Use of flow-based assays to monitor drugs with antithrombotic, anticoagulant or anti-inflammatory potential

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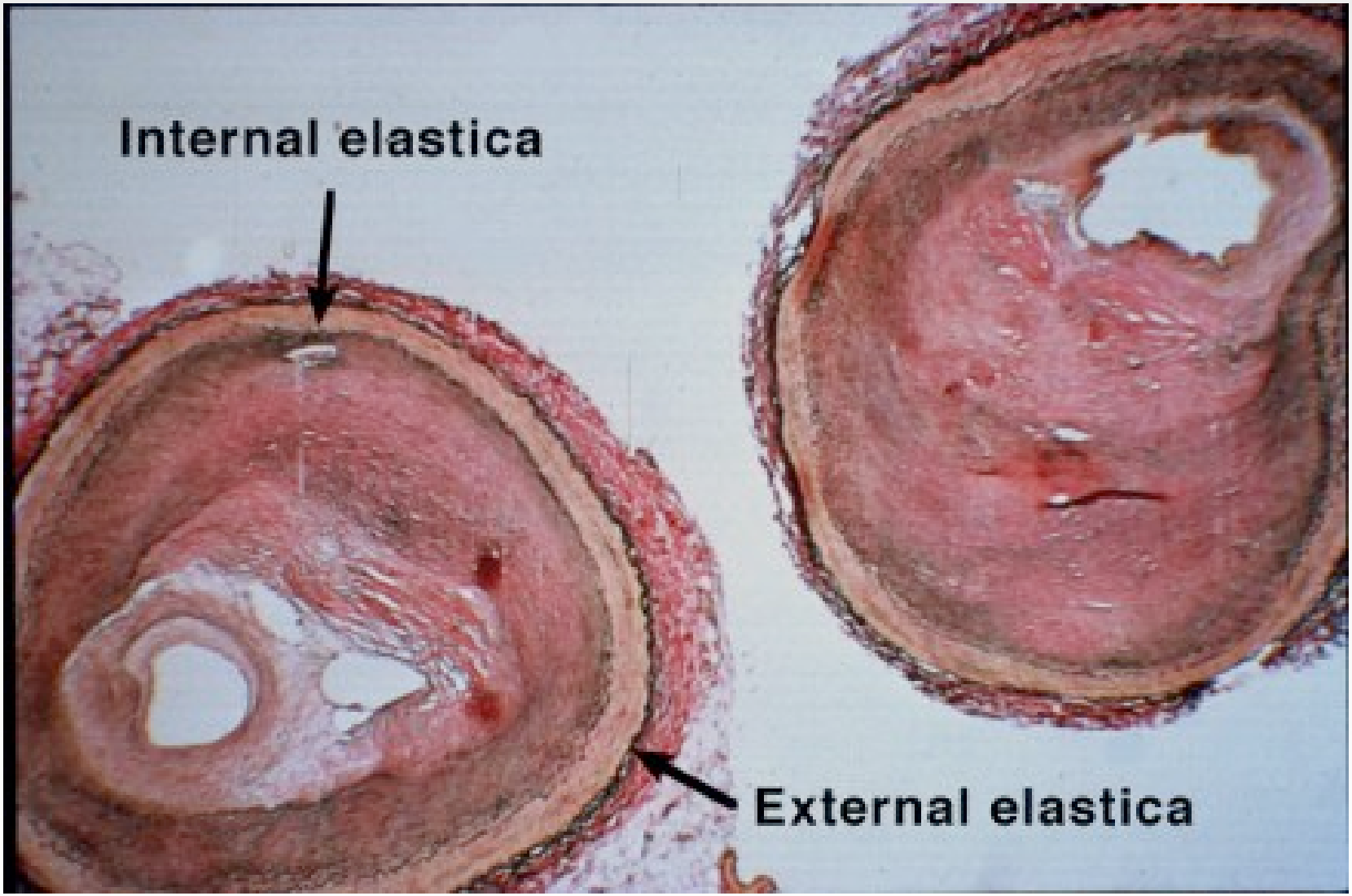
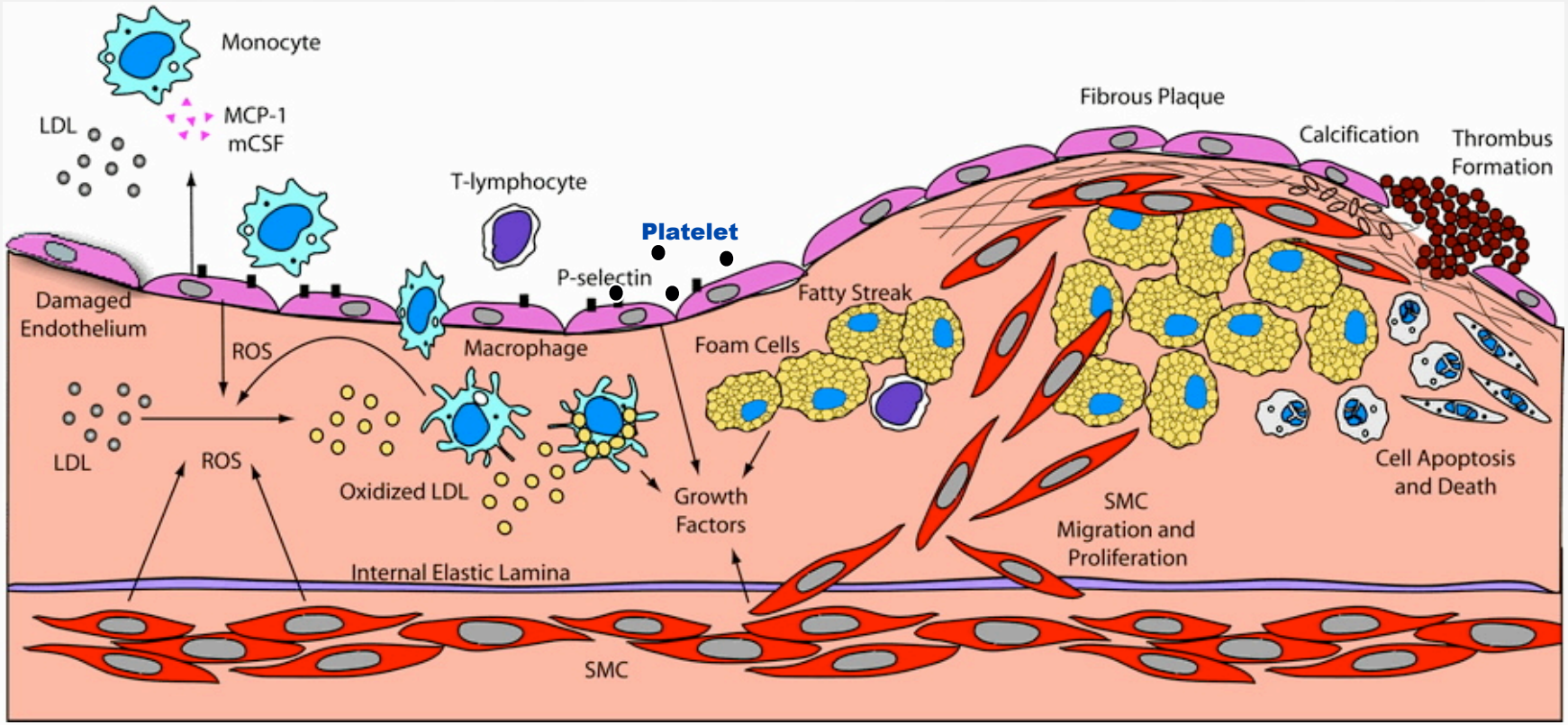
Atherosclerosis and thrombosis



Atherosclerosis and thrombosis



Madamanchi ATVB (2005)

Atherosclerosis and thrombosis

Primary and secondary prevention of cardiovascular disease
antithrombotic therapies, all with risk of bleeding

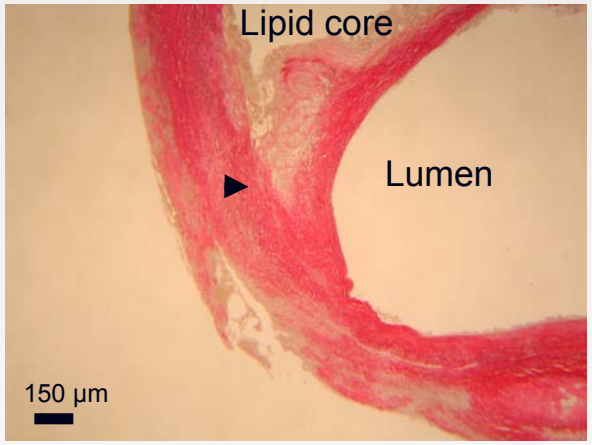
Antiplatelet drugs and biopharmaceuticals
COX (aspirin)
ADP receptor blockers (clopidogrel)
integrin blockers (peptides, mAb)

Anticoagulant drugs
oral anticoagulants (coumarins)
heparins
factor inhibitors

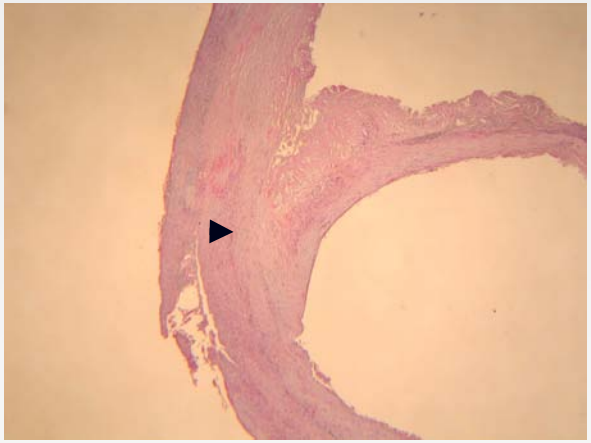
=> **Validated laboratory assays**, testing only platelet or coagulation function
- no integrative assays
- no bedside assays
- no tests incorporating blood flow

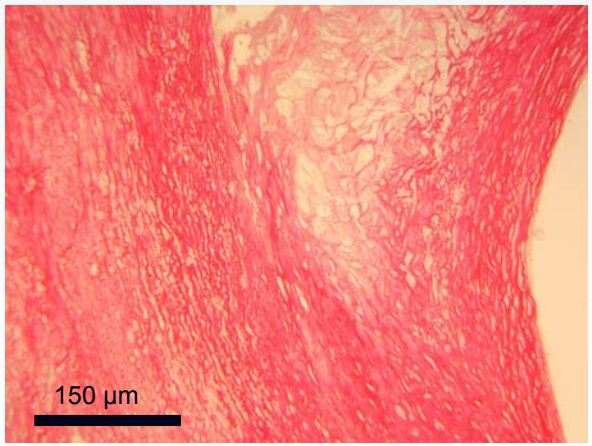
Atherosclerosis and thrombosis

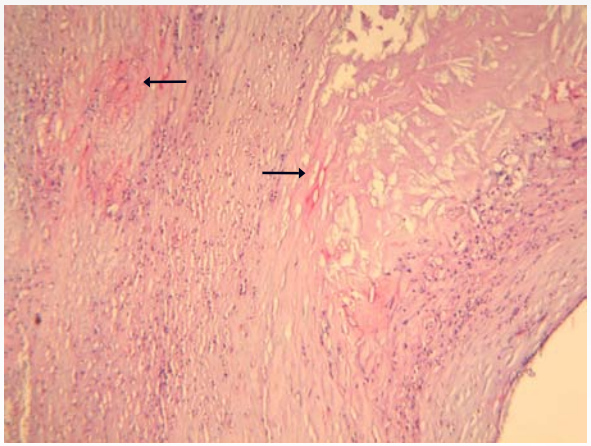
Collagen (Sirius Red)



Tissue factor staining







Cosemans, Atherosclerosis (2003)

Atherosclerosis and thrombosis

Question 1:

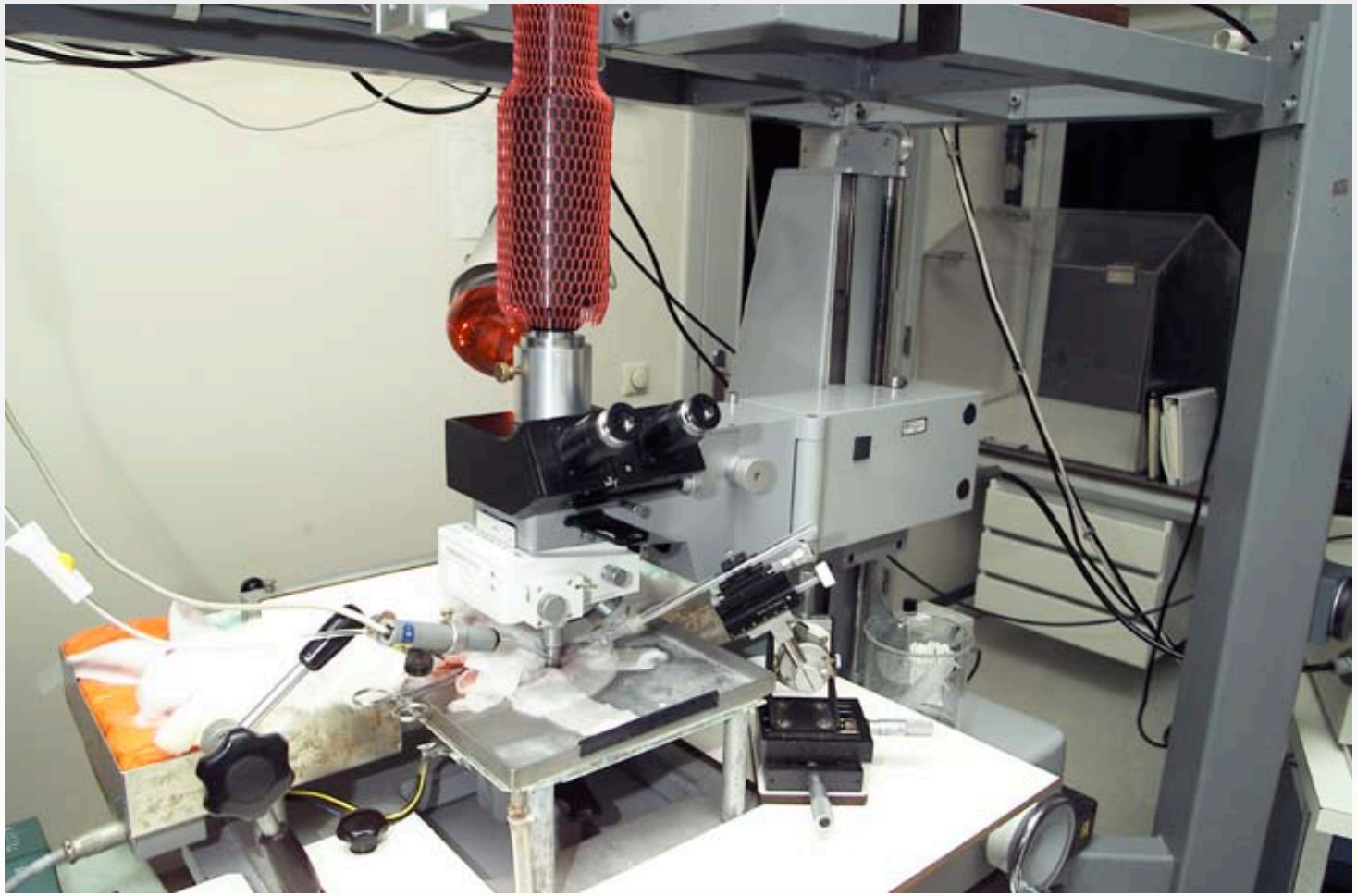
Which are the main thrombogenic components on a ruptured plaque?

- collagen
- von Willebrand factor
- tissue factor (coagulation)
- fibrin
- ...

Thrombus formation in animal models
in vivo

Mechanical puncture of rabbit mesenteric arteriole

haemostasis model: thromboembolic process (1999)



Mechanical puncture of rabbit mesenteric arteriole

haemostasis model: thromboembolic process (1999)

Vehicle control

Infused thrombin inhibitor (melagatran)

Calcium response
(Fluo-3-loaded platelets)

Van Gestel, ATVB (2003)

Free radical-induced damage of mouse mesenteric arteriole

arterial thrombosis model (2003)

- **Bengal rose / illumination**
- **Laser irradiation**
- **$FeCl_3$ application**

Venule

Arteriole (5 min)

Munnix (2005)

Free radical-induced damage of mouse mesenteric arteriole

arterial thrombosis model (2003)

- **Bengal rose / illumination**
- **Laser irradiation**
- **$FeCl_3$ application**

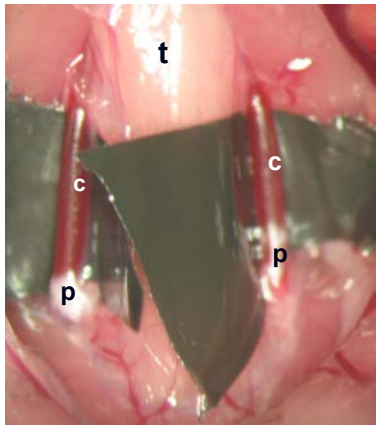
Collagen-R $\alpha 2\beta 1^{-/-}$ (5 min)

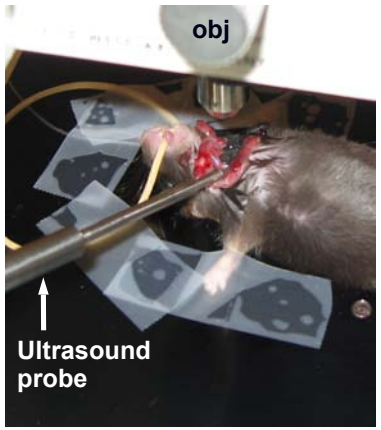
wildtype (5 min)

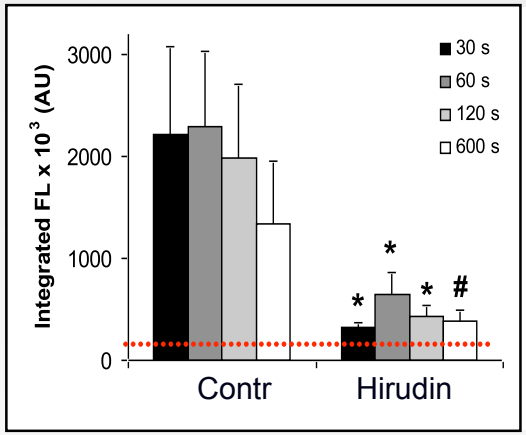
Kuijpers Thromb Haemost (2007)

Ultrasound-induced rupture of carotid plaque in apoE$^{-/-}$ mice

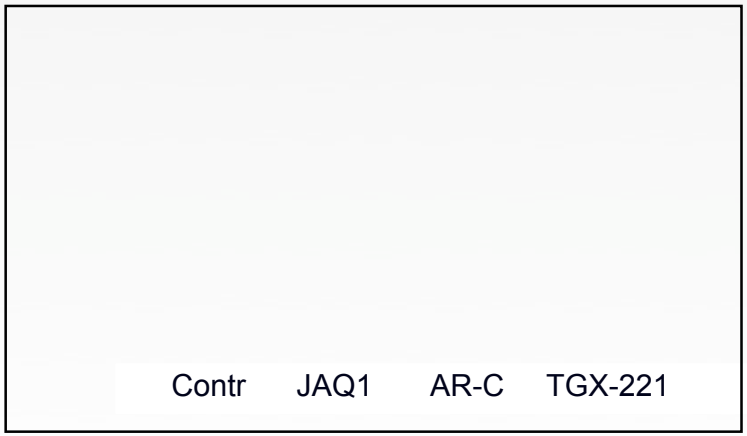
atherothrombosis model in mouse carotid artery (2007)







Control mouse



Kuijpers, JTH (2008)

Thrombus formation in mouse models *in vivo*

- small / large arteries
- healthy / atherosclerotic arteries
- mechanical, laser, ligation damage
- free radical induced damage

Question 2:

Which are the main thrombogenic components of the damaged vessel wall?

- collagen
- von Willebrand factor
- tissue factor (coagulation)
- membrane phospholipids (coagulation)
- ...

Thrombus formation in mouse models *in vivo*

- small / large arteries
- healthy / atherosclerotic arteries
- mechanical, laser, ligation damage
- free radical induced damage

Question 3:

Which are the main prothrombotic factors in the blood?

platelet activation:
- (collagen), von Willebrand factor, fibrinogen
- autocrine ADP, ATP, Gas6, TxA_2
- thrombin, ...

coagulation activation:
- (tissue factor), coagulation factors
- platelet membrane phospholipids
- thrombin, fibrin(ogen), ...

Thrombus formation in mouse models *in vivo*

- small / large arteries
- healthy / atherosclerotic arteries
- mechanical, laser, ligation damage
- free radical induced damage

Question 4:

By which interventions or genetic changes is the thrombotic process affected?

platelet activation:
- (collagen receptors), von Willebrand factor, fibrinogen
- autocrine ADP, ATP, Gas6, TxA_2
- thrombin, ...

coagulation activation:
- (tissue factor), coagulation factors
- platelet membrane phospholipids
- thrombin, fibrin(ogen), ...

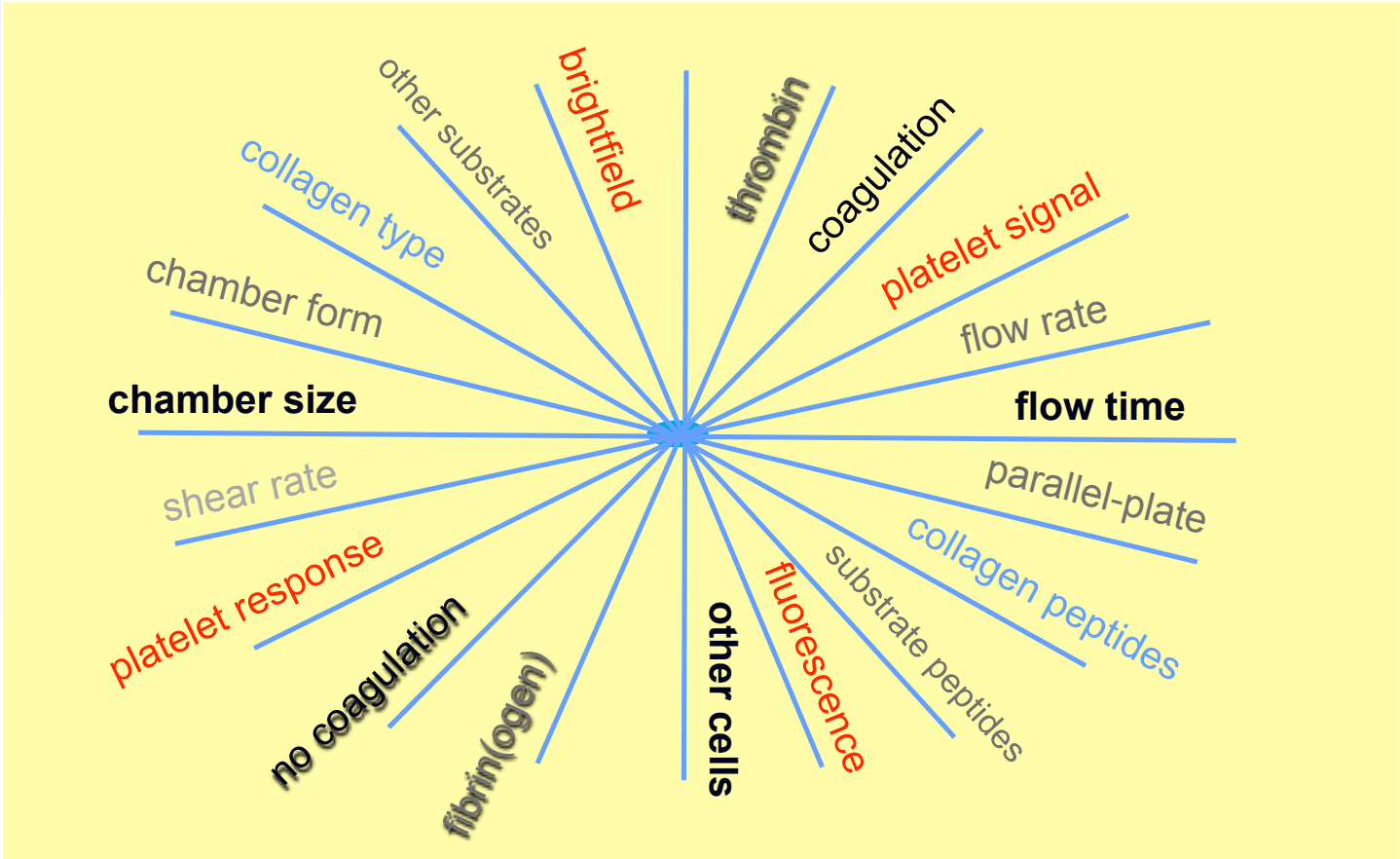
integrin block
ADP-R block
aspirin

oral anticoagulation
heparins
thrombin inhibitor

Use of flow chambers for *ex vivo* measurement of thrombus formation

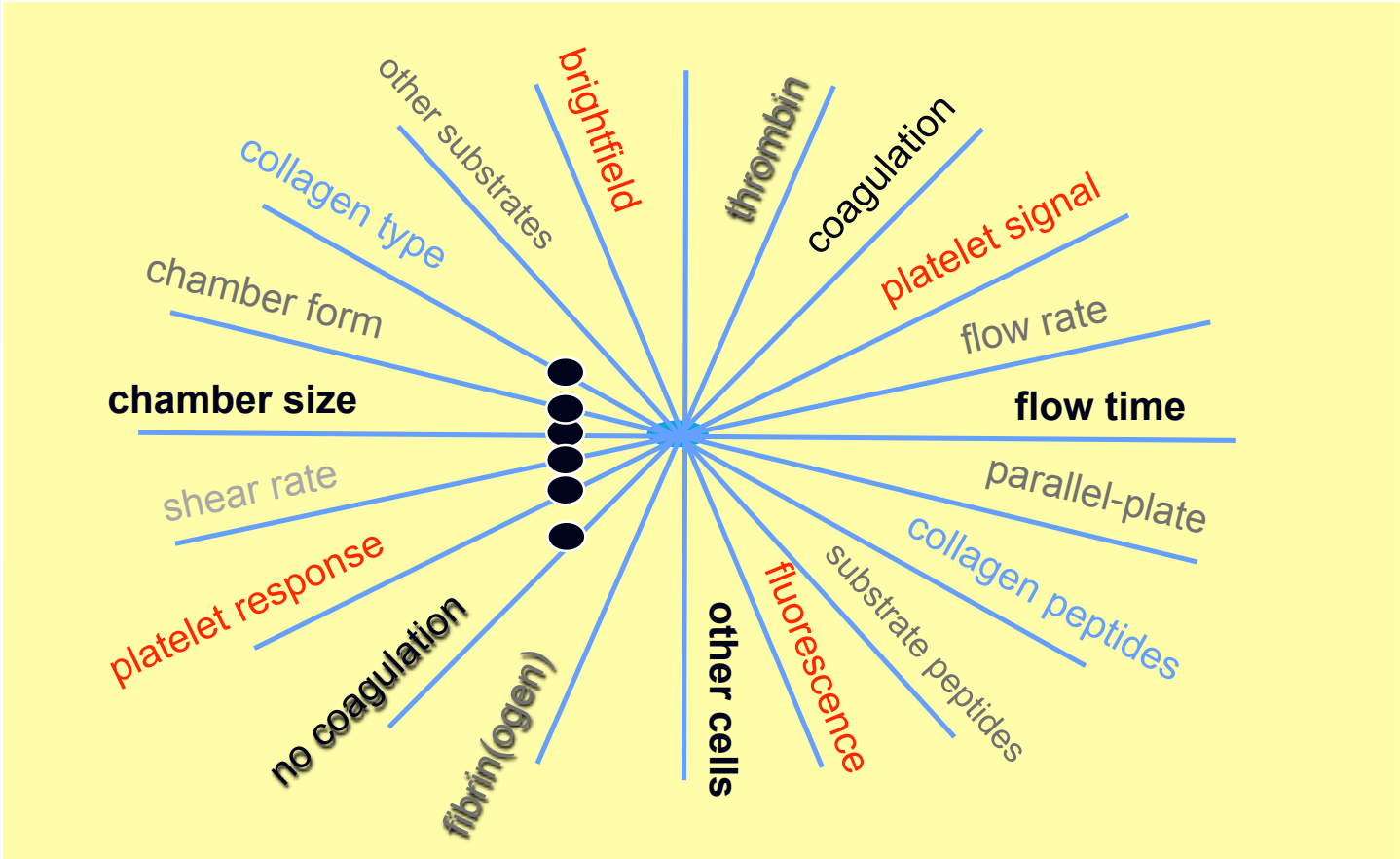
Thrombus formation in flow chambers

multiple choices in design, substrate and measurement



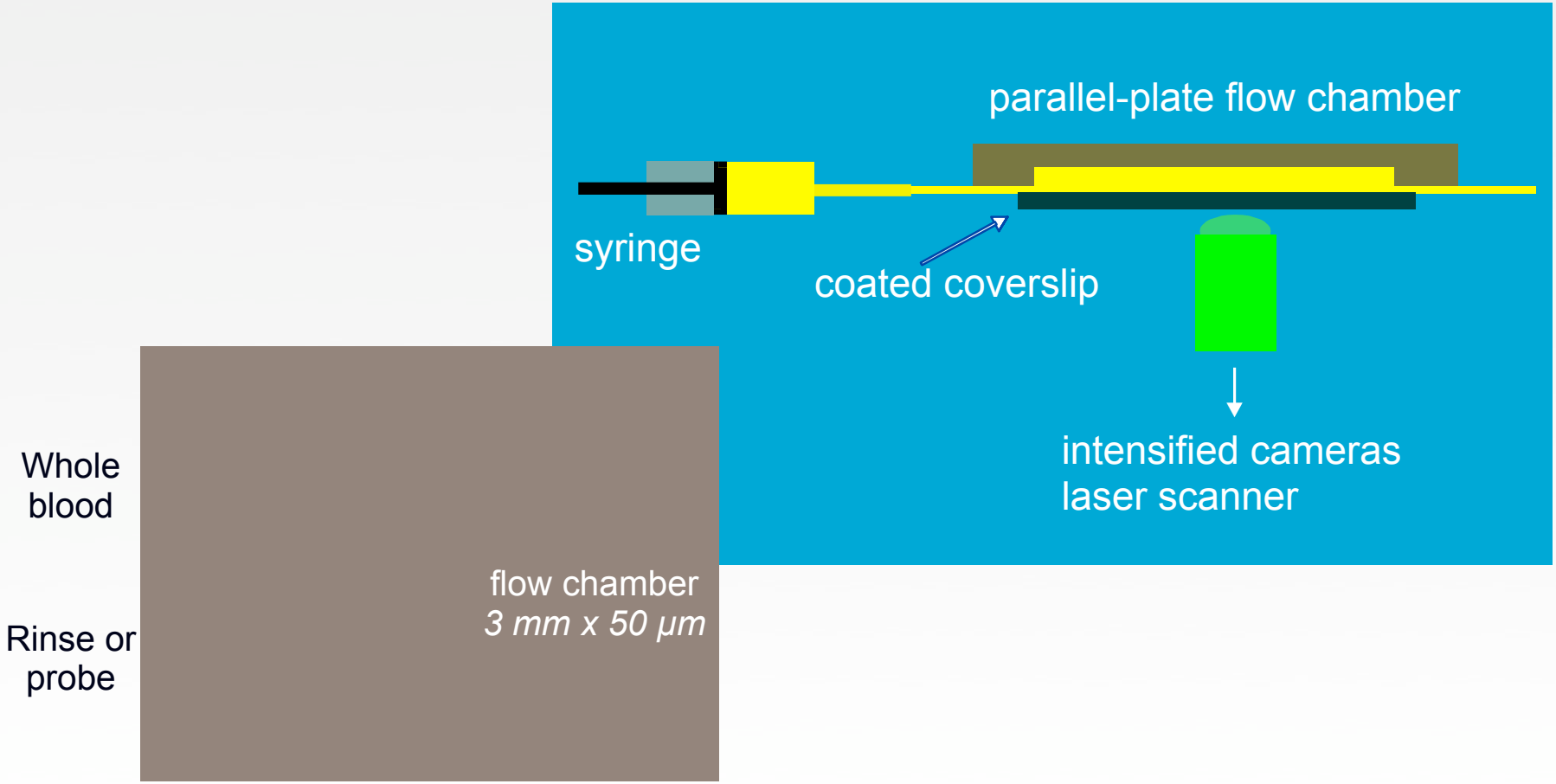
Thrombus formation in flow chambers

multiple choices in design, substrate and measurement



Pulse-free flow of whole blood over collagen type I

Anti-coagulated blood perfused at shear rate of 150 – 1700 s^{-1}



Pulse-free flow of whole blood over collagen type I

Imaging of thrombi by conventional microscopy
or by multiphoton laser-scanning microscopy

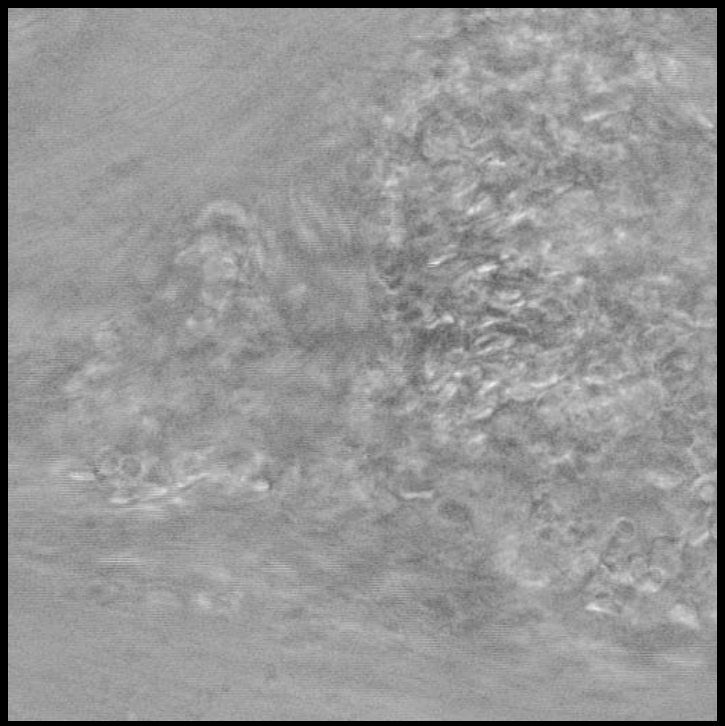
Whole blood

Rinse or probe

flow chamber
3 mm x 50 µm

TPLSM: 3 fluorescent colours

Collagen-induced thrombus formation at arterial shear rate (1000 s^{-1})



no coagulation

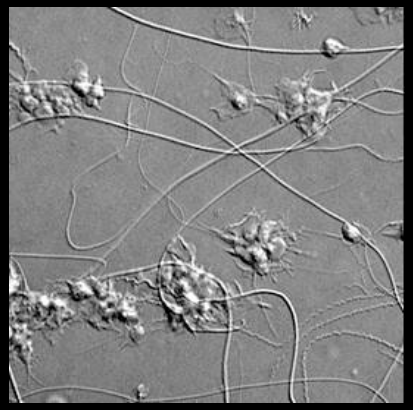
coagulation

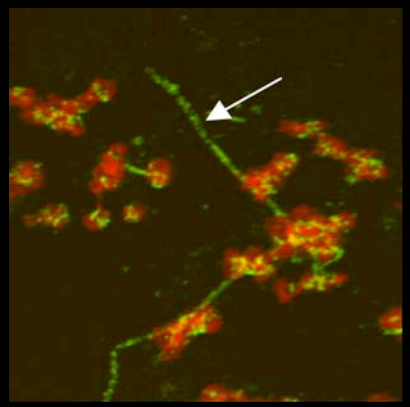
Collagen-induced thrombus formation at arterial shear rate (1000 s^{-1})

calcein-labeled platelets

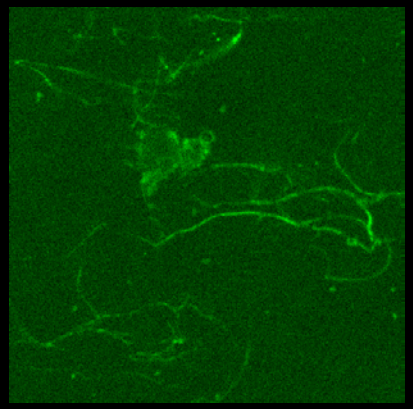
fibrinogen label

Collagen-induced thrombus formation at arterial shear rate (1000 s^{-1})

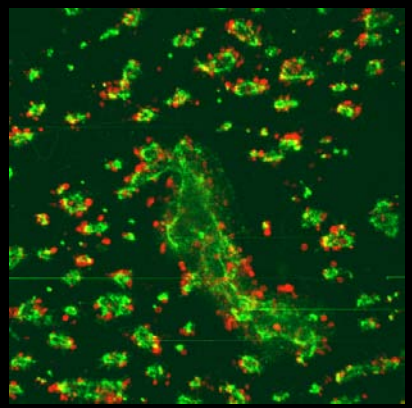




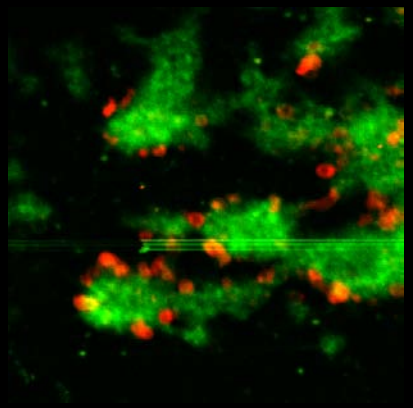
vWF



factor XII



integrin αIIbβ3*



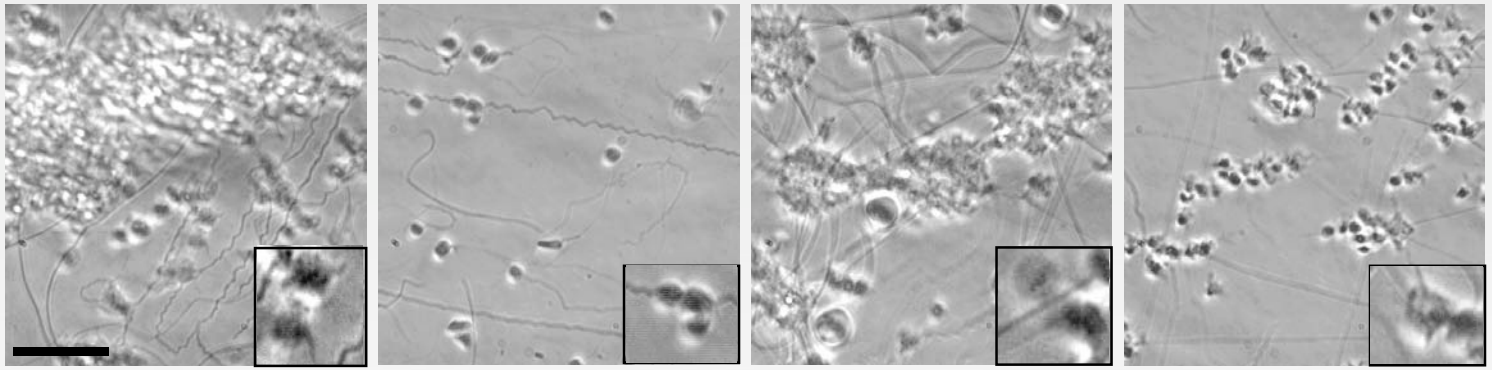
P-selectin

Role of immunoglobulin-type collagen receptor GPVI on platelets

Collagen-induced thrombus formation at arterial shear rate (1000 s^{-1})

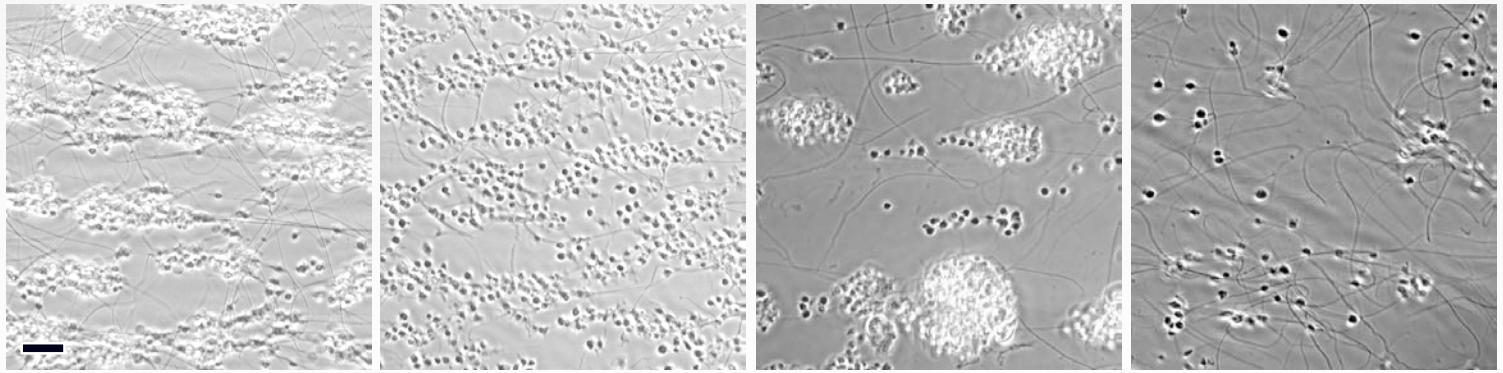
role of platelet collagen receptors and autocrine agonists

Mouse



Wildtype | GPVI/FcRγ def | α2β1 def | Gqα def

Human



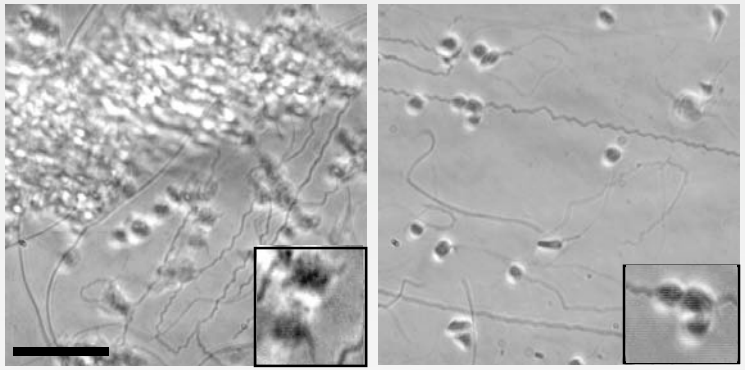
Control | anti-GPVI | anti-α2β1 | - ADP / TxA_2

Kuijpers, FASEB J (2003), Siljander, Blood (2004)

Collagen-induced thrombus formation (1000 s^{-1})

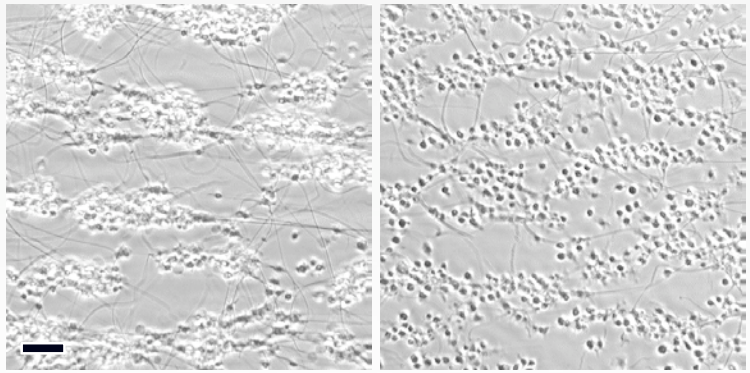
role of platelet collagen receptors and autocrine agonists

Mouse

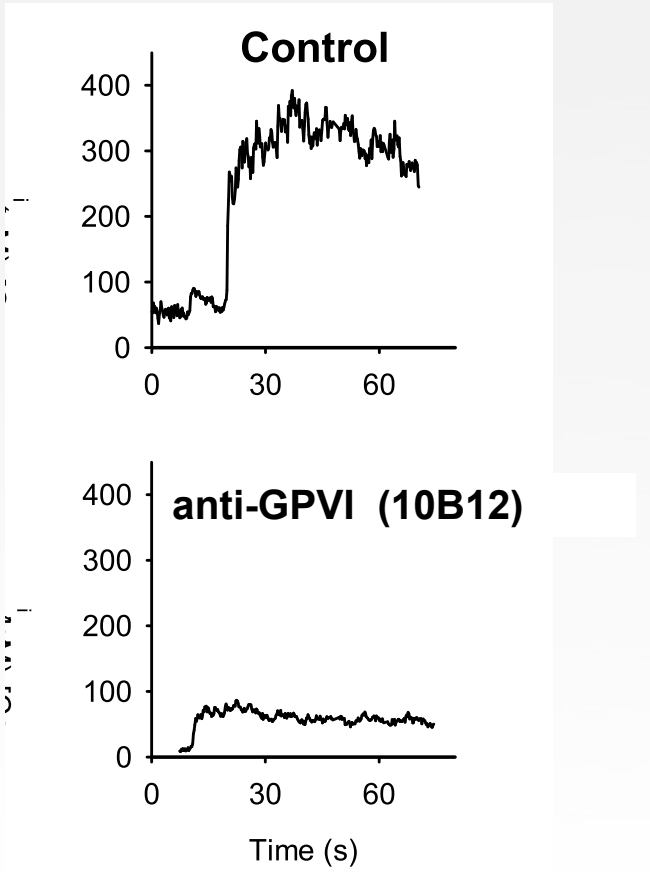


Wildtype GPVI/FcRγ def

Human

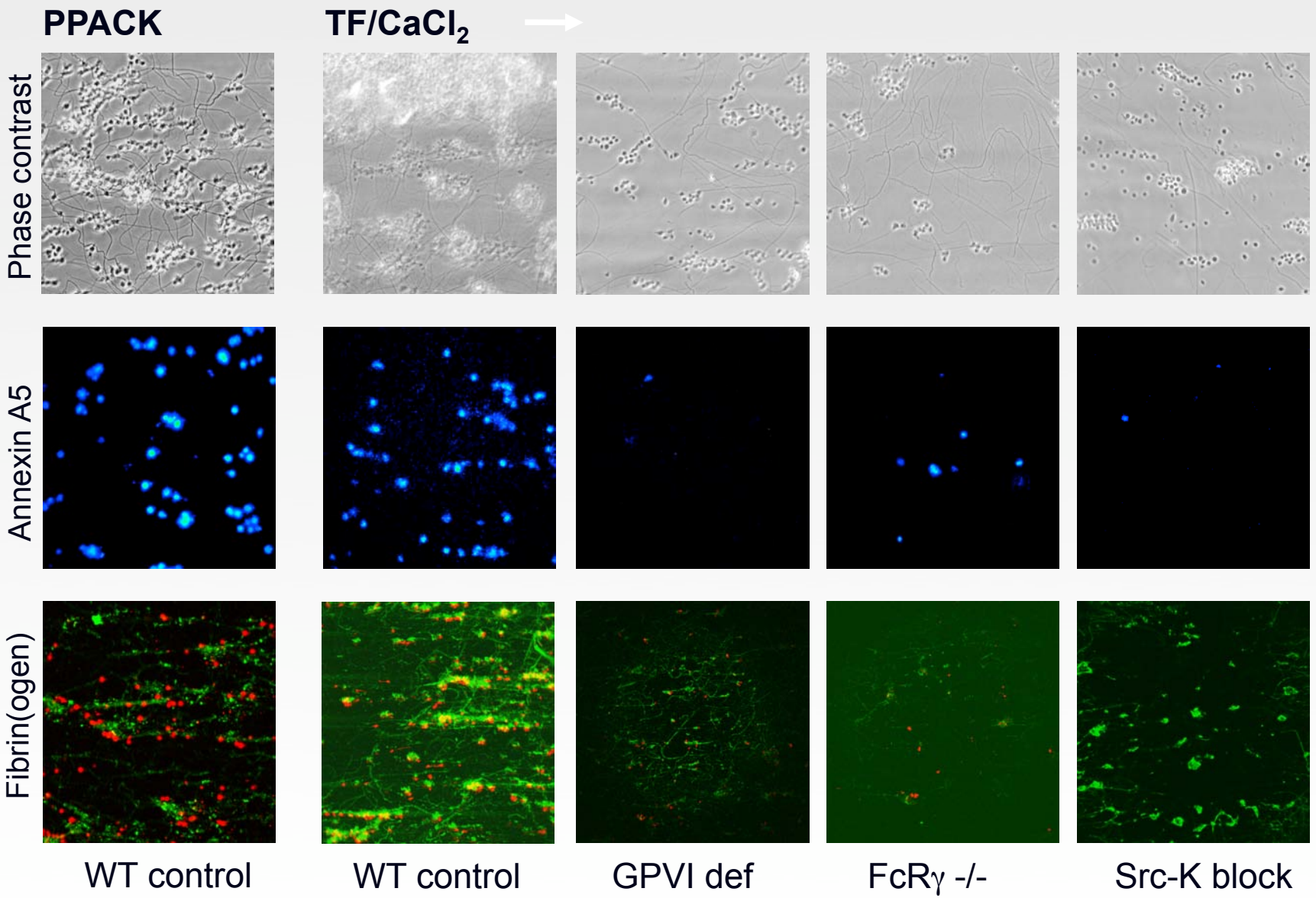


Control anti-GPVI



Kuijpers, FASEB J (2003), Siljander Blood (2004)

GPVI signalling in thrombus formation on collagen
blood from wildtype mice, coagulation with TF (1000 s^{-1})



Mouse proteins and factors contributing to thrombus formation under flow condition .... there are many more ....

Positive (platelet)

GPIb-V-IX, GPVI, $\alpha 2\beta 1$, $\alpha IIb\beta 3$, $P2X_1$, $P2Y_1$, $P2Y_{12}$, Par4, TP, Axl, Sky, Mer, CD36

Gi2, G13, Gq, FcRγ chain, LAT, Fyn, Lyn, Src, Syk, Erk, Rho-K, MLCK, Orai1, STIM1

PLC$\gamma 2$, PLCβ, PKCα, PKCβ, PI3Kα, PI3Kβ, PI3Kγ, Rab1b, Rac1, talin, kindlin-3

Negative (platelet)

IP, EP, PECAM-1, Gs

PKCδ, PKCθ, AC

Plasma

Fibrinogen, vWF, Gas6, prothrombin, factors V, VII, VIII, X, XI, XII, XIII, TFPI, AT

Thrombus formation in flow chambers

- human or mouse blood
- low or high shear rate
- suitable staining and detection

Questions 5:

Using flow assays to find novel prothrombotic proteins and factors?

- platelet activation
- coagulation activation

Testing pharmaceuticals for antithrombic properties?

Monitoring the efficacy of (novel) antithrombotic drugs
in patients?

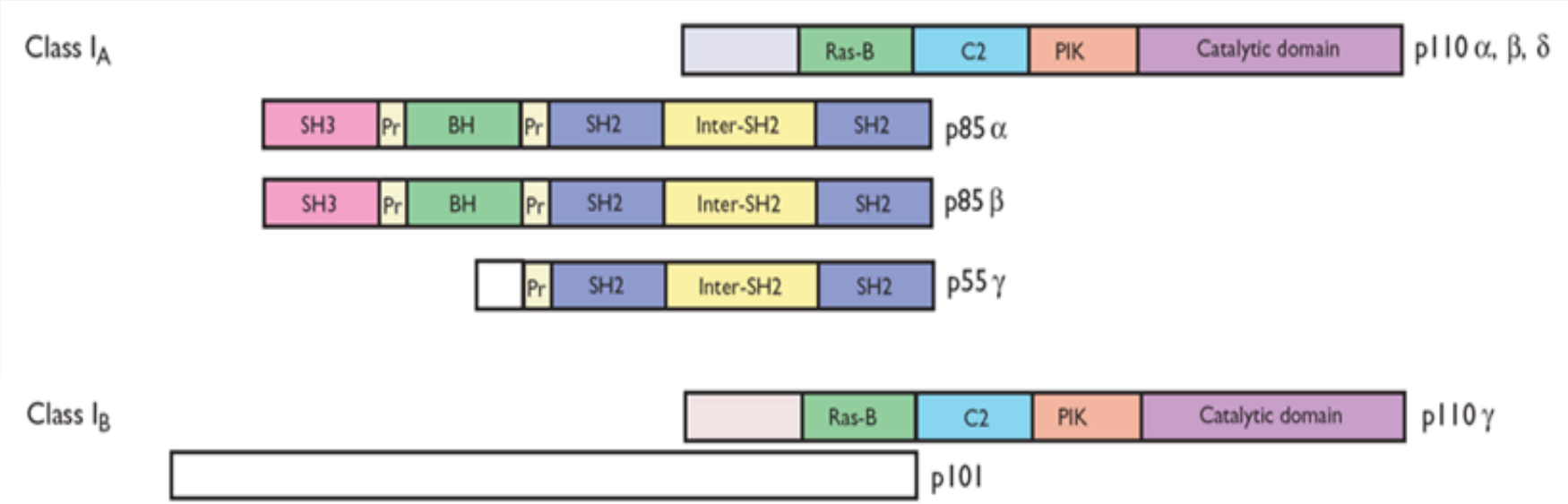
Role of phosphoinositide 3-kinase (PI3K) isoforms in platelets

PI3K isoforms present in platelets

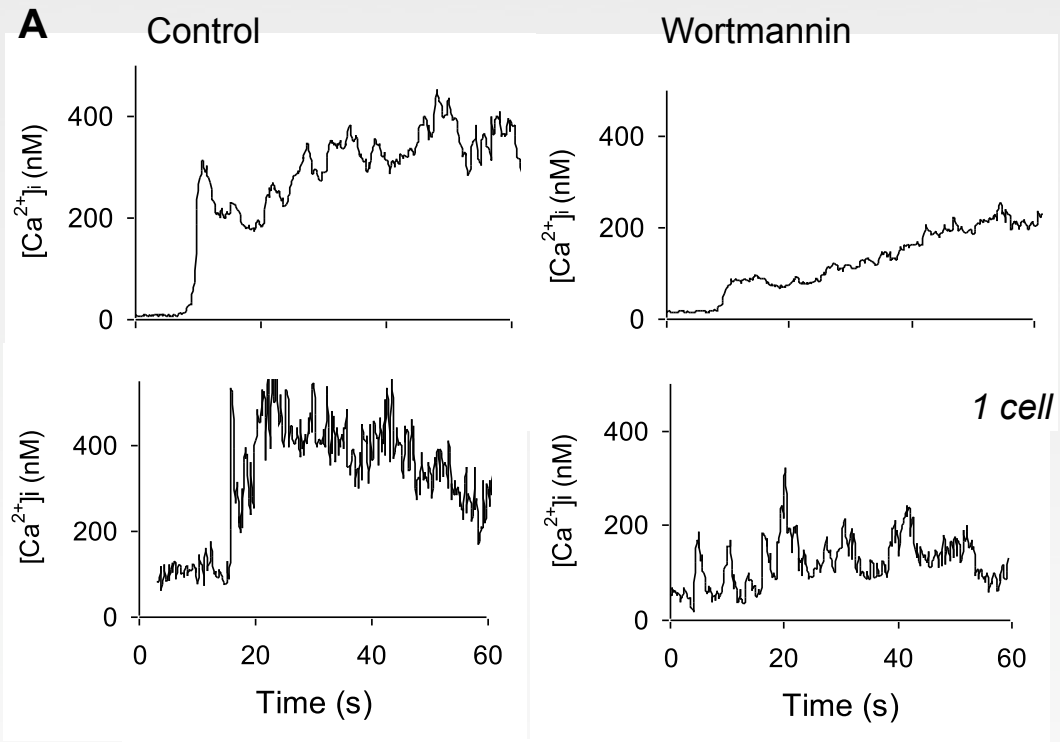
- PI3K isoforms have a catalytic and regulatory subunit
- Platelets express various PI3K isoforms

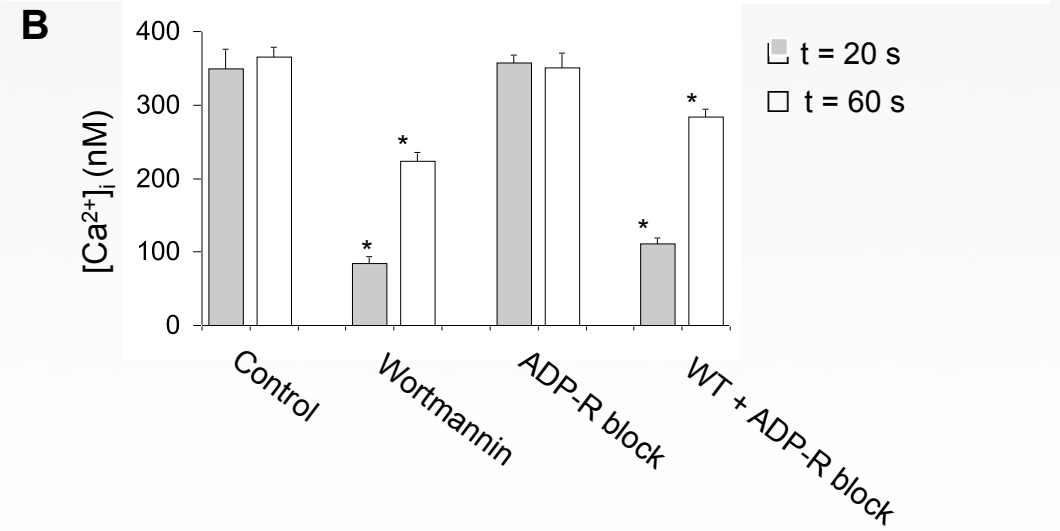
  Class Ia: p110α, β and little p110δ

  Class Ib: p110γ



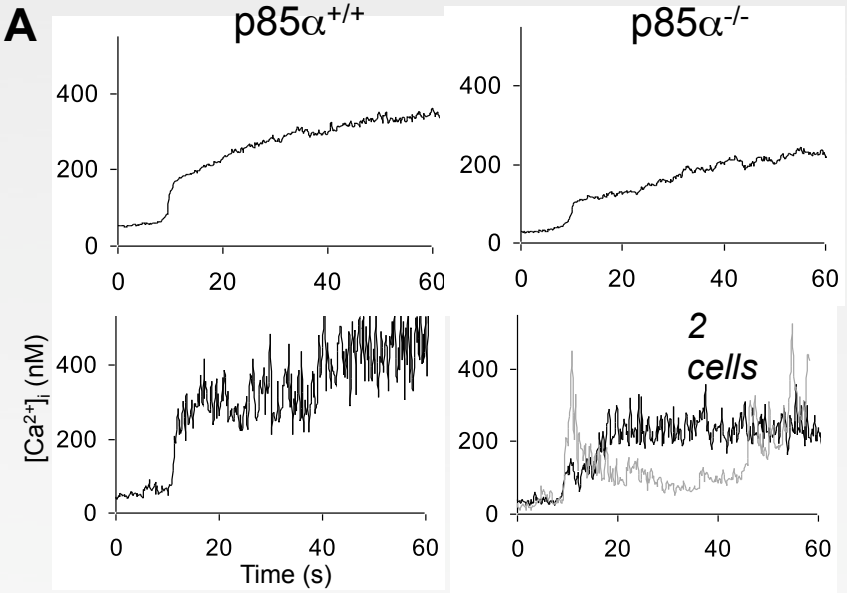
Effect of PI3K inhibition on human platelet Ca^{2+} responses under flow

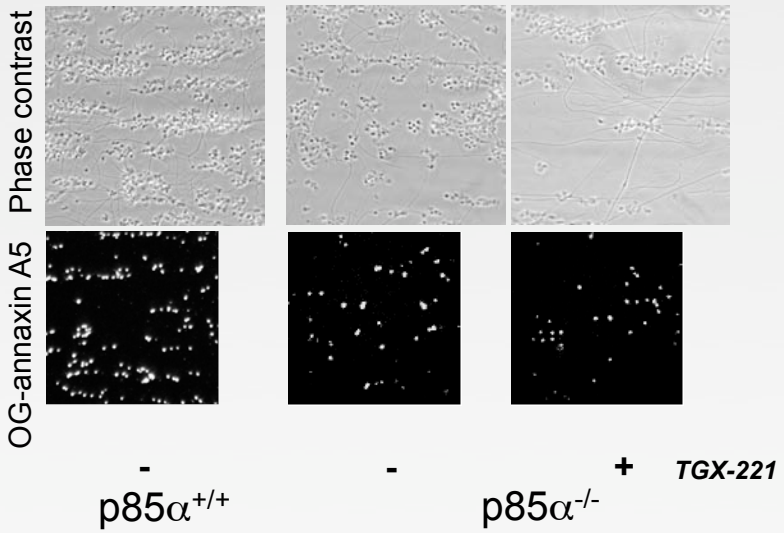


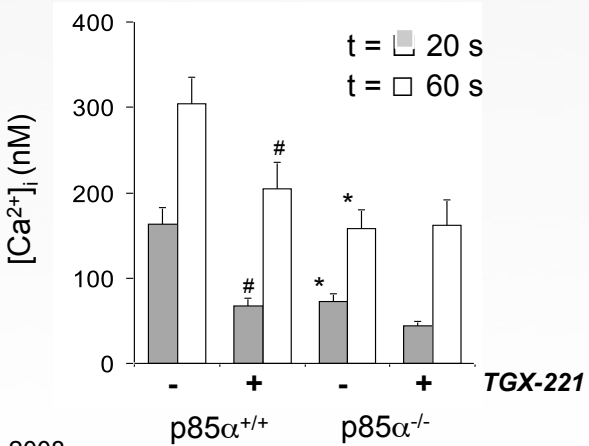


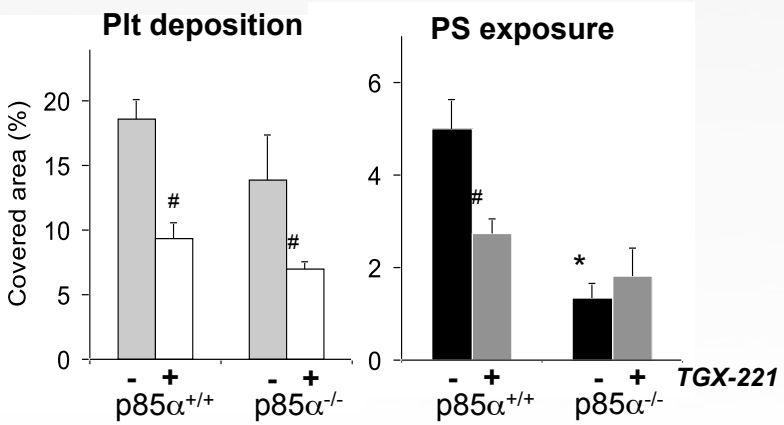
Munnix / Gilio (2008)

Mouse PI3Kα and PI3Kβ contribute to GPVI-induced thrombus formation under flow









Munnix / Gilio, 2008

Mouse PI3Kβ and PI3Kγ contribute to ADP-dependent thrombus stability under flow

- Collagen-dependent thrombus formation
- Shear rate 1000 s^{-1}

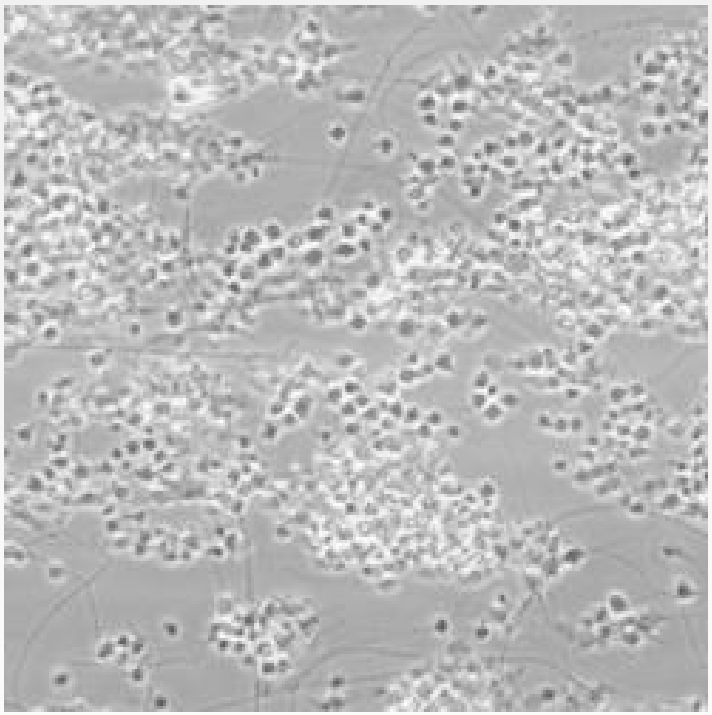
Wildtype **PI-3Kγ -/-**

Cosemans, Blood 2006

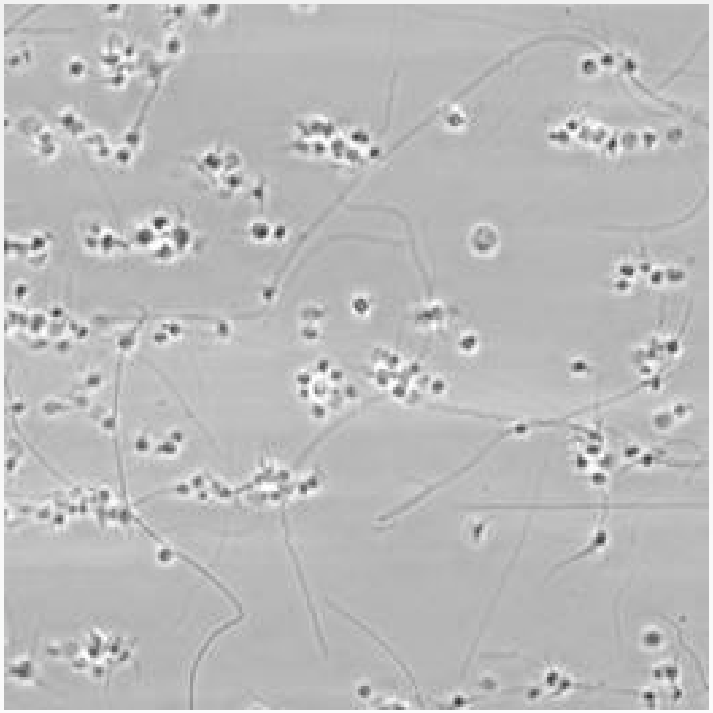
Role of protein kinase C (PKC) isoforms in platelets

Human PKC is required for thrombus formation under flow

Phase contrast



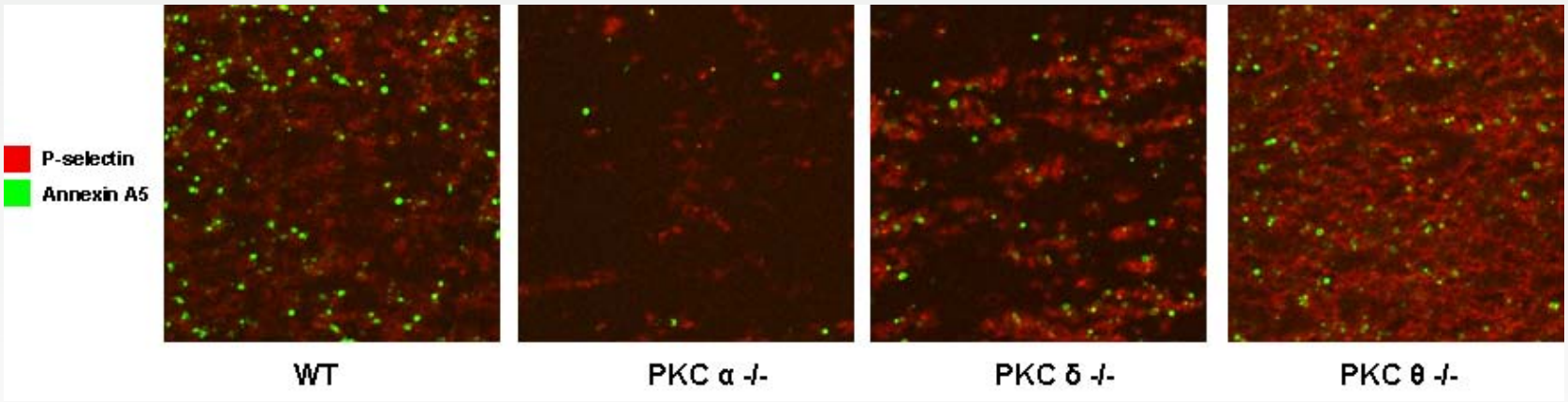
Vehicle



RO318425

Strehl, J Biol Chem (2007)

Thrombus formation under flow is differently regulated by PKC α and PKC δ/θ isoforms

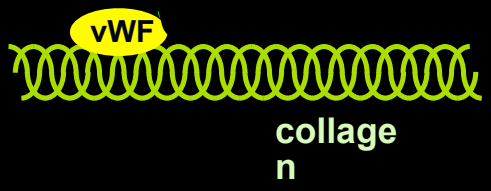


Gilio (2008)

The stages of thrombus formation

tethering and stable adhesion

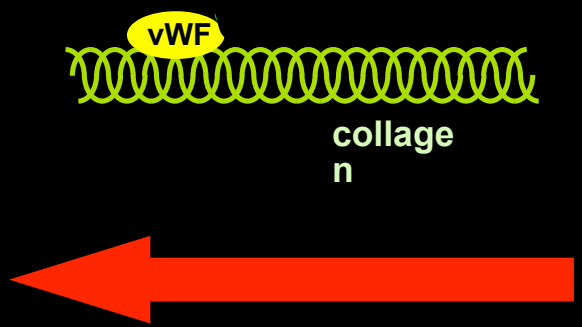
tethering



The stages of thrombus formation

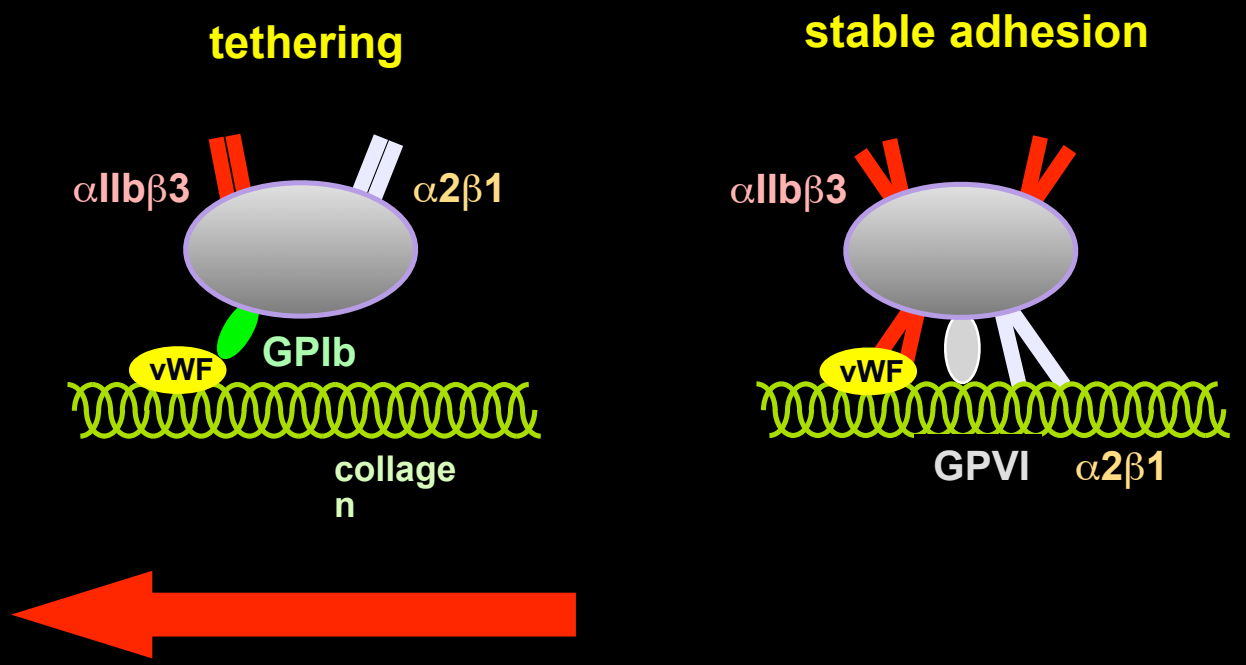
tethering and stable adhesion

tethering



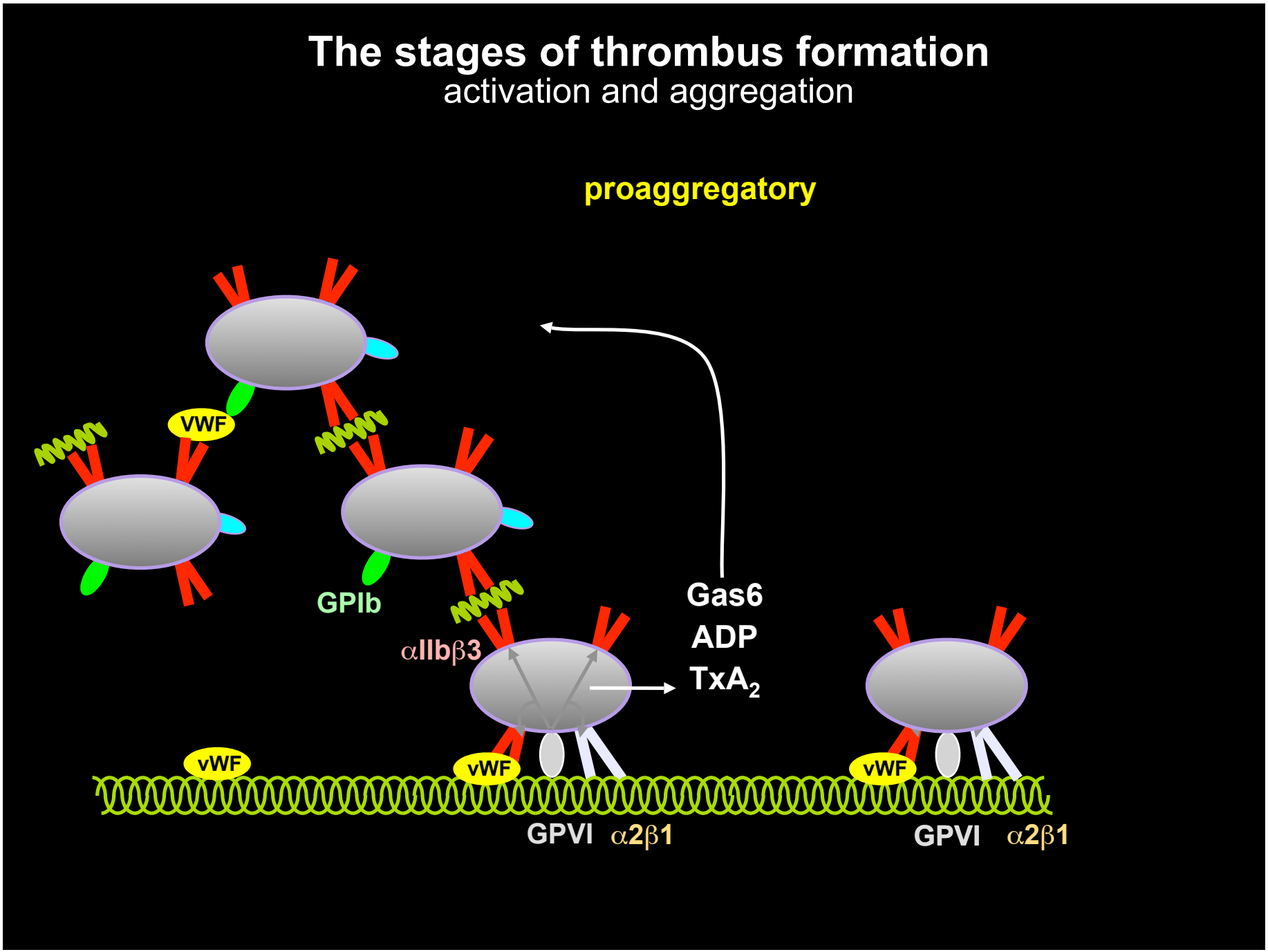
The stages of thrombus formation

tethering and stable adhesion



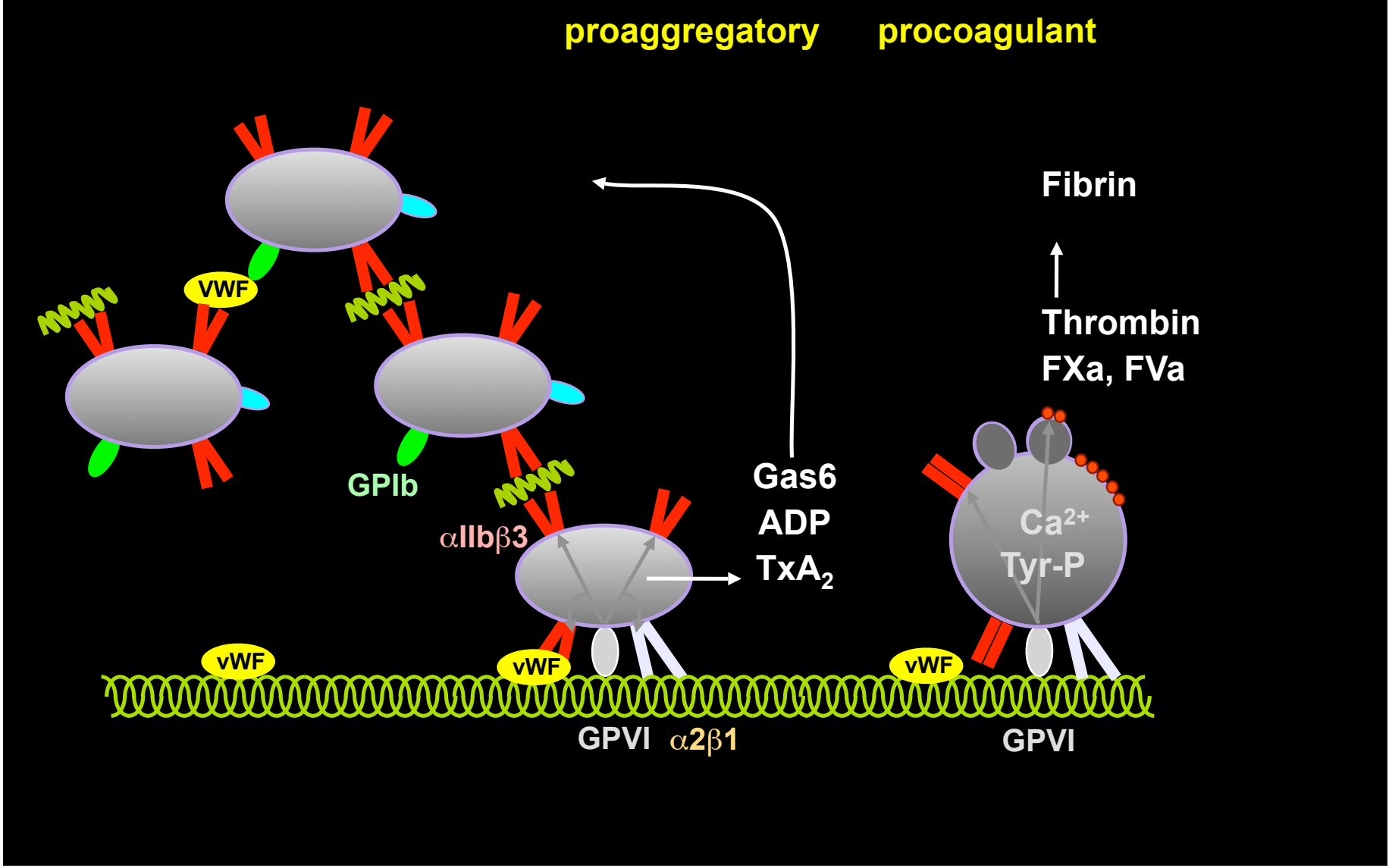
The stages of thrombus formation

activation and aggregation



The stages of thrombus formation

aggregation and coagulation



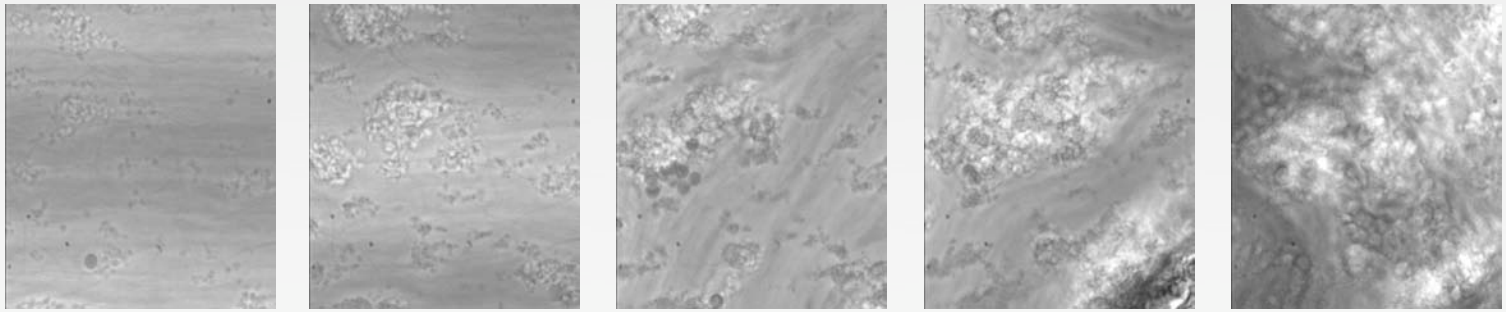
New possibilities:

contribution of thrombin and coagulation

Thrombus formation and clotting on collagen under flow

recalcification of citrated blood (no tissue factor)

Wildtype mouse

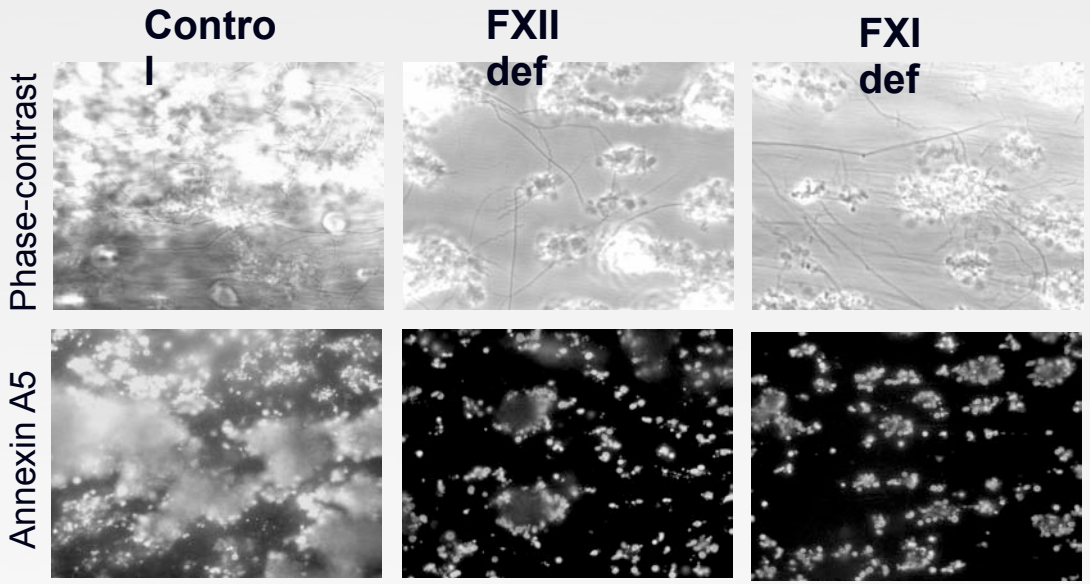


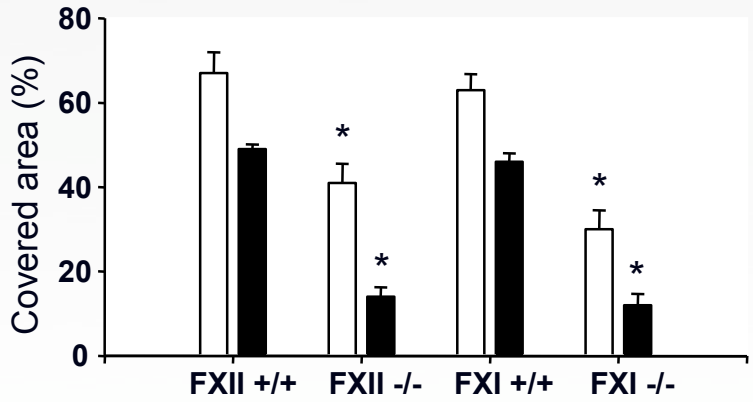
1 min | 2 min | 3 min | 4 min | 5 min

Van der Meijden, Blood (in press)

Thrombus formation and clotting on collagen under flow

recalcification of citrated mouse/human blood (no tissue factor)



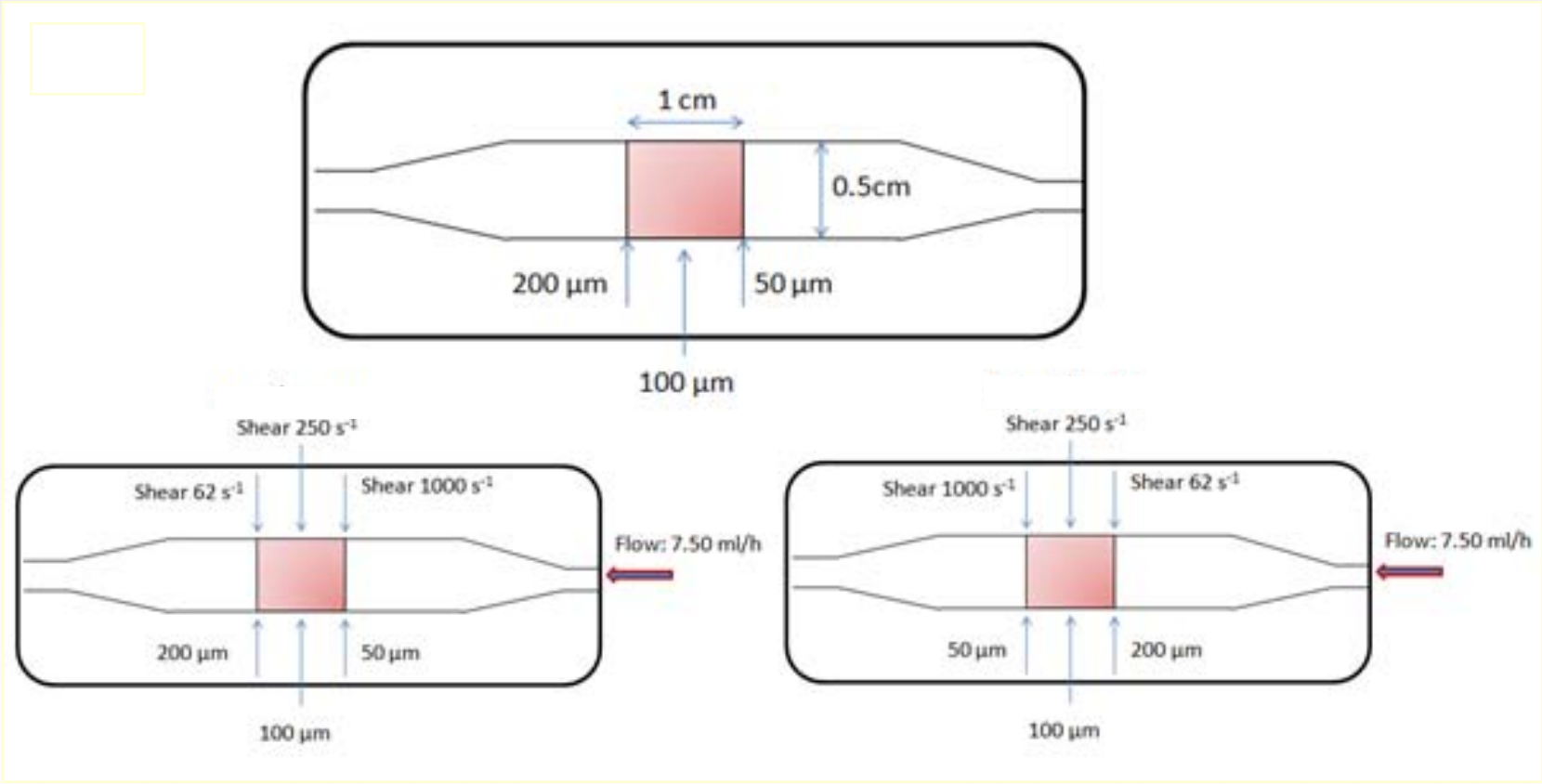


Van der Meijden, Blood (in press)

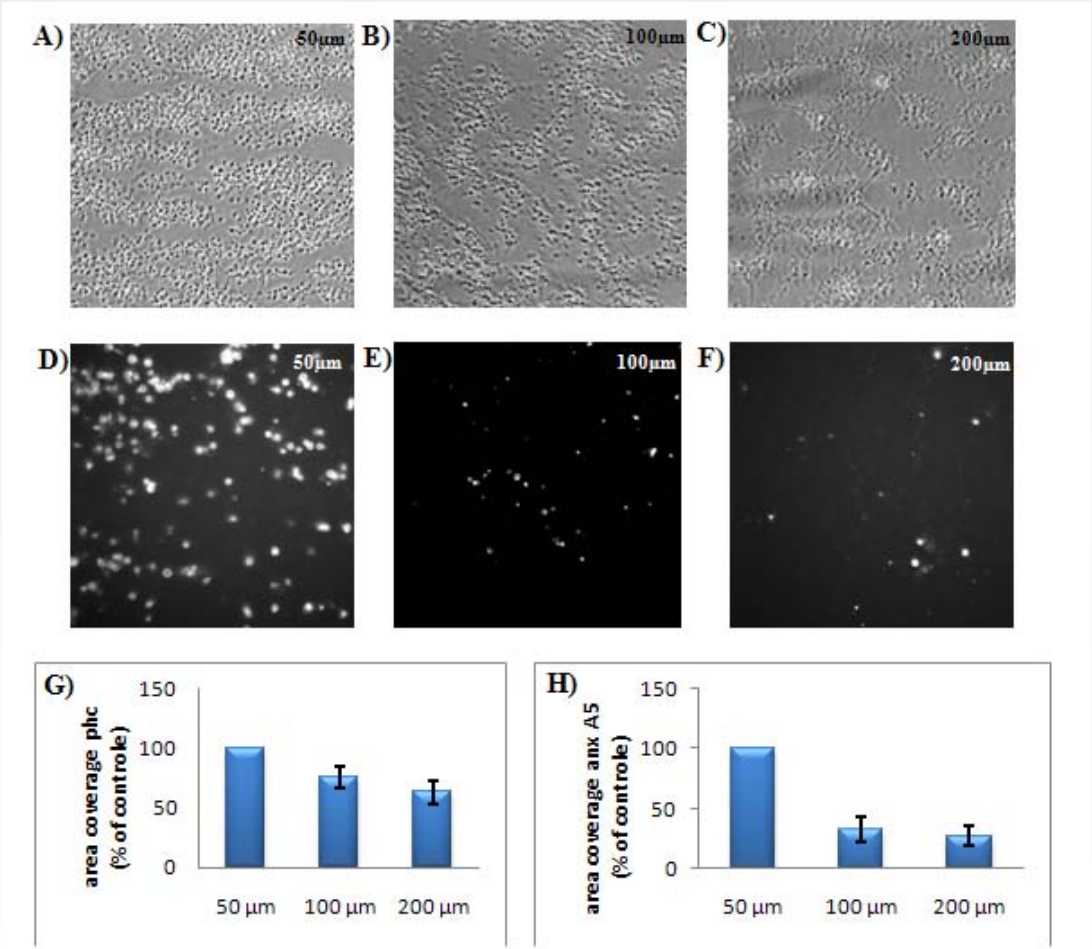
New possibilities:

simultaneous testing at multiple flow rates

Stenotic-type of flow chamber



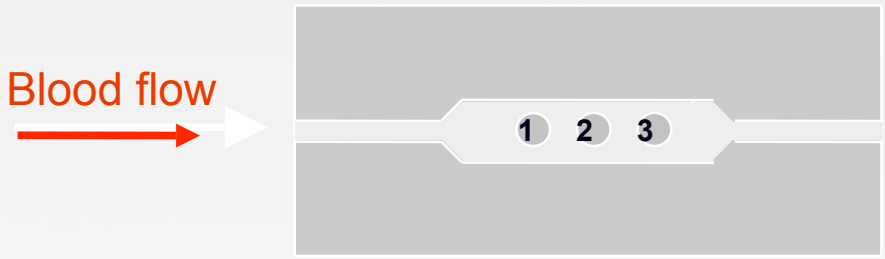
Thrombus formation decreases with flow chamber height



New possibilities:

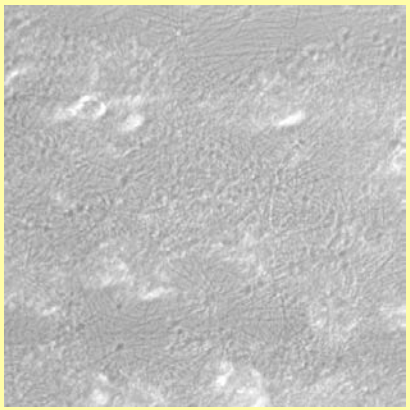
multiple thrombogenic substrates

Multiple coatings: immobilized tissue factor synergizes with collagen to trigger fibrin formation

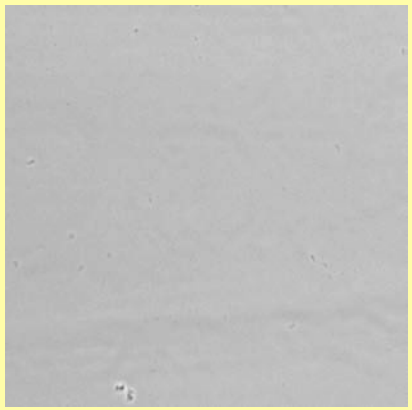


Collagen (spot 1)

Collagen + tissue factor (spot 2)



Tissue factor (spot 3)



New possibilities:

Inflammatory leukocytes on thrombus

Other blood cells

Conclusions

- There is a need for physiologically relevant integrative assays (bedside assays) to quantify the prothrombotic activity of blood.

- Flow chambers with collagen coating are valuable for measuring the multifactorial process of thrombus formation. This markedly resembles thrombus formation in vivo.

- Such flow chambers have a high potency for monitoring the efficacy of old and new antithrombotic drugs.

- They make it possible to test drug interactions, etc.

Conclusions (3R)

- Blood from GM animals can be tested *in vitro* instead of *in vivo*.
- Human blood instead of animal blood can be used for drug testing.
- Still limitations in analysis power.
- Higher capacity test systems need to be developed for 3R purposes.


CARIM

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