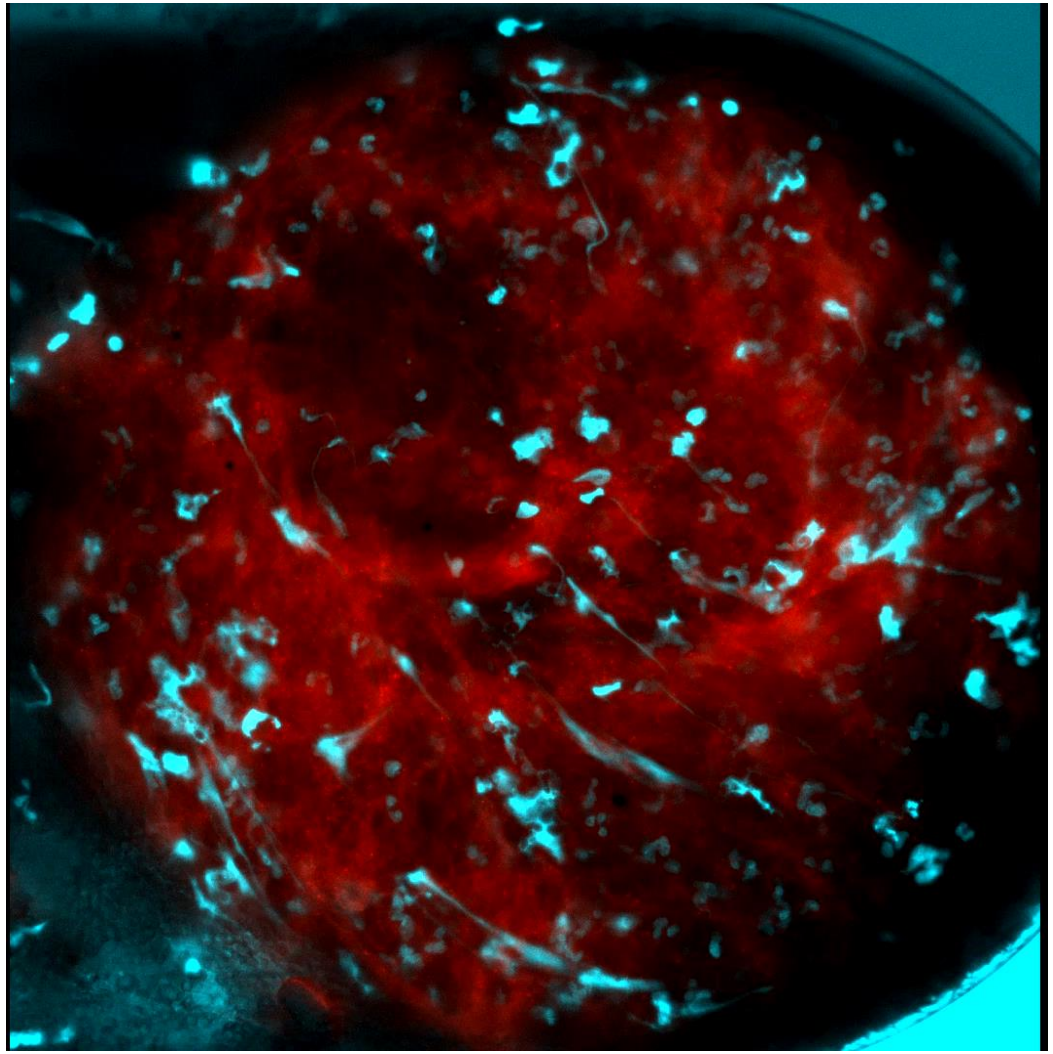
In flow thromboinflammation and NETosis are exacerbated by hyperglycemia: reproduces *in vivo* observations



Fibrin
DNA



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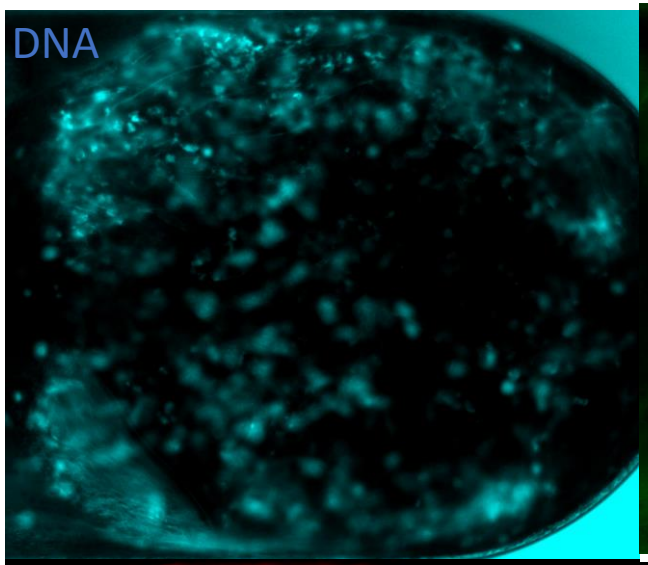
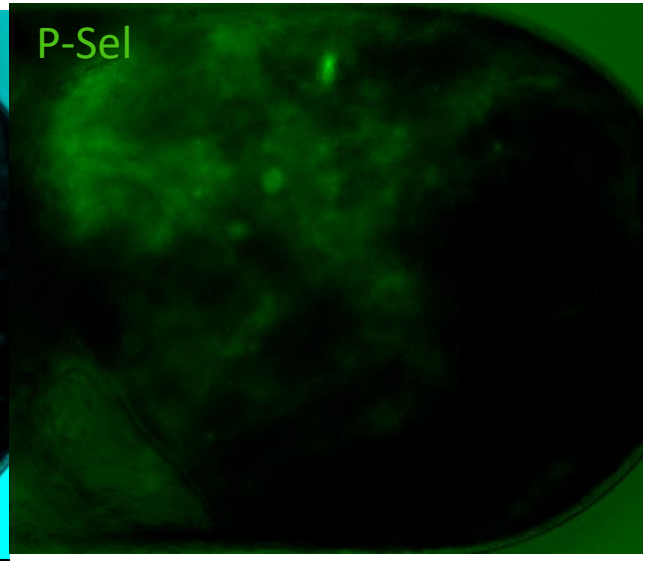
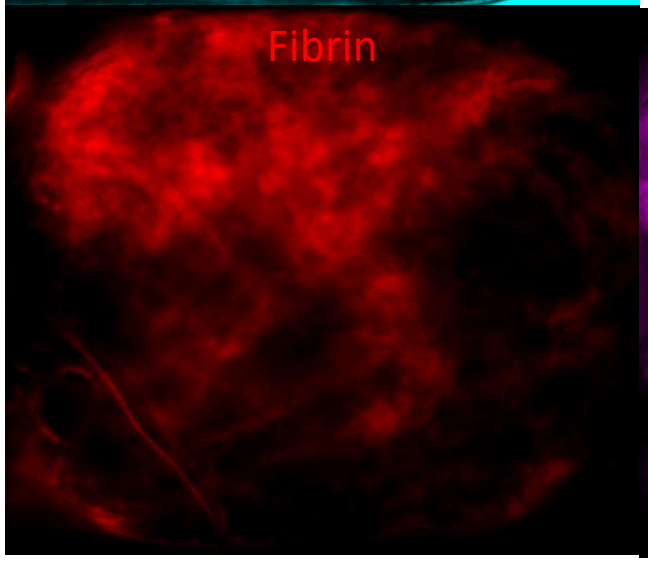
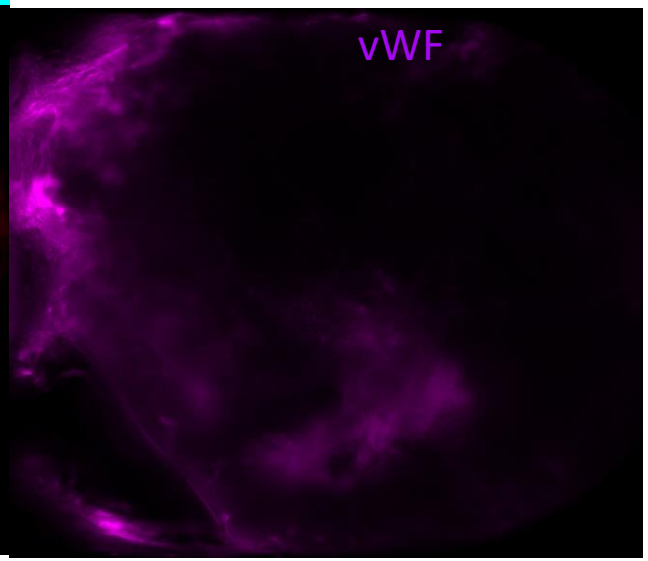
Final thrombus composition

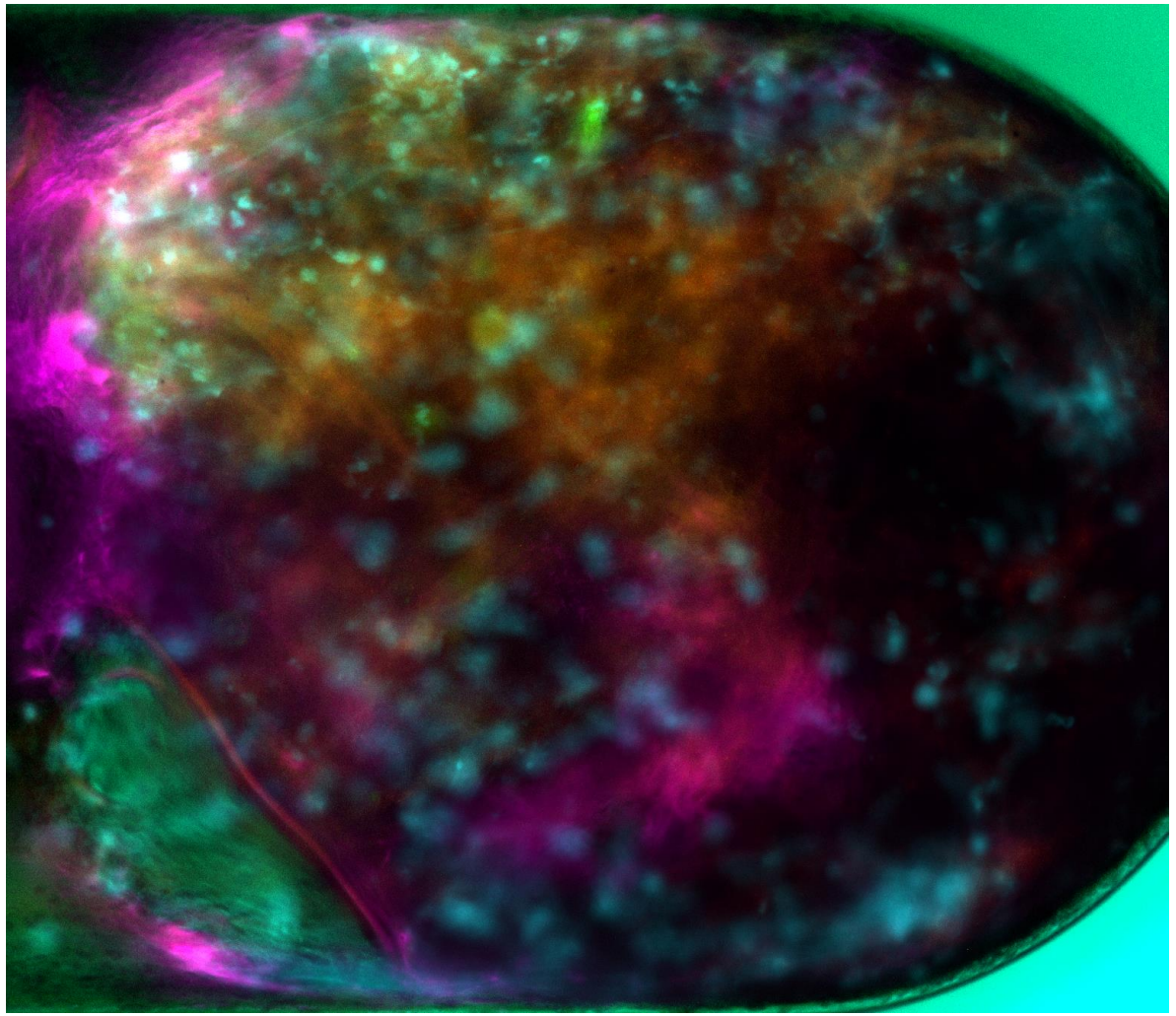








Overlay



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Perfusion (200 sec-1) of recalcified human whole blood onto activated HUVECs

Conclusions

- In vitro reproduction of microvascular thromboinflammation is feasible with common flow chambers

➢ Suitable for the study of platelet procoagulant activity

➢ Recruitment and activation of neutrophils

➢ In flow generation of neutrophils and NETs-rich thrombi: the most resistant to thrombolytics

➢ Suitable for testing of anti-thromboinflammatory strategies and thrombolytic drugs

Acknowledgments

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Optimisation Thérapeutique en Neuropsychopharmacologie

Université Paris Cité

INSERM UNIT 1144

HÔPITAL FONDATION Adolphe de ROTHSCHILD LA RÉFÉRENCE TÊTE ET COU

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Demiana Fanous

Sabrina Mavouna

Mehdi Khobzi

Jean-Philippe Desilles

Varouna Syvannarath

Lucas Di Meglio

Mialitiana SoloNomenJanahary

Sébastien Dupont

Fatima Zemali

Véronique Ollivier

Nathalie Kubis

Mikael Mazighi

& The Stroke Unit from Rothschild Foundation Hospital

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