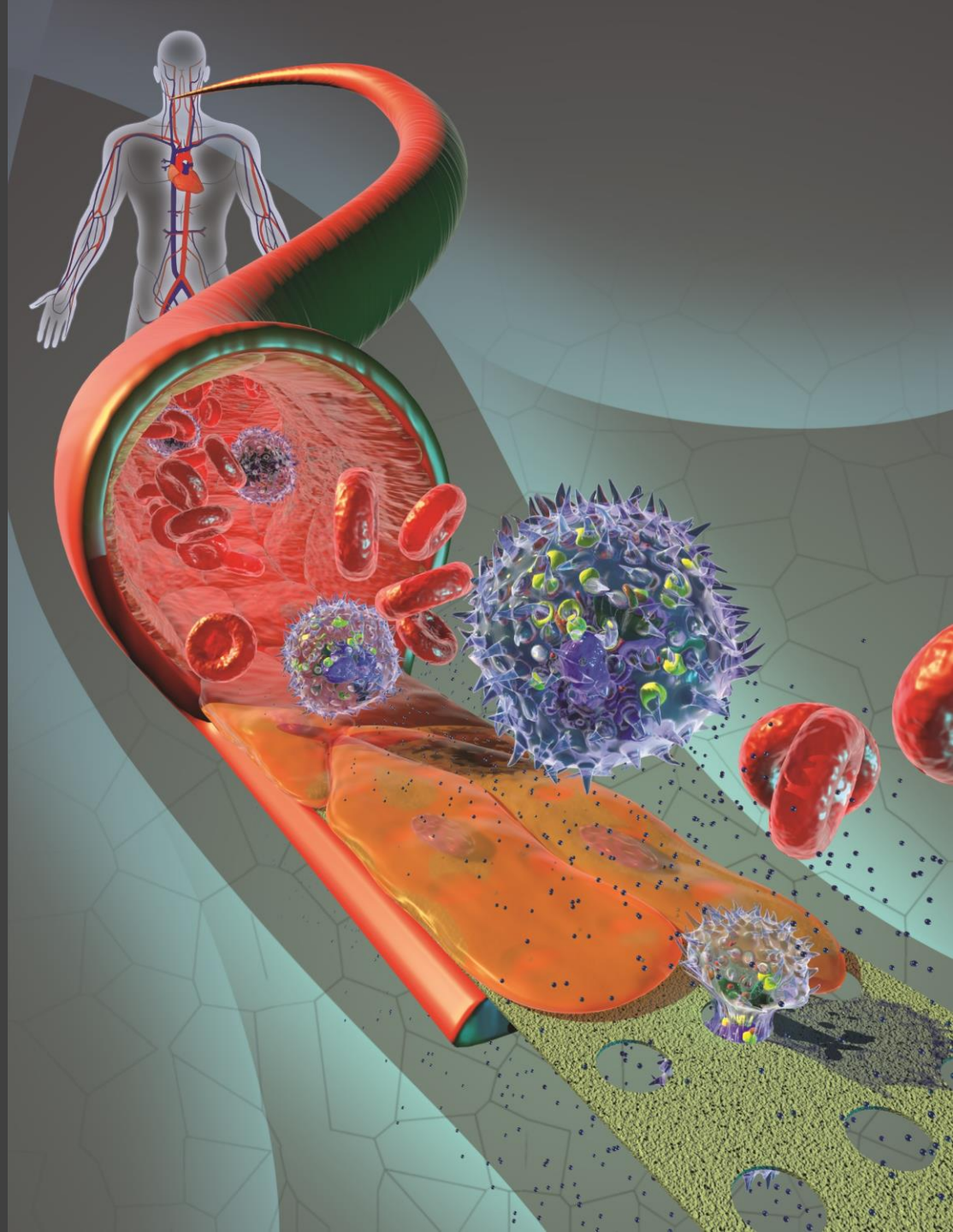
Pulmonary Microvasculature Mimetics for Investigating the Role of Endothelial Apicobasal Polarity in Inflammation and Sepsis

Proposal Presentation

Alec Salminen

April 16th, 2019



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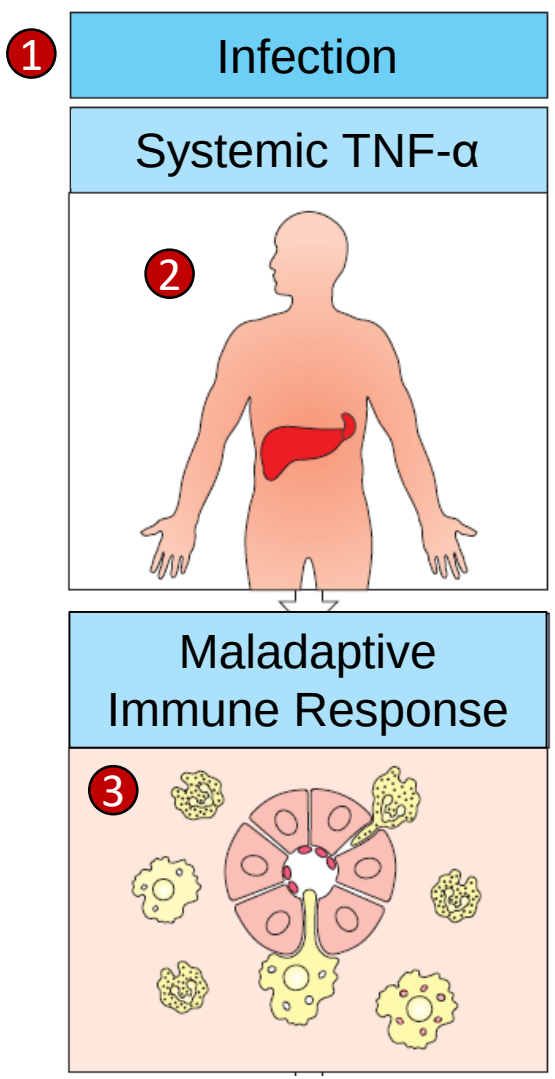
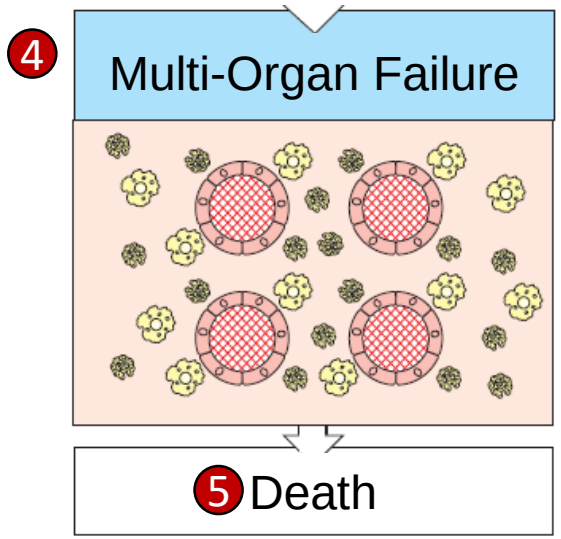
Pathophysiology of Sepsis



Janeways Immunobiology 9th



Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection

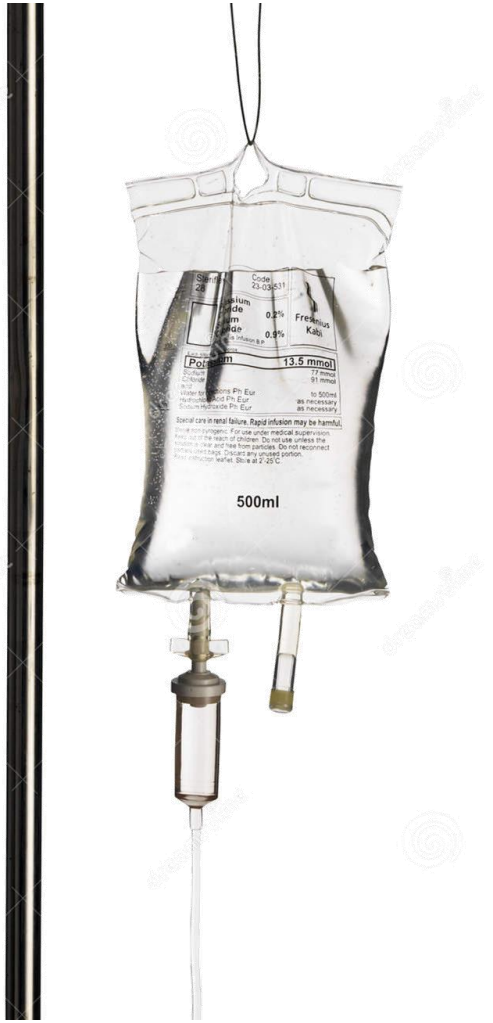
Third International Consensus Definitions for Sepsis and Septic Shock **2016**

Sepsis in The Clinic

Global sepsis cases total ~31.5 million each year, with ~5.3 million resulting in death (**15-25% mortality**)

In the USA, this amounts to ~**250,000 deaths** annually

Clinical treatment mainly consists of managing symptoms (fluid replacement, ventilation) and antibiotics



Epidemiology: Hotchkiss et al., Nature Reviews Disease Primers 2016

Defining Sepsis in The Clinic: SOFA Score

The **sequential organ failure (SOFA) score** is utilized in the clinic to predict and track patient outcome due to complications of sepsis

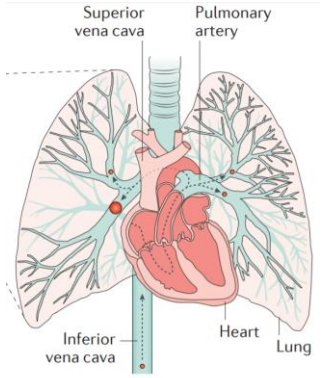


TABLE 1. SEQUENTIAL ORGAN FAILURE SCORE

	0	1	2	3	4
Ventilation: $PaO_2:FiO_2$, mm Hg	>400	≤400	≤300	≤200[a]	≤100[a]
Coagulation: platelets × $10^3/mm^3$	>150	≤150	≤100	≤ 50	≤ 20
Liver: bilirubin, mg/dL (micromol/L)	<1.2 (<20)	1.2–1.9 (20–32)	2.0–5.9 (33–101)	6.0–11.9 (102–204)	>12.0 (>204)
Cardiovascular	No hypotension	Mean arterial pressure <70 mm Hg	Dopamine ≤5 or dobutamine (any dose)[b]	Dopamine >5 or epinephrine ≤0.1 or norepinephrine <0.1[b]	Dopamine >15 or epinephrine >0.1 or norepinephrine >0.1[b]
Central nervous system: Glasgow Coma Scale	15	13-14	10–12	6–9	< 6
Renal: creatinine, mg/dL (micromol/L) or urine	<1.2 (<110)	1.2–1.9 (110–170)	2.0–3.4 (171–299)	3.5– 4.9 (300–440) or <500 mL/day	> 5.0 (>440) or <200 mL/day

[a]With ventilatory support.
[b]Adrenergic agents administered for at least one hour (doses are given in mcg/kg/min).

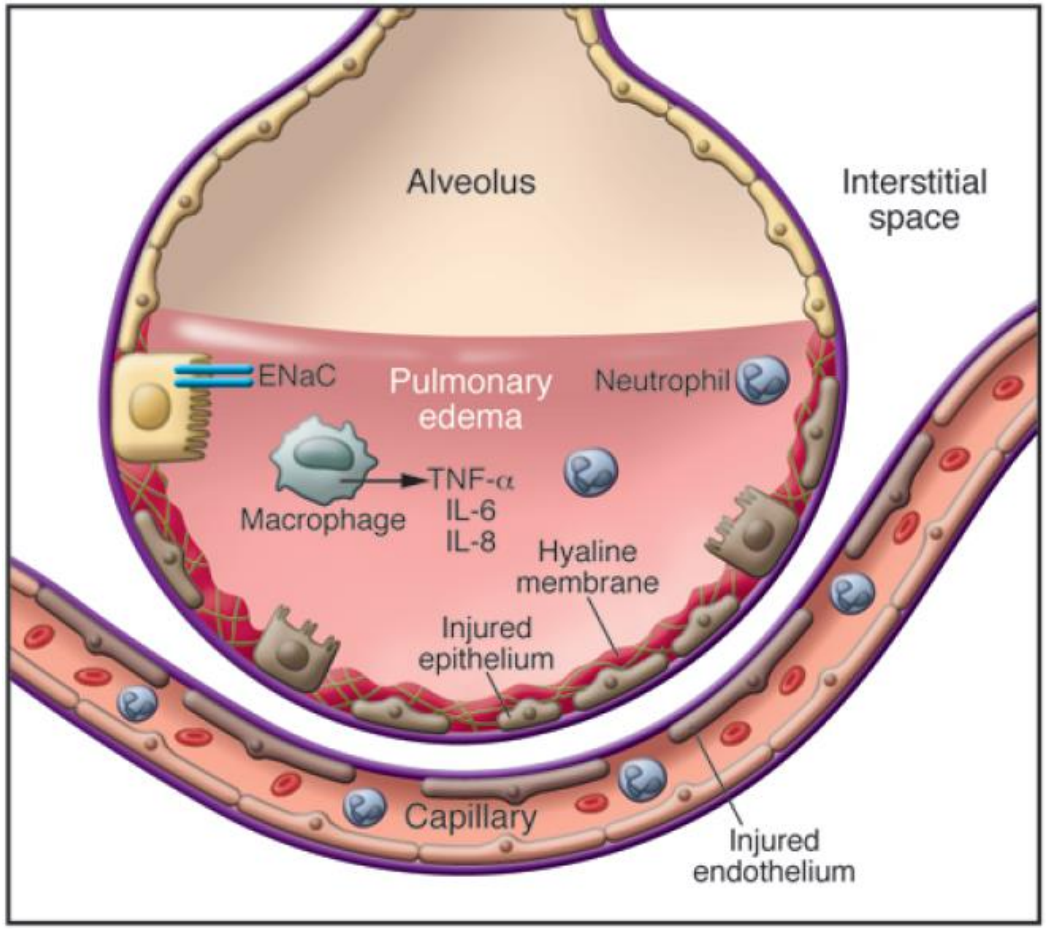
Vincent et al., Surgical Infections 2006

ARDS: The Pulmonary Manifestation of Sepsis

Sepsis is the number 1 risk factor for acute respiratory distress syndrome (ARDS)

ARDS Pathophysiology is defined by:

- Dysregulated inflammation
- Inappropriate accumulation and **activity of leukocytes**
- Uncontrolled coagulation
- Altered **permeability of endothelial** and epithelial barriers of the alveoli



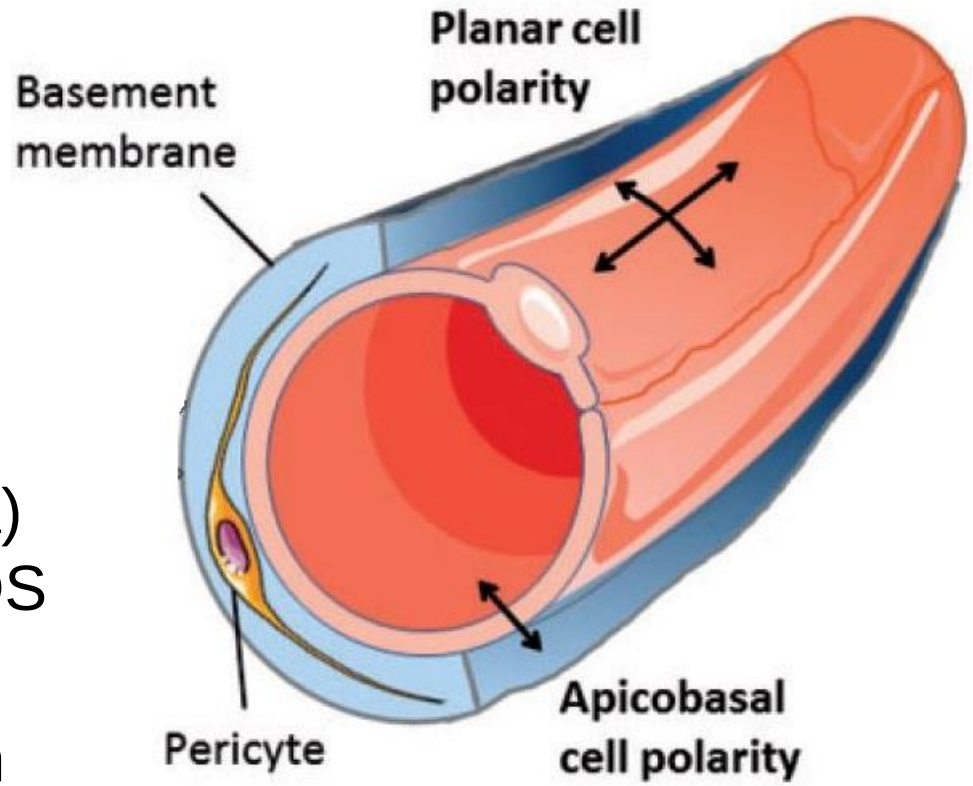
Info and Graphic: Matthay et al., J Clin Invest. 2012

Endothelium Function and Polarity

The pulmonary endothelial cell barrier regulates **leukocyte extravasation** and **edema**

Endothelial cell interactions with inflammatory cytokines (e.g. TNF-α) and pathogen derived endotoxins (e.g. LPS from gram-negative bacteria) can alter these factors, leading to sepsis and ARDS

How do we therapeutically target dysregulation on an endothelial cell level?



Graphic adapted from: Worzfeld et al., JCBFM 2016

Targeting the Immune Response – For Better or For Worse?

LPS (Endotoxin)

Signals through membrane bound Toll-like receptors (**TLR-4**)

Blocking/Transgenic Studies:

- TLR-4 Antagonist
- TLR-4 LOF Mutation
- HA-1A
- J5

Result: Murine resistance to systemic LPS induced sepsis; susceptibility to LPS bearing pathogens

TNF-α (Cytokine)

Signals through membrane bound TNF receptor family (**TNFR1** and TNFR2)

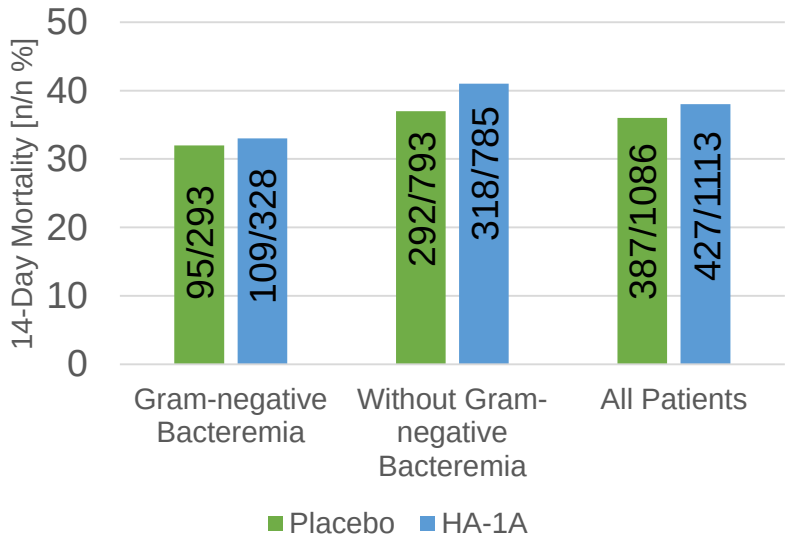
Can be blocked at many levels:

- Systemic (IV Soluble TNFR1)
- Ab (Monoclonal or Polyclonal)
- Receptor Antagonist

Result: Murine resistance to sepsis; high susceptibility to primary (local) infections

Anti-Endotoxin Therapeutics

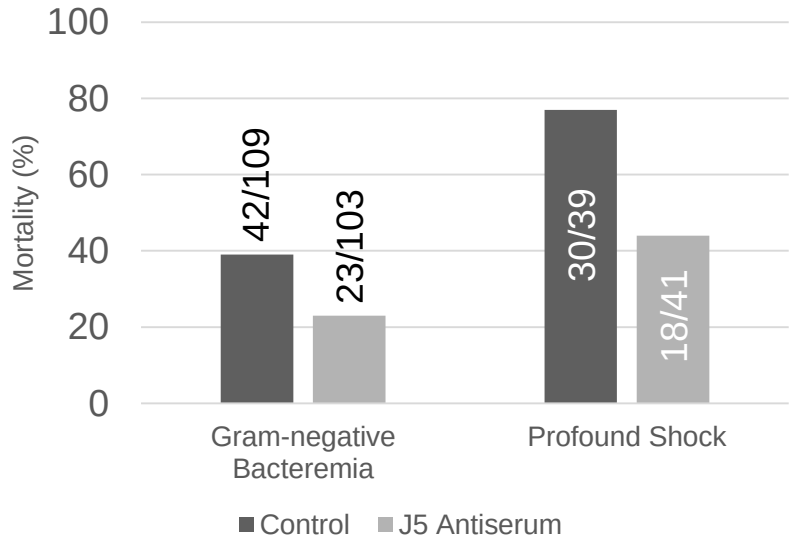
Human Anti-Lipid A Clinical Trails



<u>Conclusion</u>: not effective, inclusion criteria must be reassessed

Graphic adapted from: McCloskey et al., Annals of Internal Medicine 1994

J5 Antiserum (LPS Vaccine)

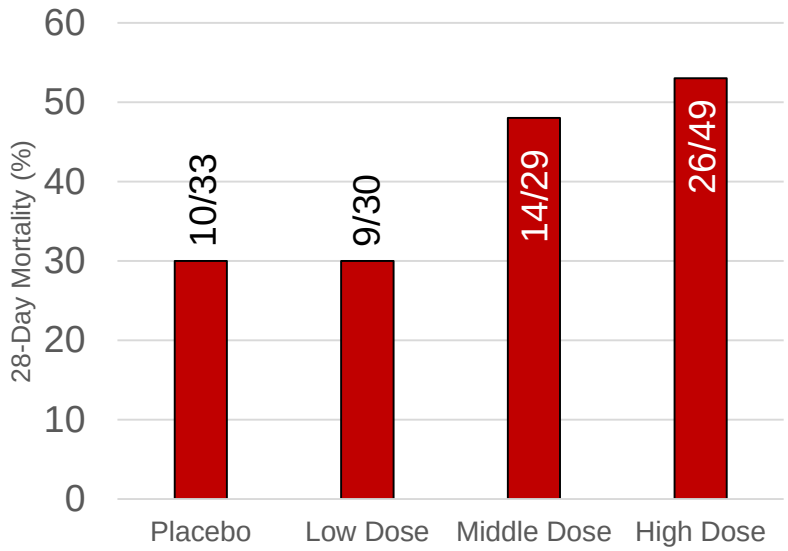


<u>Conclusion</u>: effective; subsequent studies **disprove** results

Graphic adapted from: Ziegler et al., NEJM 1982

Anti-Cytokine Therapeutics

TNFR:Fc Fusion Protein
[Etanercept]



Conclusion: not effective, may increase mortality?

Graphic adapted from: Fisher et al., NEJM 1996

Hypothesis: Anti-TNF-α therapy is opening the door for opportunistic pathogens

Overarching Problem

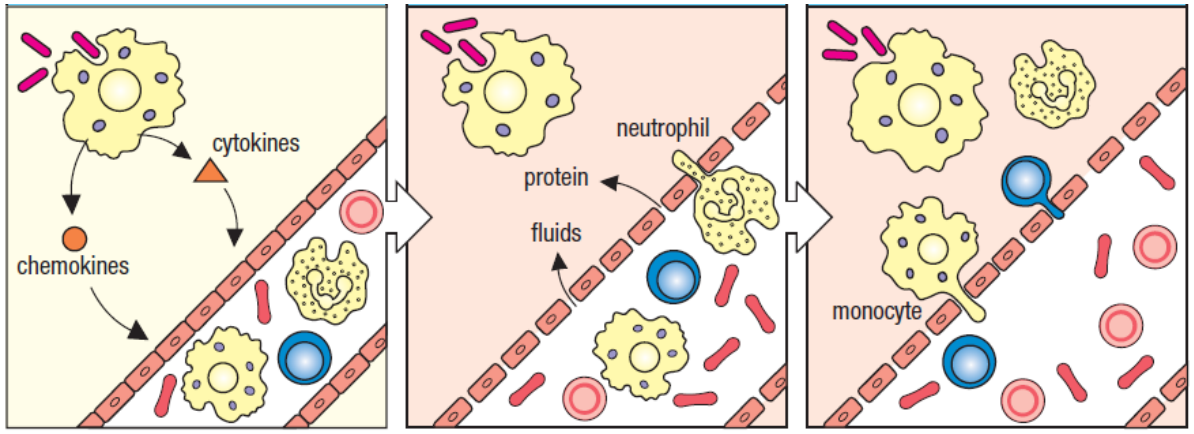
- Sepsis therapeutics, past and present, struggle to balance remedial and pathological immunosuppression
- Developing novel therapeutics *in vivo* is time consuming and expensive

Guiding Hypothesis

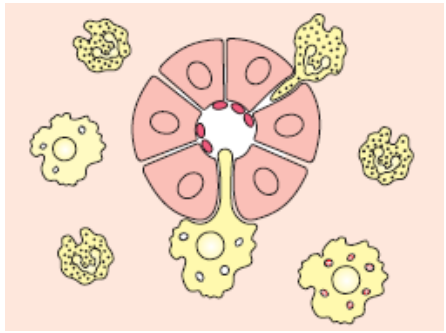
Pulmonary microvasculature mimetics can be utilized to study the distinguishing characteristics of a host response to a **primary infection** versus a **systemic inflammatory response**

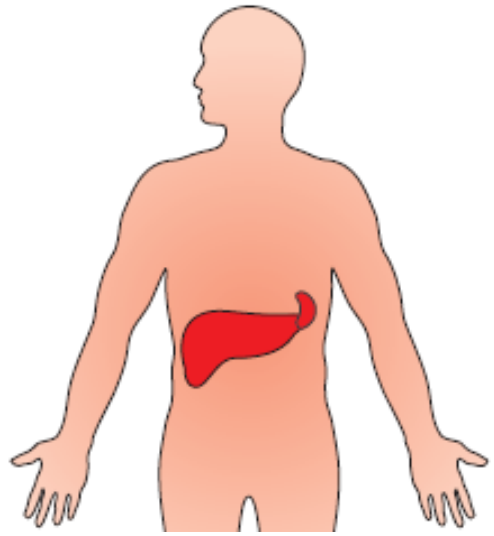
Rationale

Local Infection: **Abluminal** Cytokine



Sepsis/SIRS: Systemic/**Luminal** Cytokine





Specific Aims

1. Develop a facile microvasculature mimetic to quantify small molecule and neutrophil permeability in the presence of physiological shear
2. Elucidate the effects of polarized cytokine and endotoxin delivery on neutrophil dynamics and endothelial cell biology
3. Demonstrate the polarized dependence of S1P in regulating pulmonary vasculature permeability and neutrophil dynamics following septic insult *in vitro*

Specific Aims

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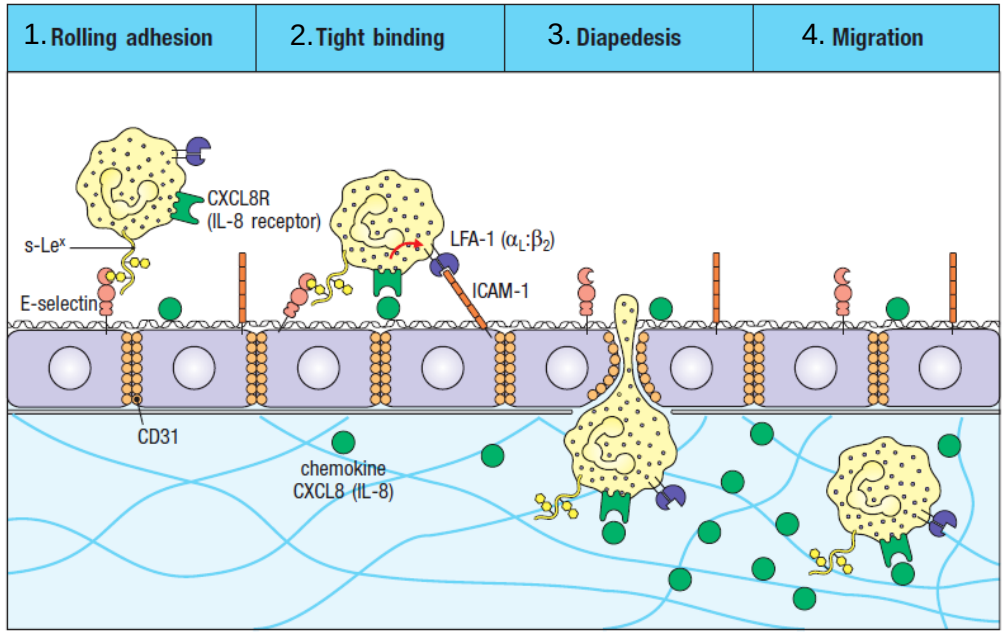
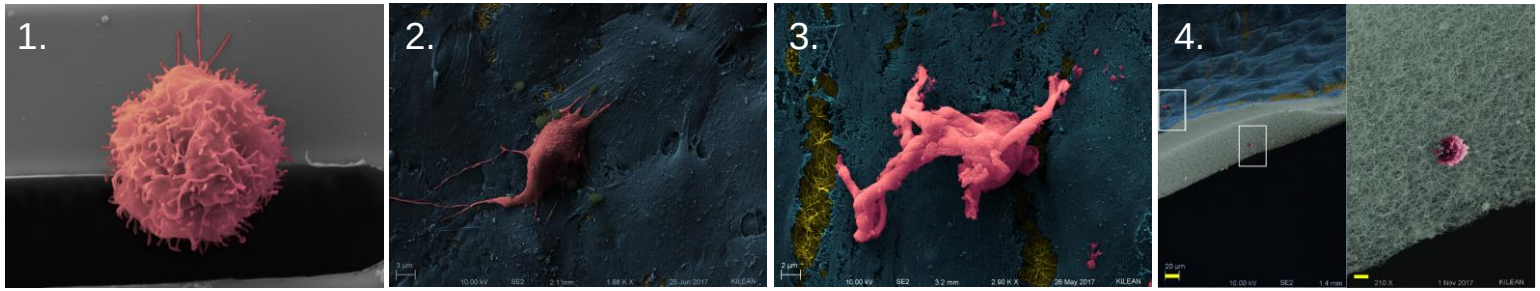
Bringing the Pulmonary Microvasculature to the Lab Bench



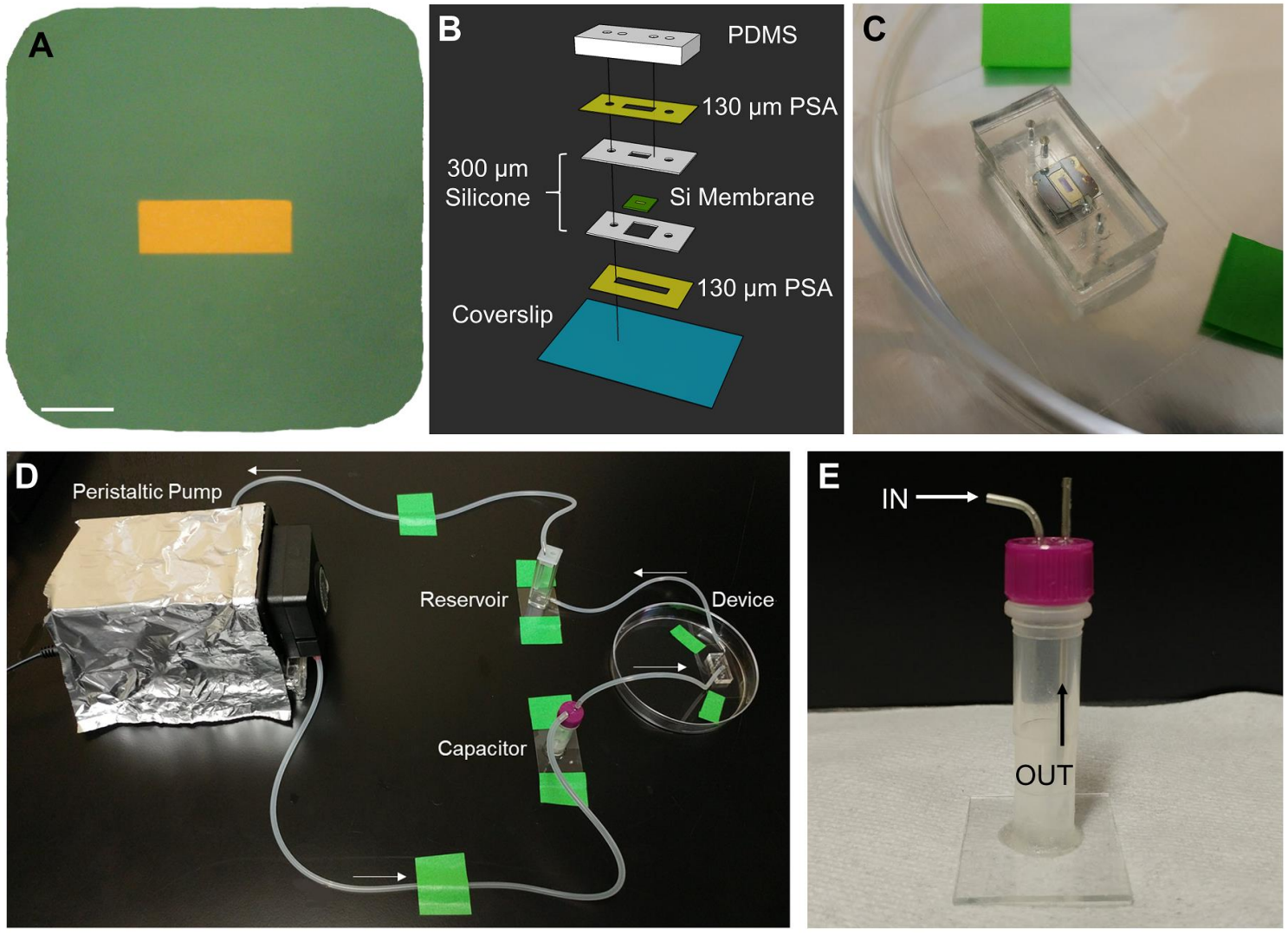


Success Metrics

I. Contain defined luminal and abluminal media compartments
II. Allow for shear priming of microvascular endothelial cells on a porous substrate
III. Permit phase contrast tracking of live PMNs
IV. Facilitate continuous *in situ* permeability measurements

Schematic: Janeways Immunobiology 9th

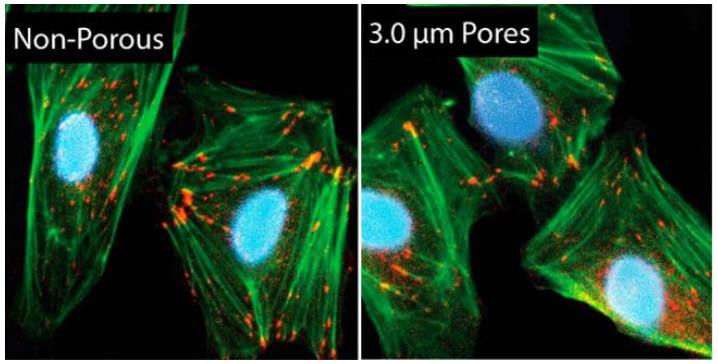
μSiM-PVB: Microfluidic Silicon Membrane Enabled Pulmonary Vascular Barrier

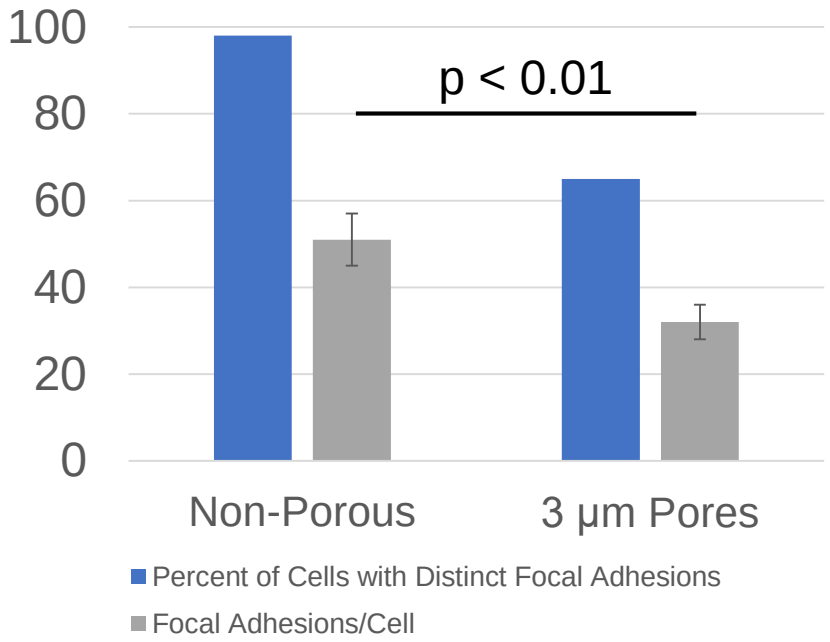


- ✓ Silicon membranes enable phase contrast imaging
- ✓ Layer-by-layer fluidic assembly features defined luminal and abluminal channels
- ✓ Custom flow circuit permits bubble-free shear priming of cells

Salminen et al., SMALL 2019

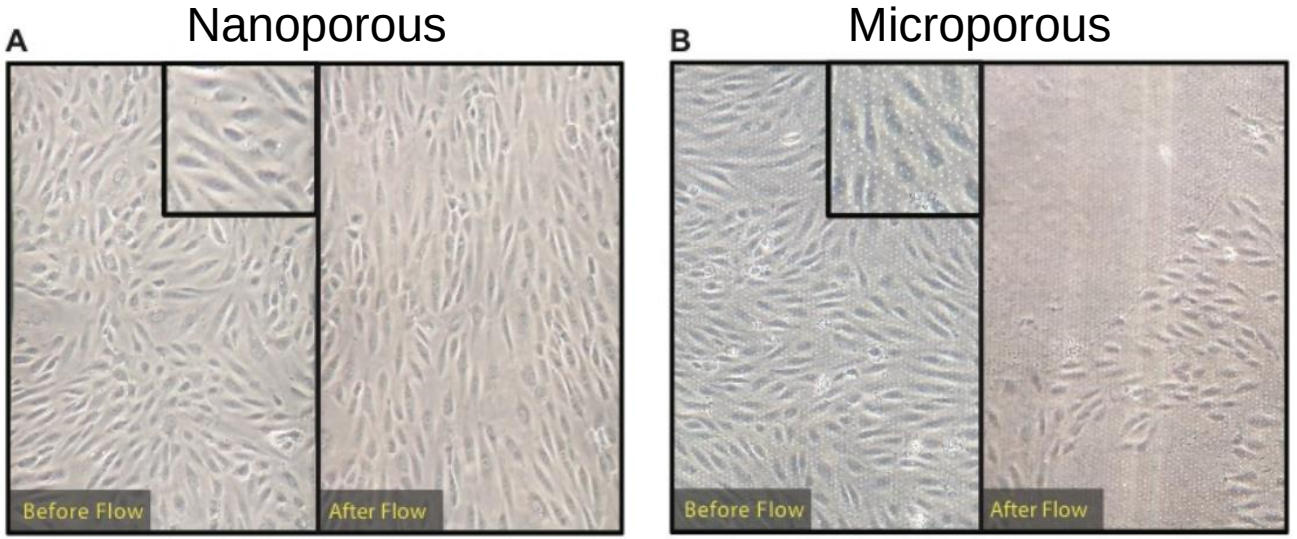
Cell Culture on Microporous Substrates





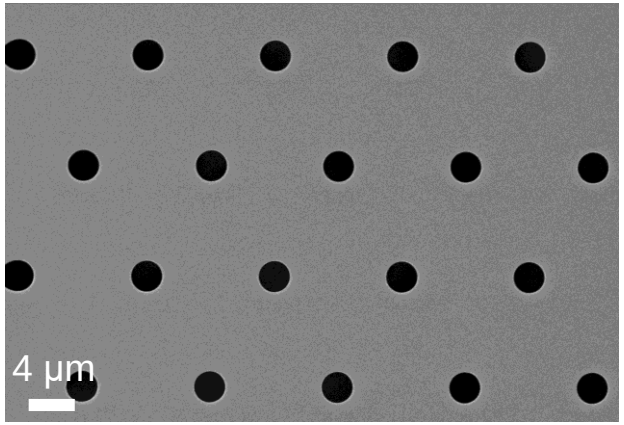
A silicon nanomembrane platform for the visualization of immune cell trafficking across the human blood–brain barrier under flow

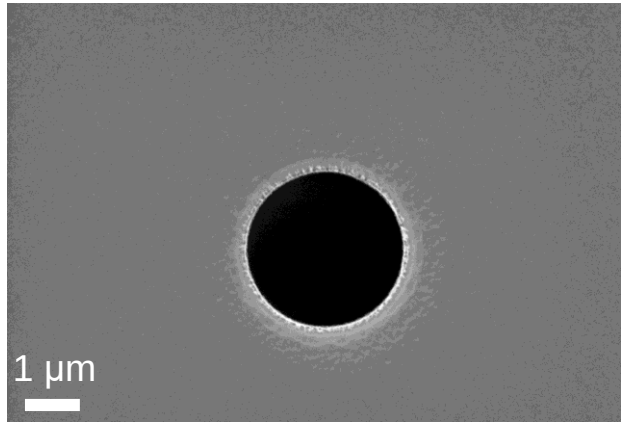
Adrien Mossu[1,*], Maria Rosito[1,*], Tejas Khire[2], Hung Li Chung[2], Hideaki Nishihara[1], Isabelle Gruber[1], Emma Luke[2], Lucie Dehouck[3], Federica Sallusto[4,5], Fabien Gosselet[3], James L McGrath[2] and Britta Engelhardt[1]



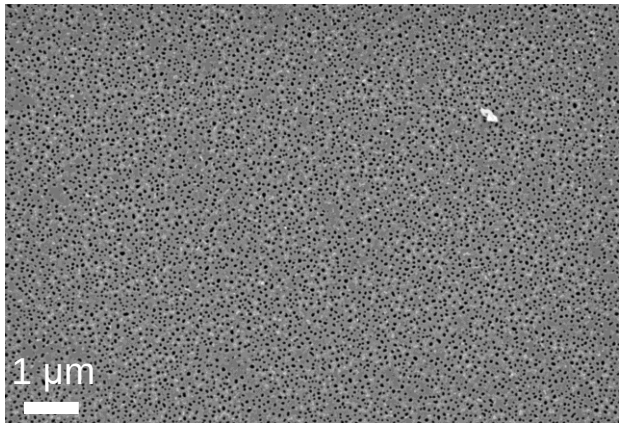
Adapted from: Casillo et al., ACS Biomater. Sci. Eng. 2017

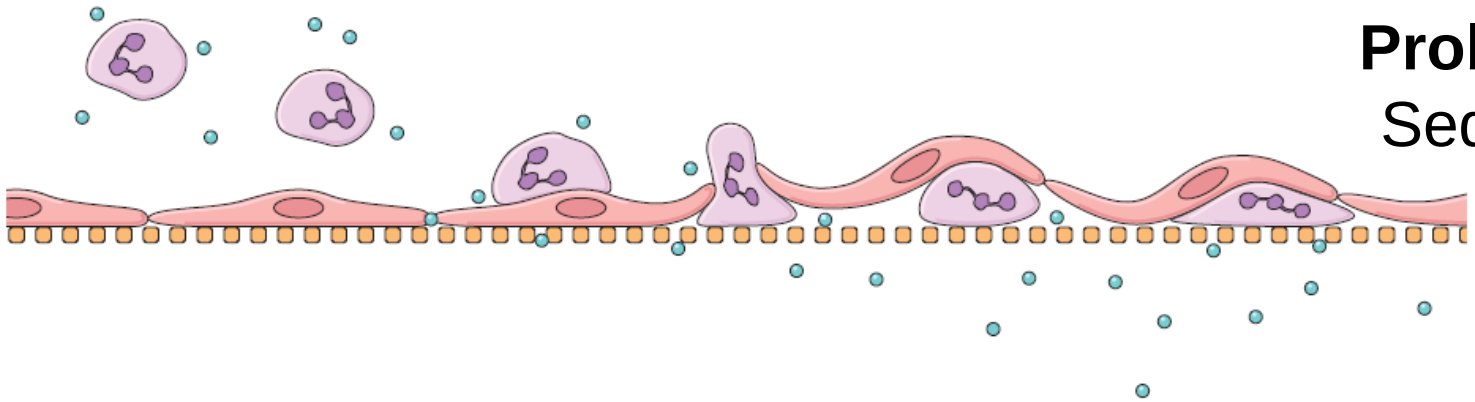
Membrane Cell Culture Paradox





Problem: Cell loss under physiological shear

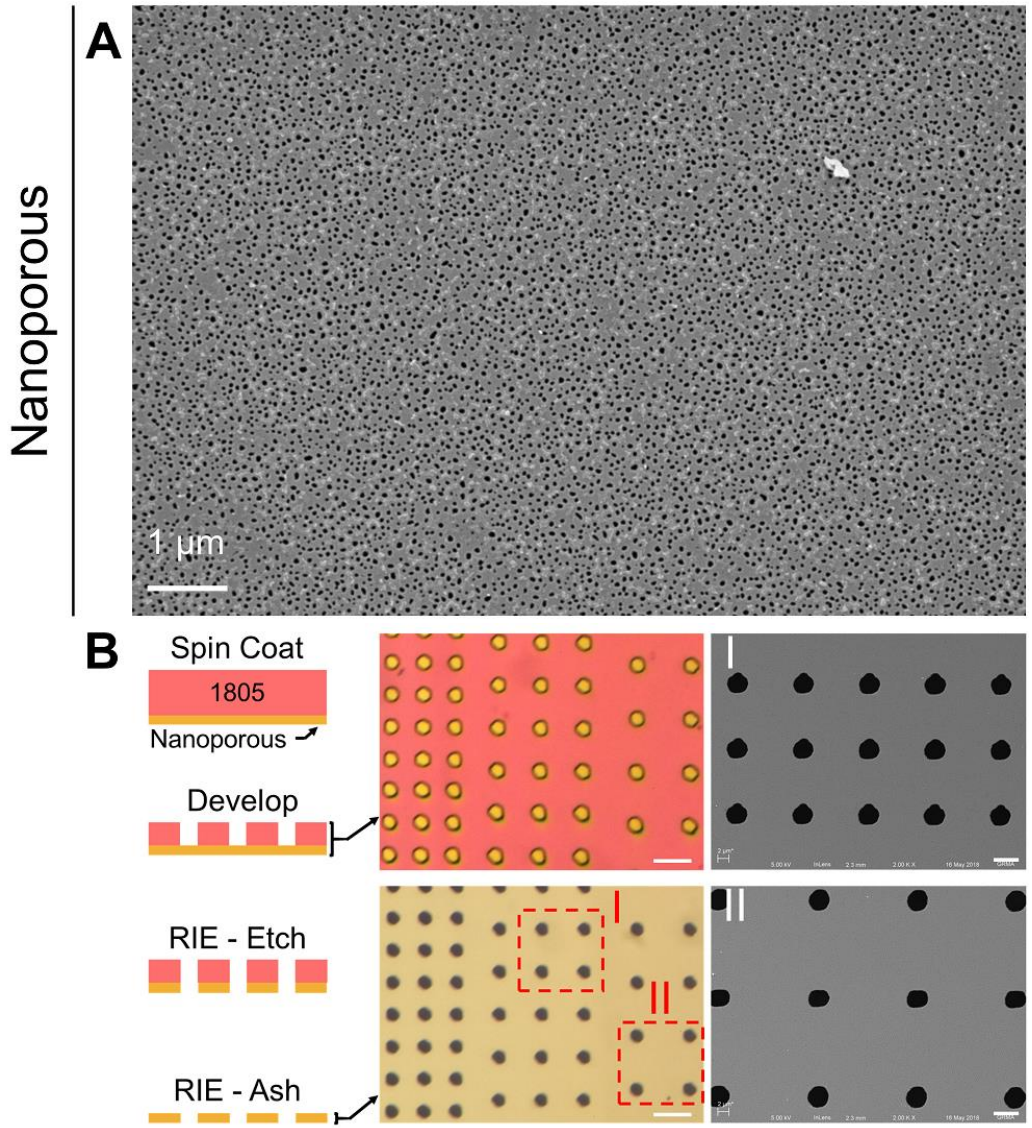


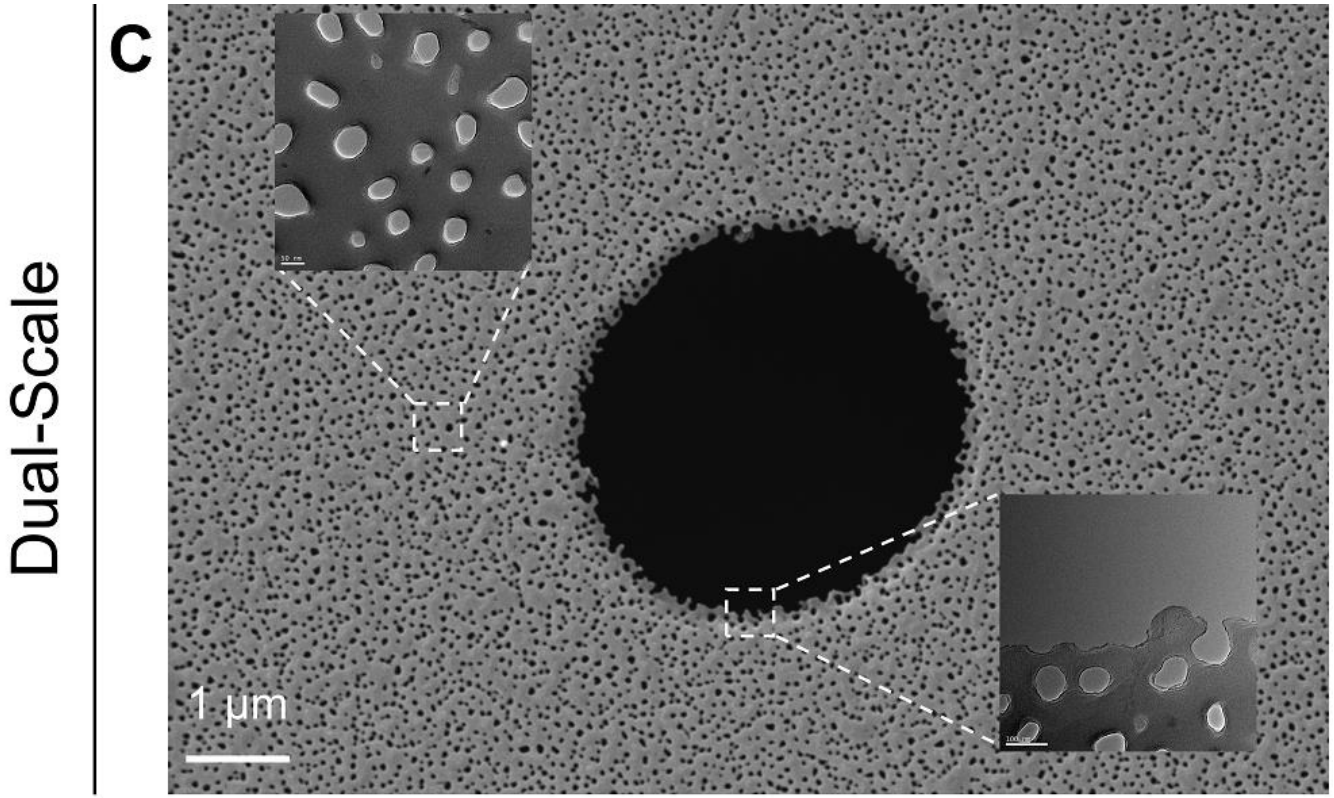


Problem: PMN Sequestration

Solution: Hierarchical pore membranes will permit abundant cell-substrate interactions without reducing permeability

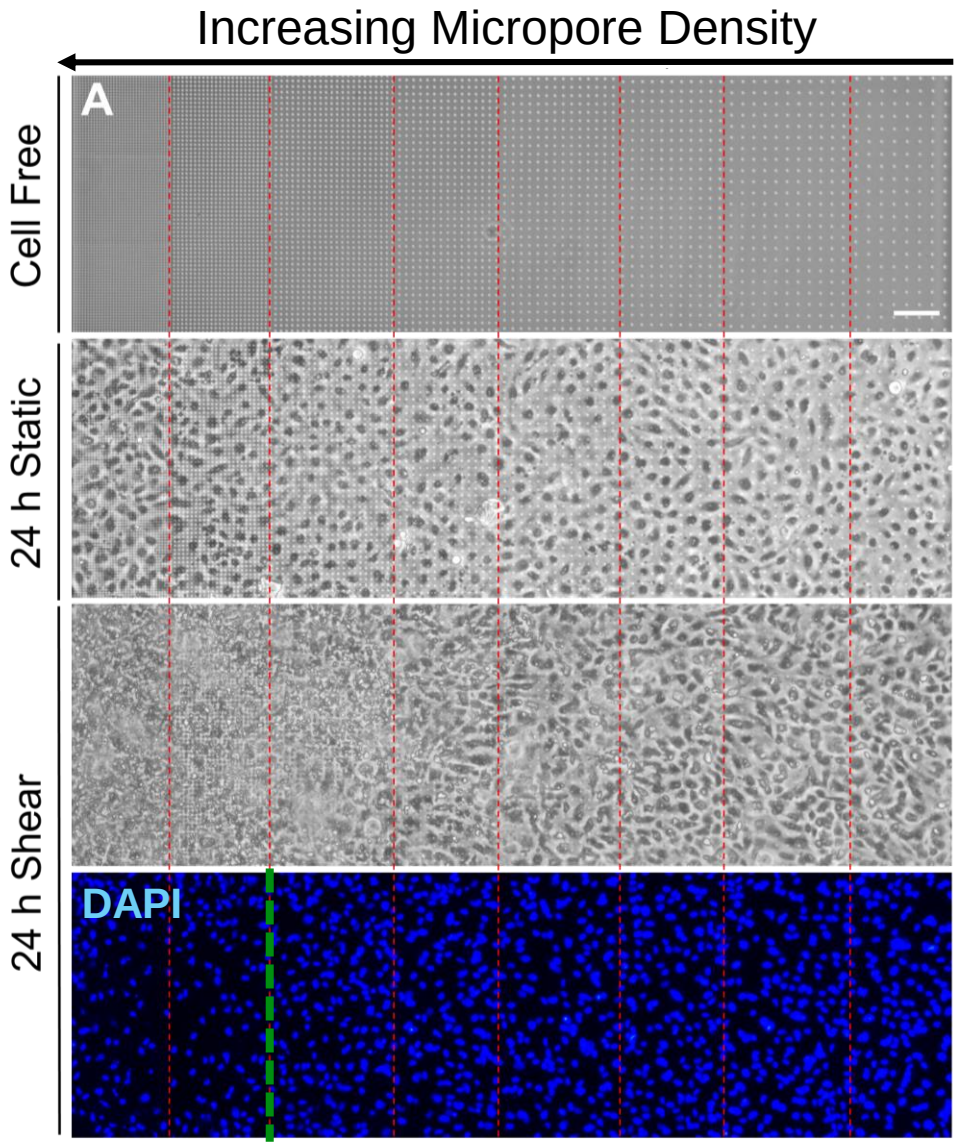
Dual-Scale (Nano- and Microporous) Membranes

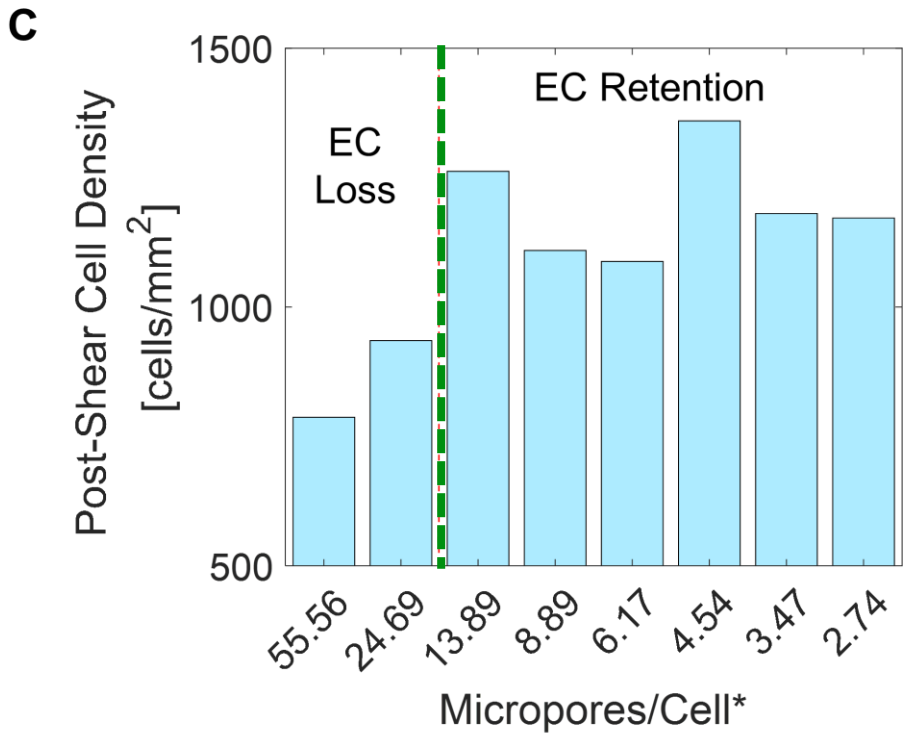




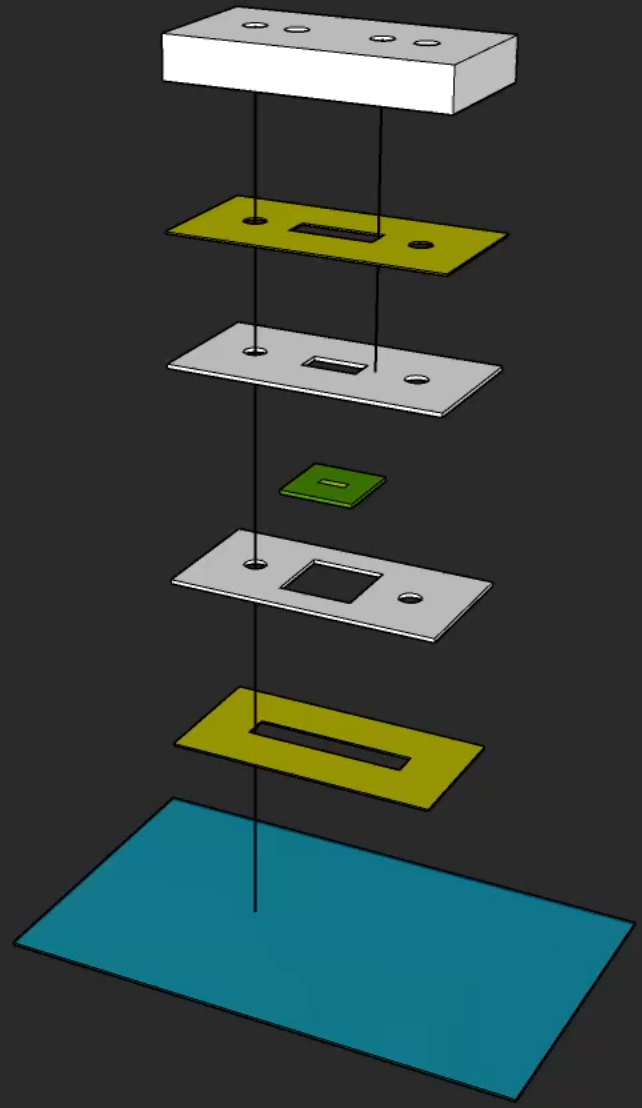
Salminen et al., SMALL 2019

Cell Culture on Dual-Scale Membranes

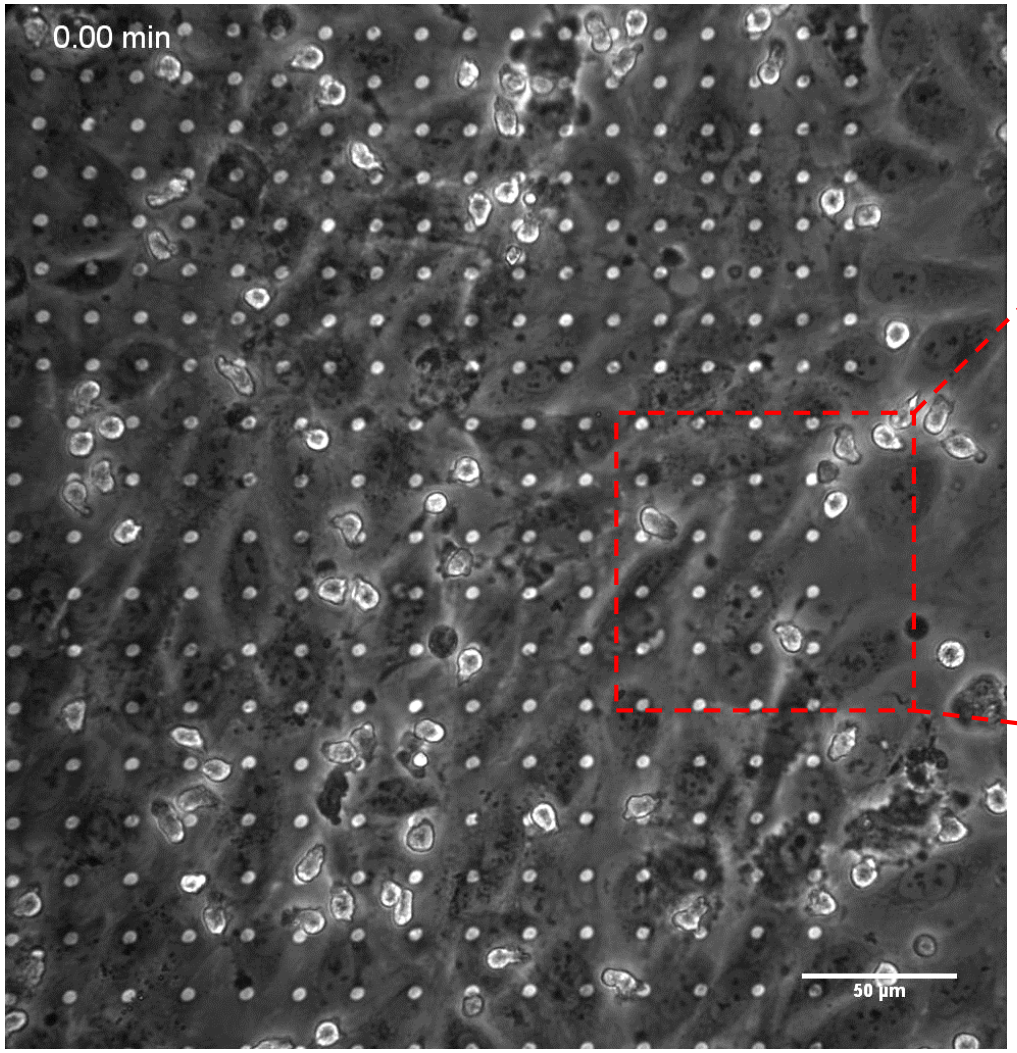


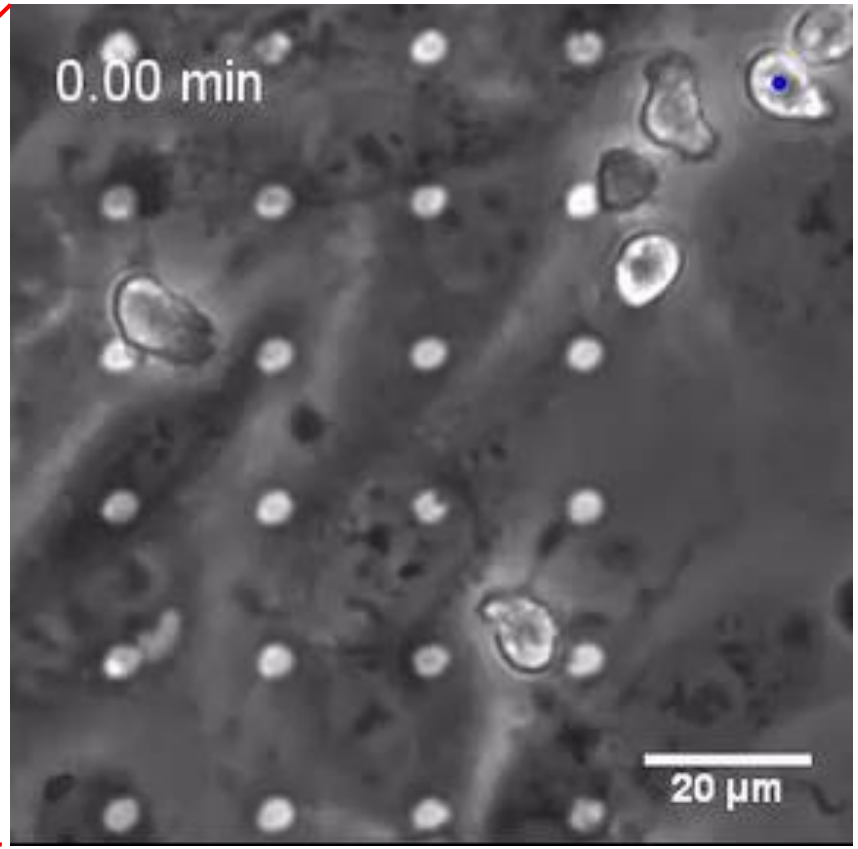


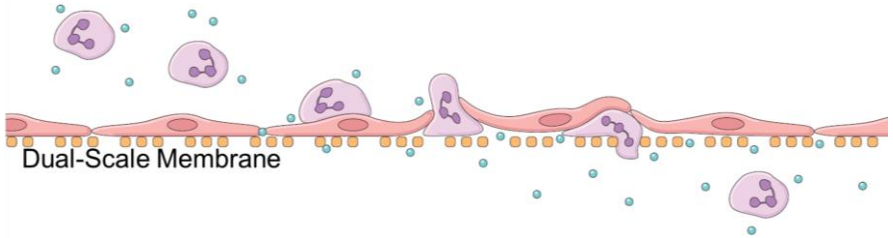
Salminen et al., SMALL 2019



Dual-Scale Membranes Permit PMN Tracking



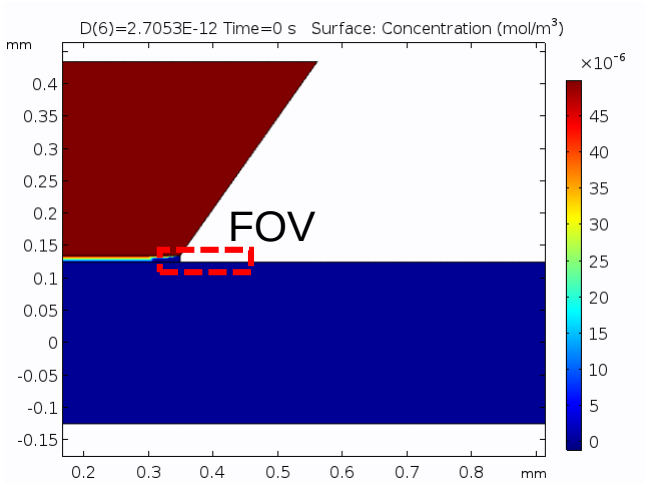




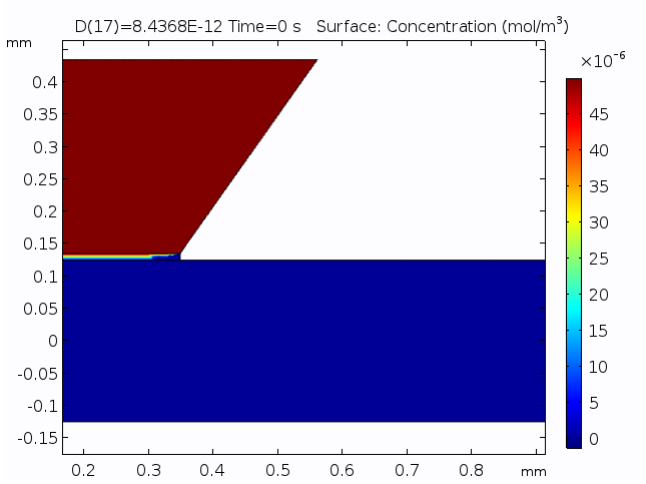
Salminen et al., SMALL 2019

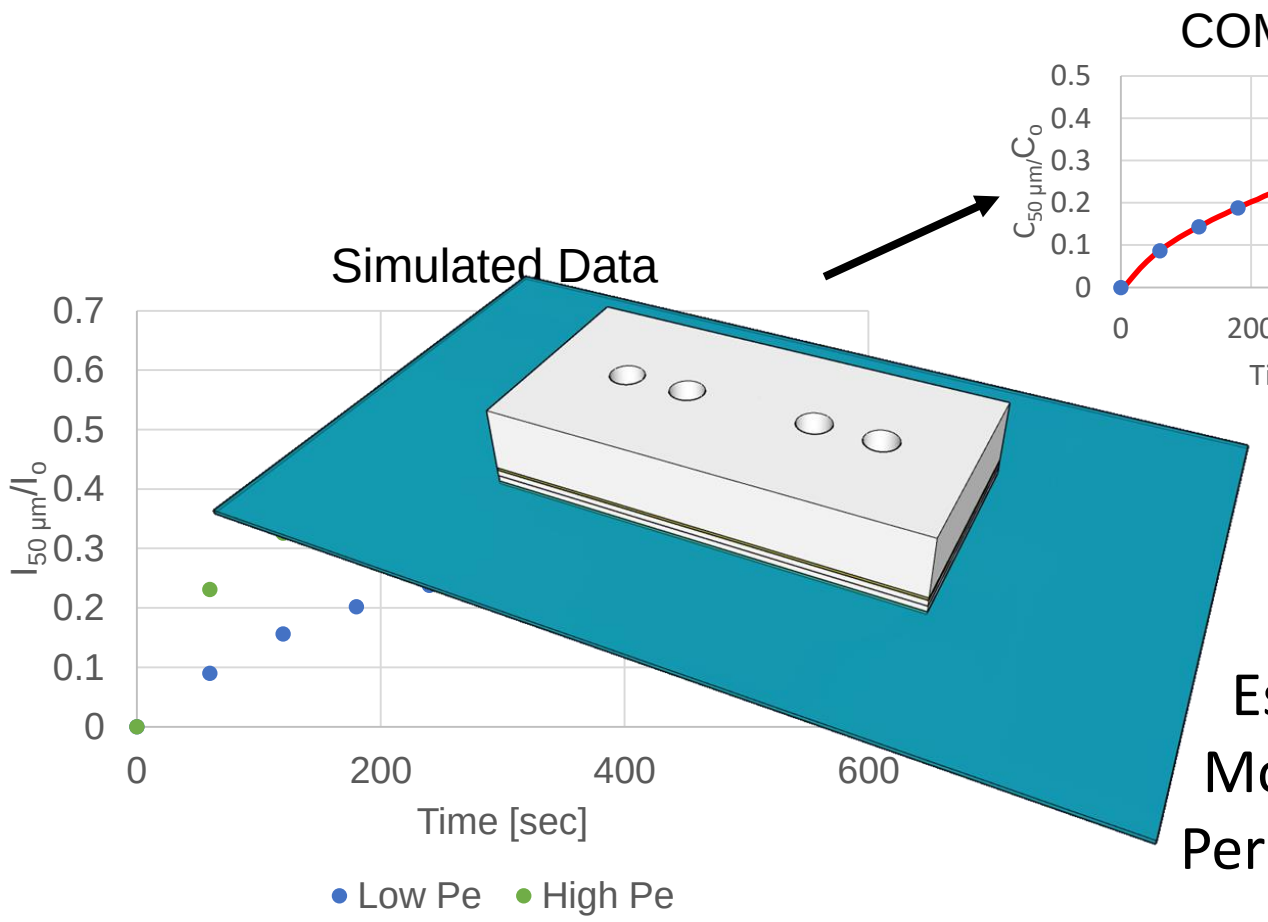
In Situ EC Permeability Assay

Low Permeability
'Healthy Monolayer'



High Permeability
'Septic Monolayer'

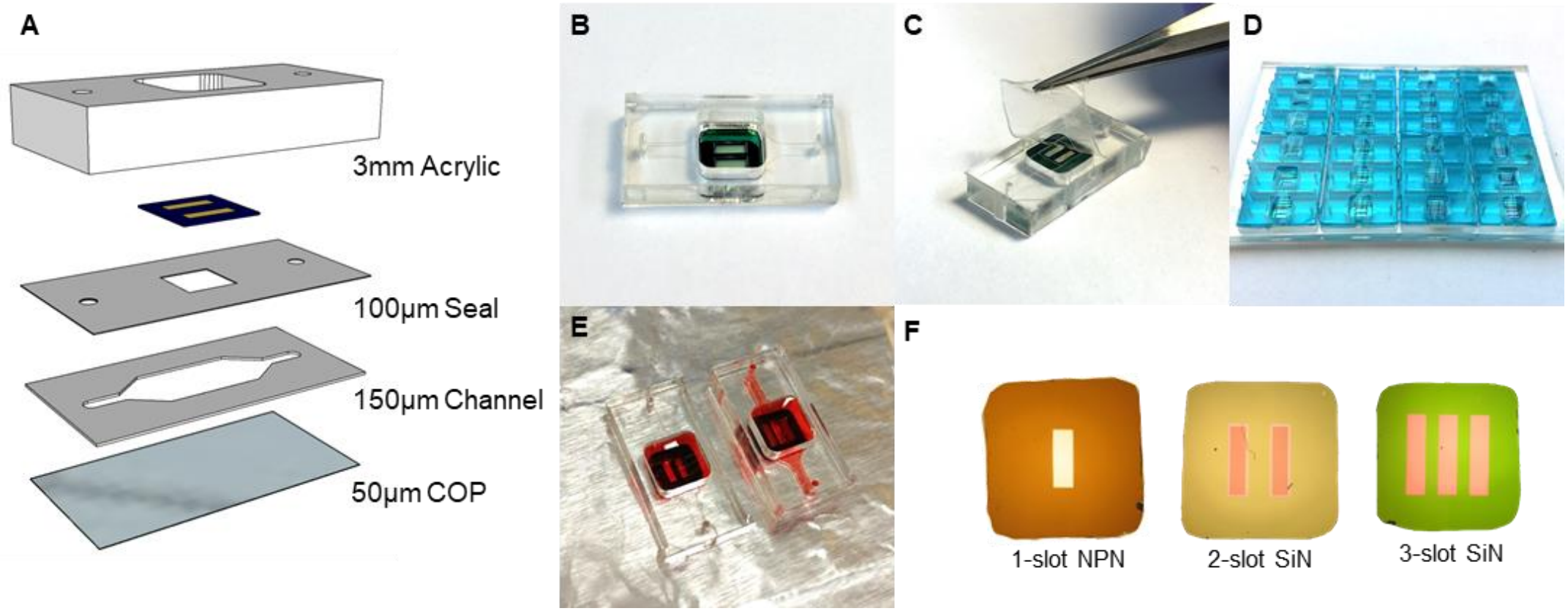




Estimate Monolayer Permeability

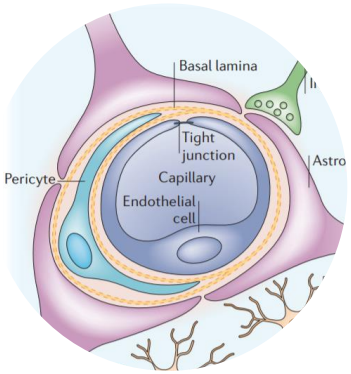
$$\frac{1}{P_{EC}} = \frac{x}{D} - \left[\frac{1}{P_{NPN}} + \frac{L}{D_{dextran}}\right]$$

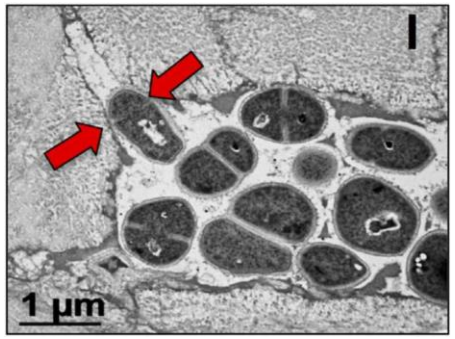
High-Throughput Manufacturing

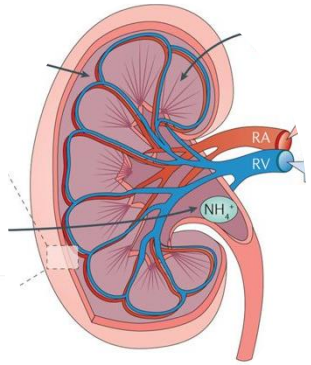


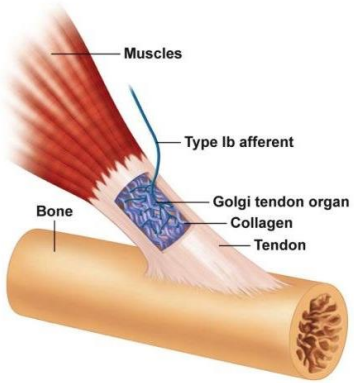
Adapted from: Masters EA, Salminen AT et al., *Unpublished* 2019

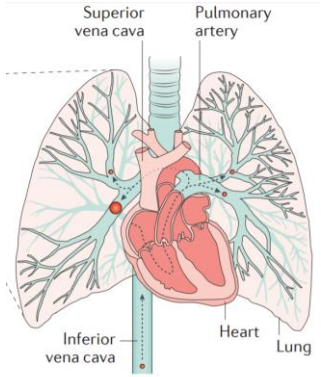
Supporting Collaborations Through High-Throughput Manufacturing







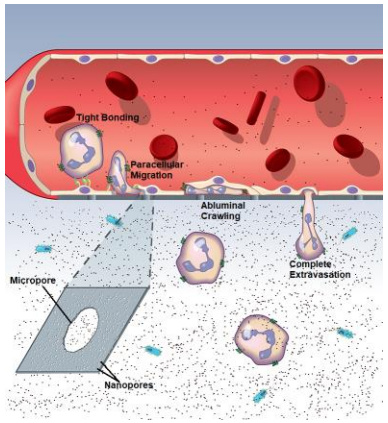




I. Dr. Edward Schwarz – University of Rochester, Rochester, NY; Microfluidic Silicon Membrane Enabled **Canalicular Array** (µSiM-CA)
II. Dr. Britta Engelhardt – University of Bern, Bern, Switzerland; µSiM- **Blood Brain Barrier** (BBB)
III. Dr. Angela Glading – University of Rochester, Rochester, NY; Microvasculature
IV. Dr. Itzik Cooper – Sheba Medical Center, Ramat Gan, Israel; BBB
V. Daan Hart – Radboud University Medical Center, Nijmegen, Netherlands; Podocyte Culture (**Kidney** on a chip)
VI. Dr. Diana Hudecz – Aarhus University, Aarhus C, Denmark; BBB
VII. Dr. Hani Awad – University of Rochester, Rochester, NY; **Tendon/Bone** Fibroblast Culture

Aim 1 Summary

✓ Designed and built a ready-to-use microfluidic system to mimic the PVB



✓ Fabricated novel dual-scale membrane that permits EC adherence and phase tracking of PMNs

✓ *In Situ* permeability measurements

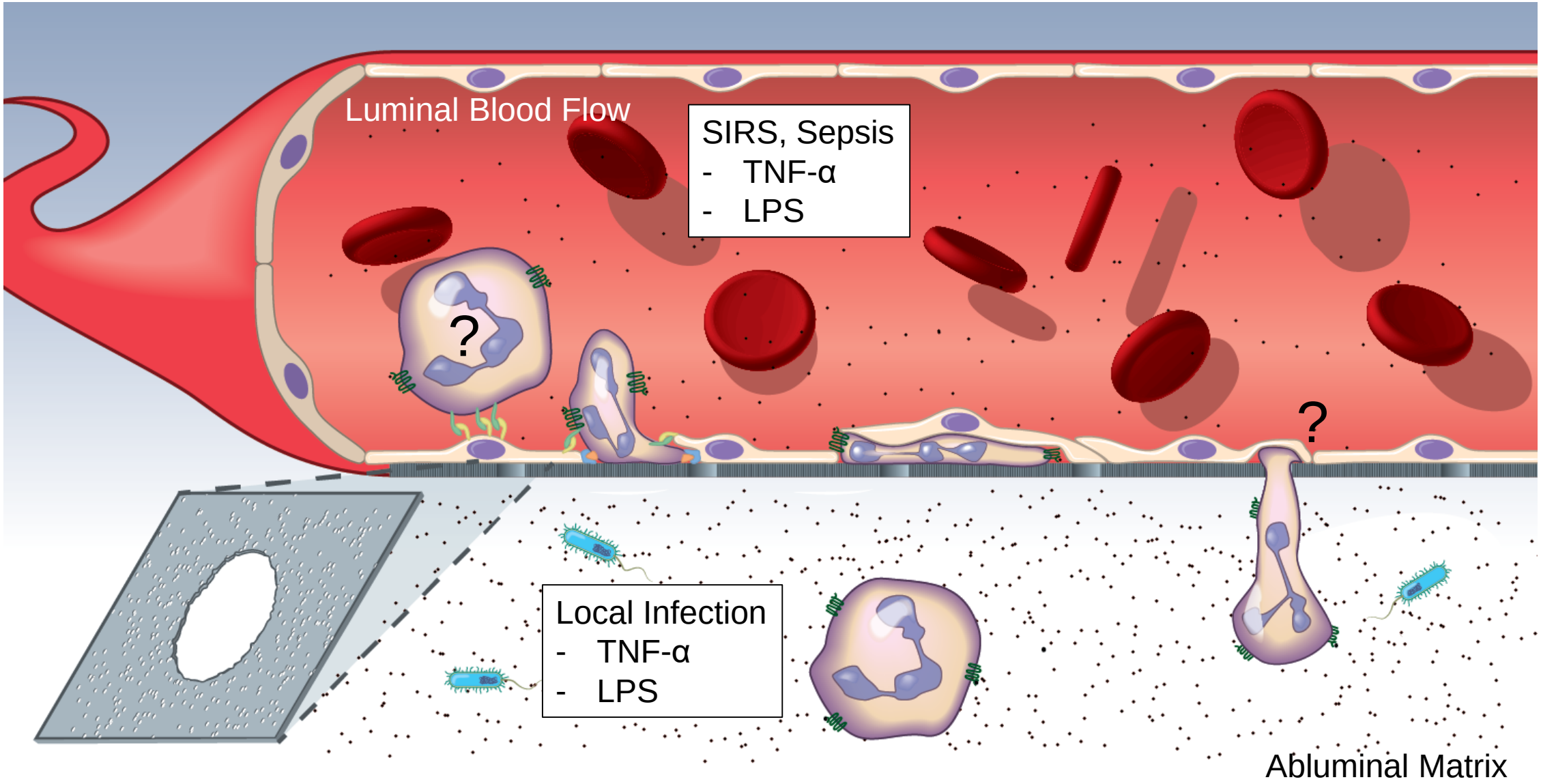
ALINE

✓ High-Throughput manufacturing

Specific Aims

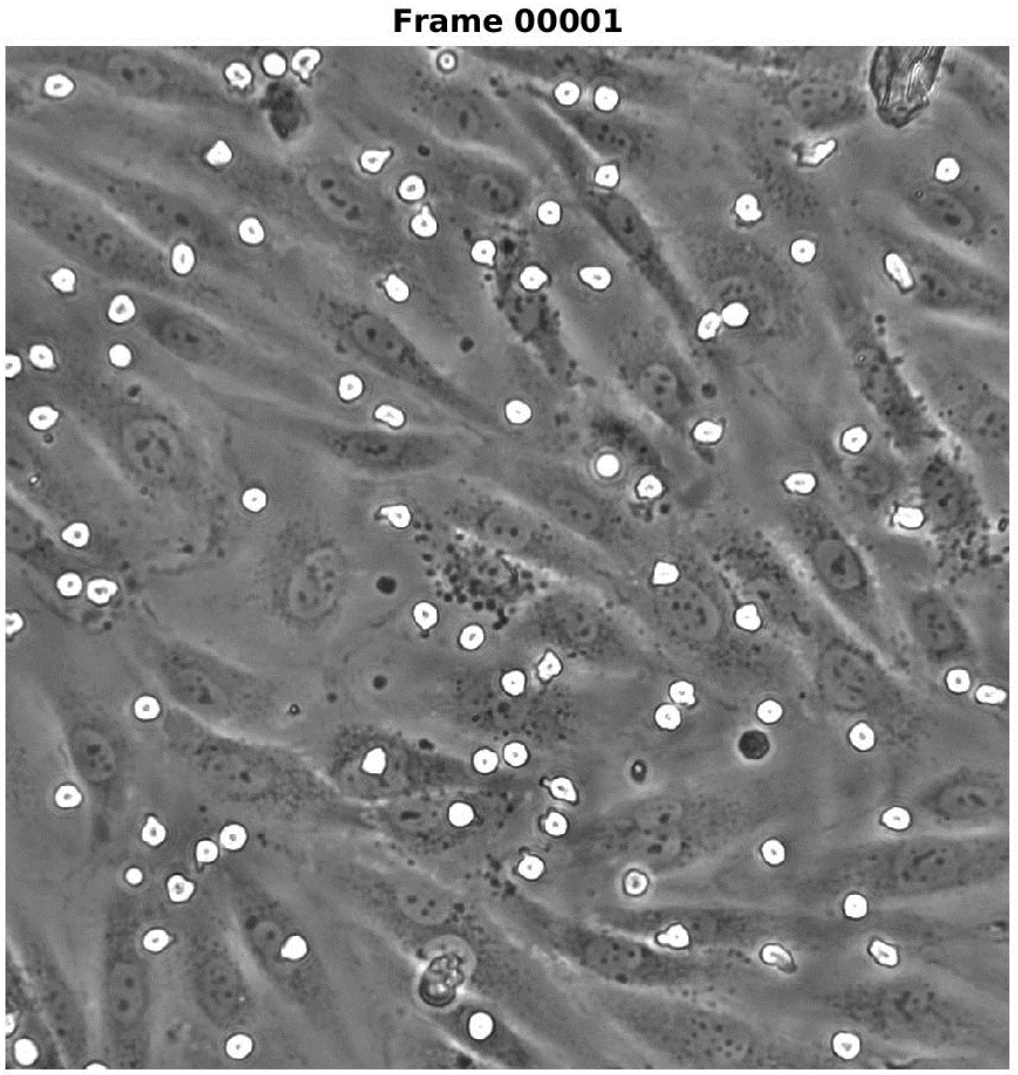
1. Develop a facile microvasculature mimetic to quantify small molecule and neutrophil permeability in the presence of physiological shear
2. Elucidate the effects of polarized cytokine and endotoxin delivery on leukocyte dynamics and endothelial cell biology
3. Demonstrate the polarized dependence of S1P in regulating pulmonary vasculature permeability and neutrophil dynamics following septic insult *in vitro*

Systemic Inflammation vs. Local Infection



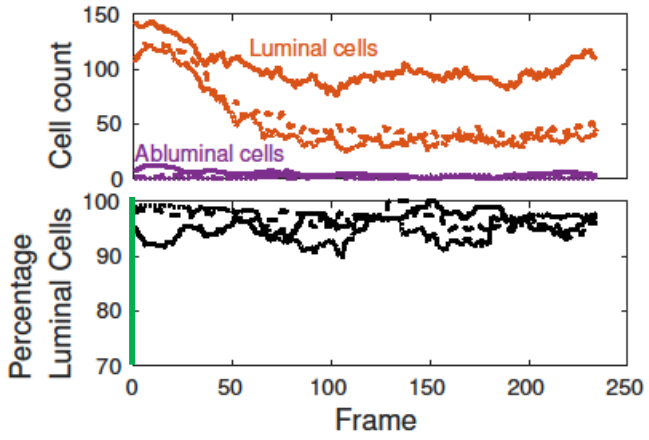
Automated Leukocyte Tracking

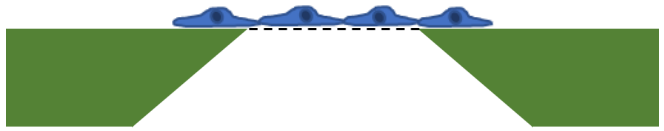
- A previously established **particle tracking algorithm** will be adapted for tracking neutrophils [Kelley Lab, University of Rochester]
- Phase contrast movies allow for **pixel intensity tracking** of luminal and abluminal leukocyte dynamics, as well as diapedesis events
- **Unbiased** quantification of leukocyte immune response

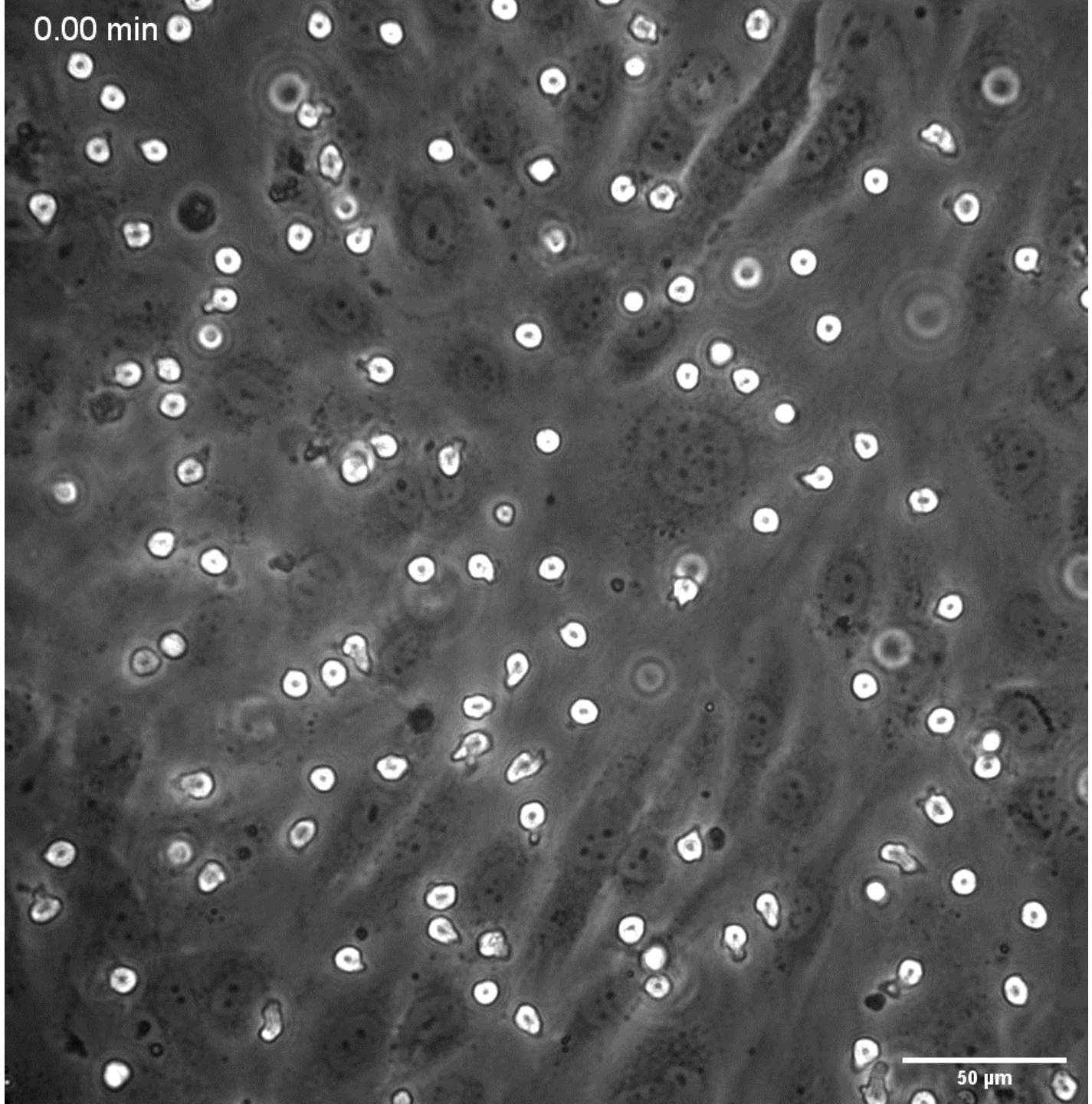


Elapsed 30 min

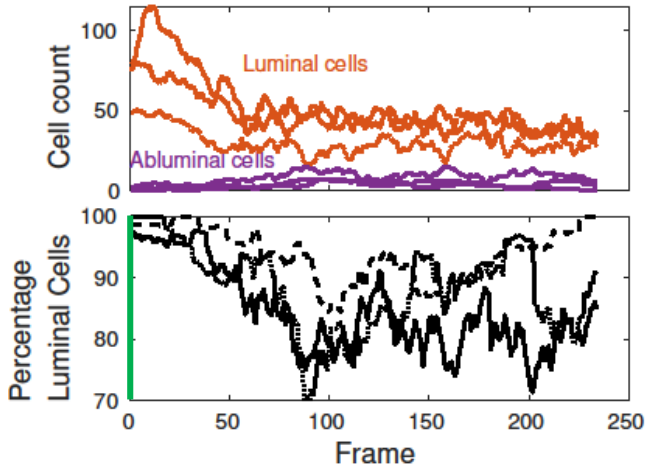
Negative Control – Neutrophils isolated from healthy donors on unstimulated HUVECs

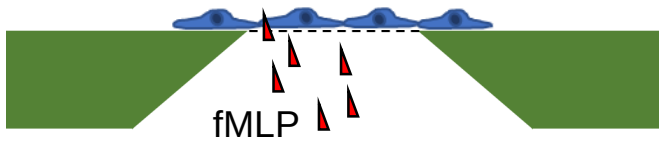


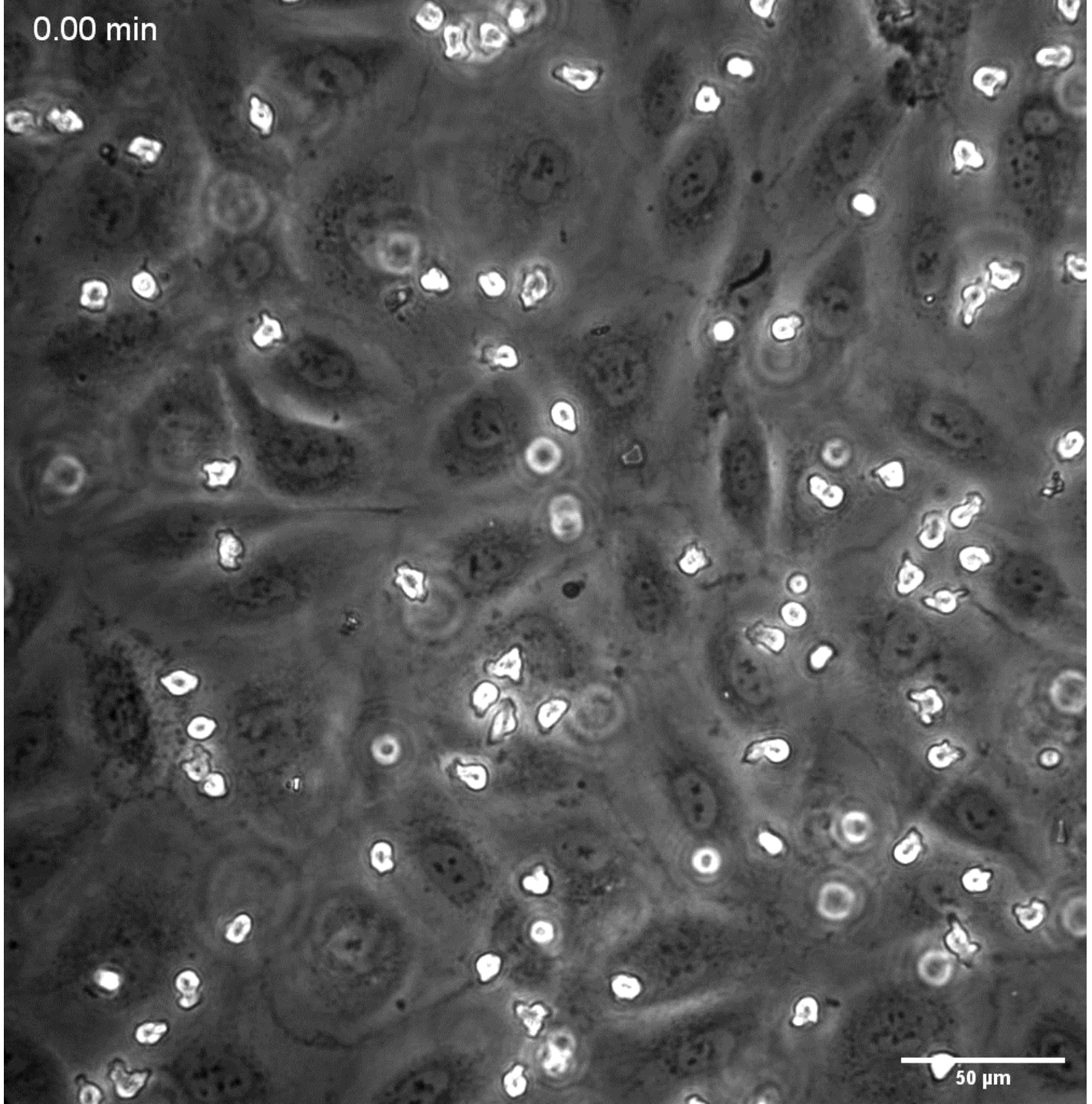




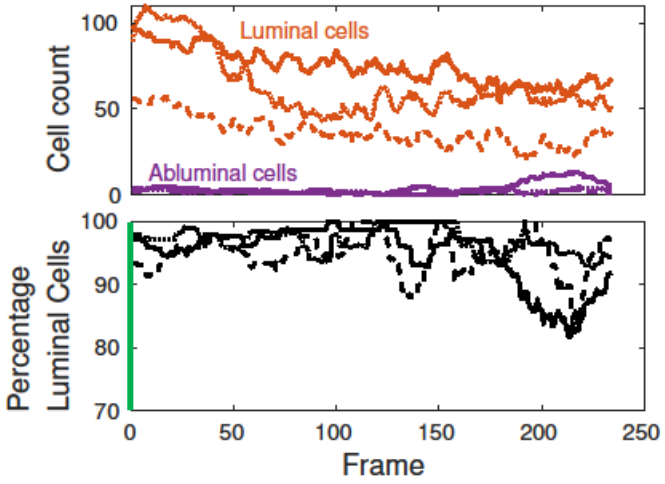
Positive Control – Neutrophils on unstimulated HUVECs with a fMLP gradient

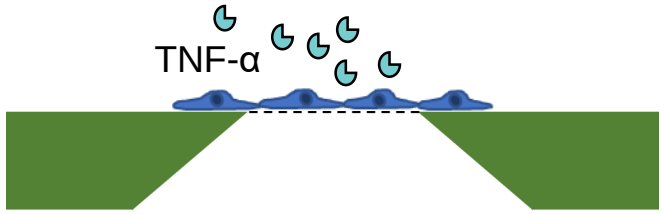


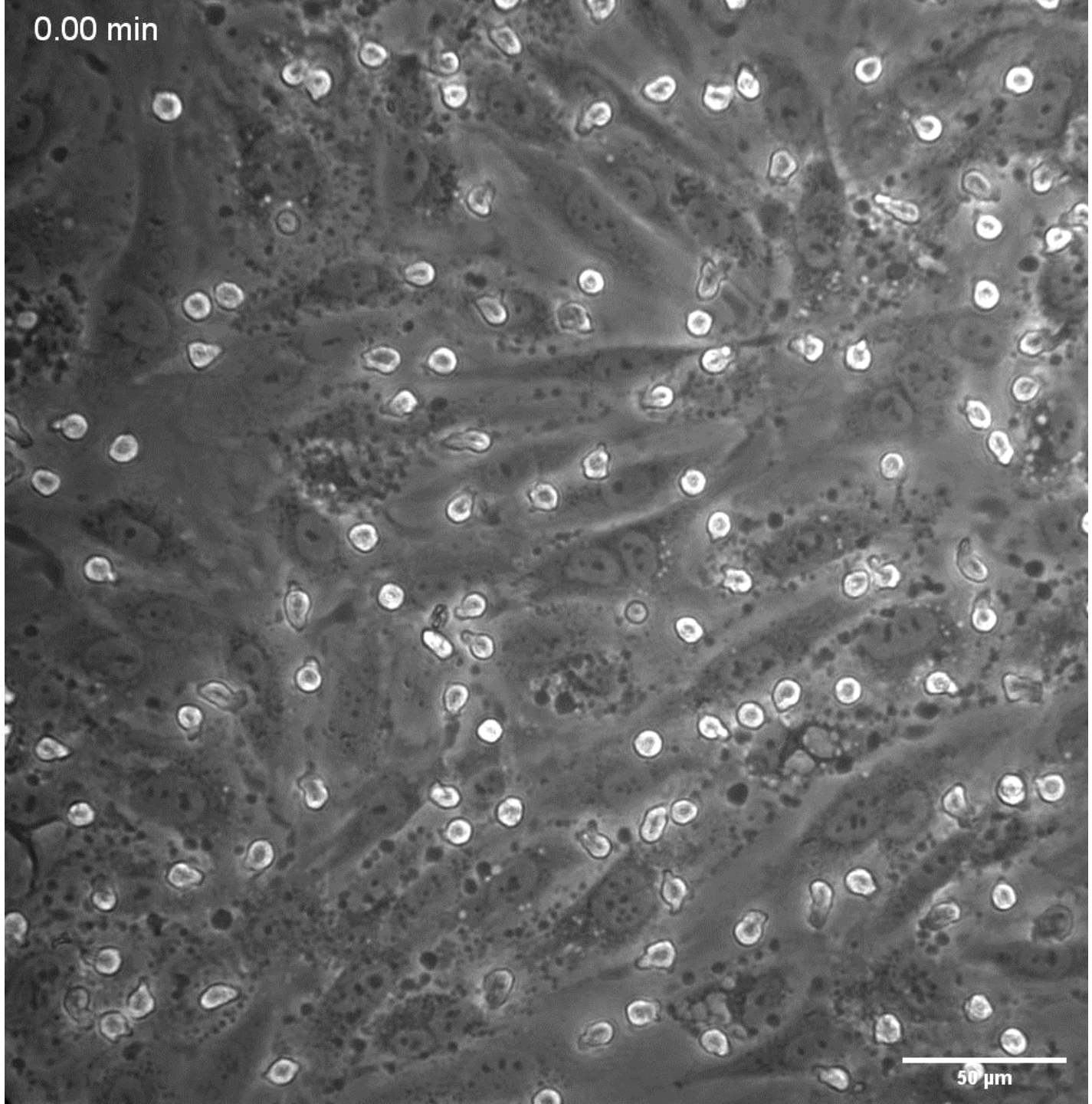




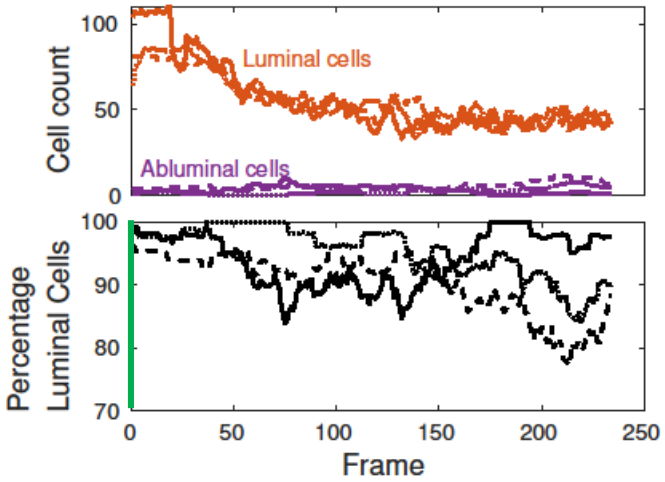
Luminal TNF-α – Neutrophils on luminally stimulated HUVECs (20 ng/mL TNF-α for 24 h)

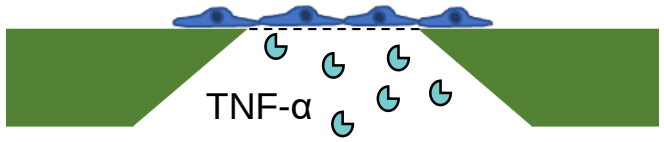


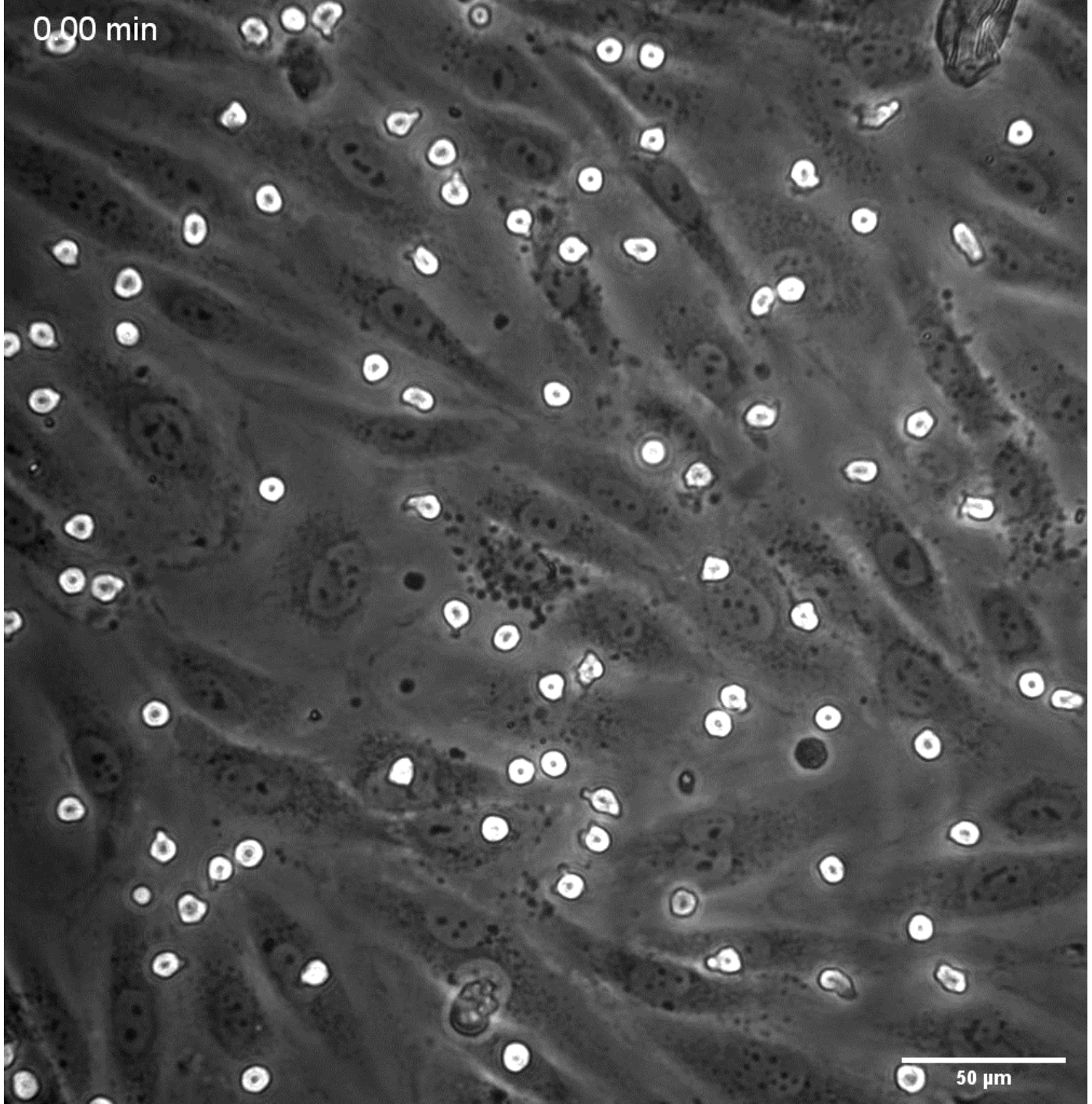




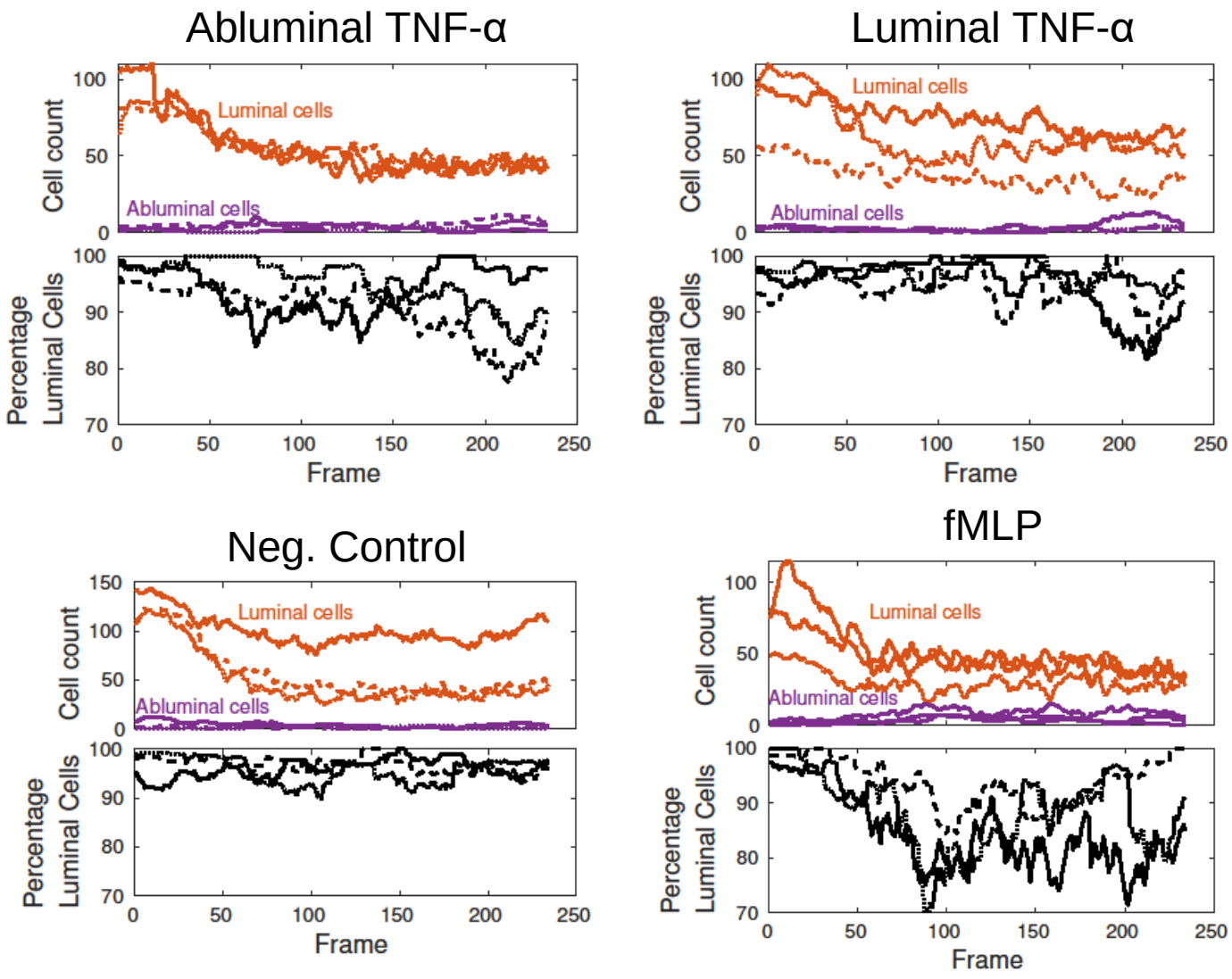
Abluminal TNF-α – Neutrophils on abluminally stimulated HUVECs (20 ng/mL TNF-α for 24 h)

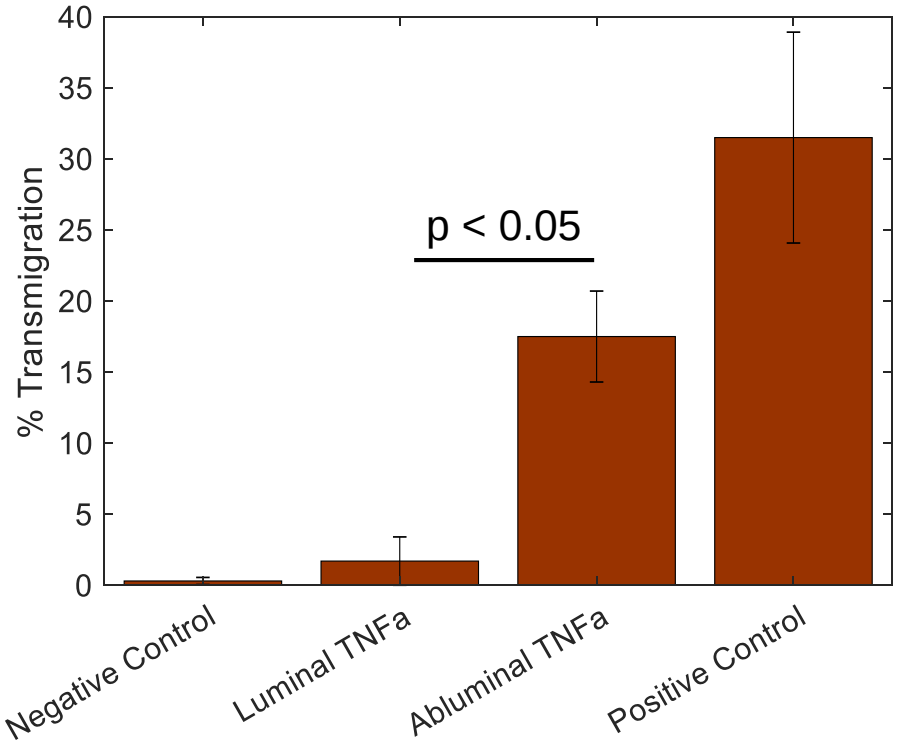




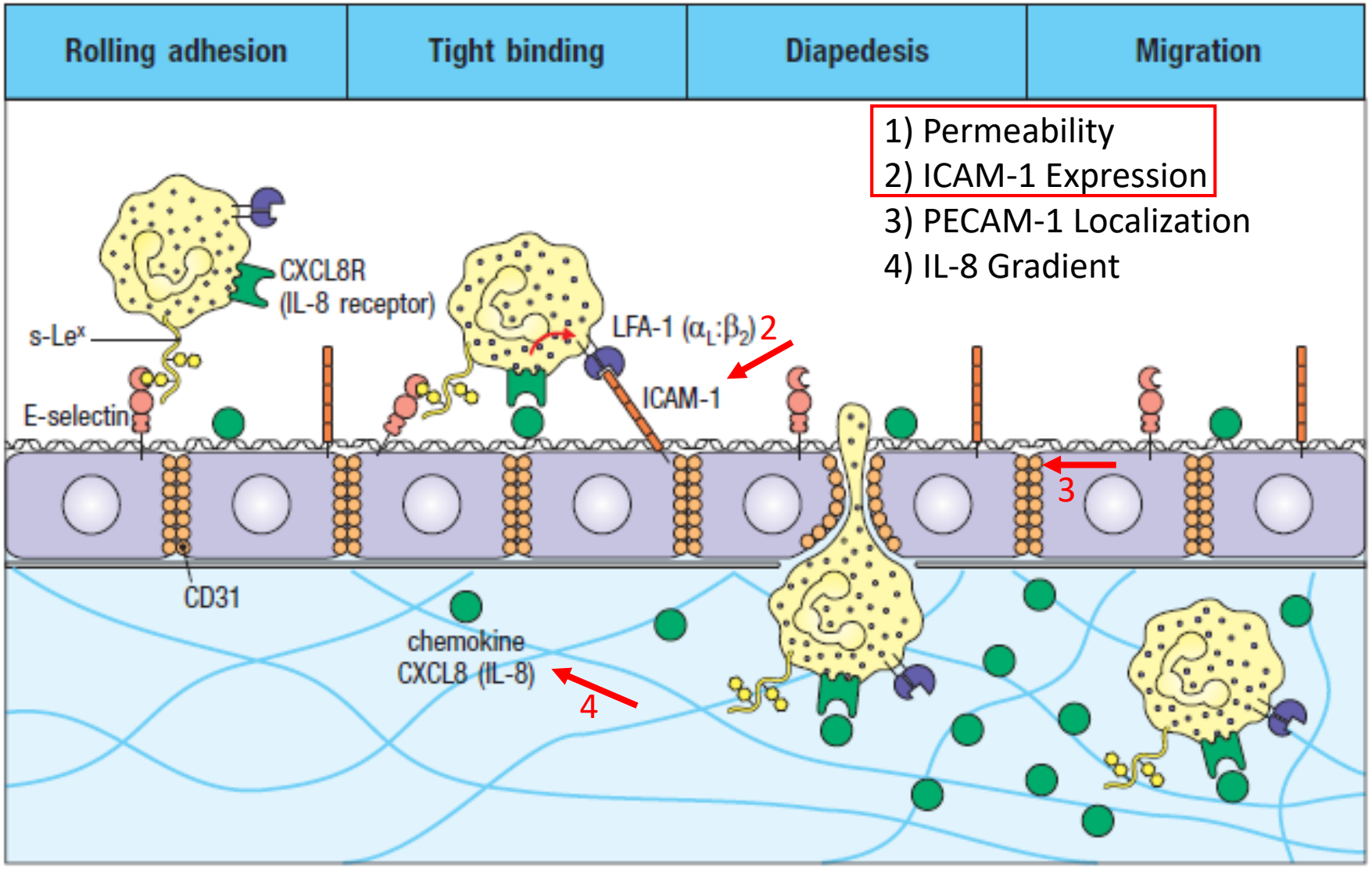


Abluminal TNF-α Induces a Potent PMN Response





Response Mechanism



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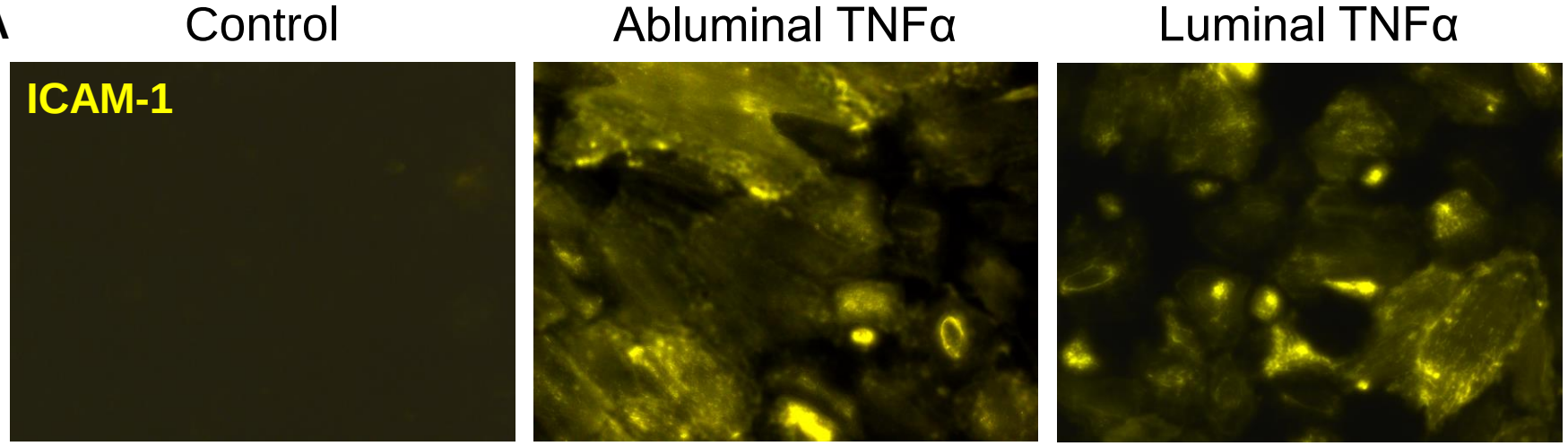
Enhanced Diapedesis Response to Abluminal TNFα is independent of ICAM-1 and EC Permeability

A



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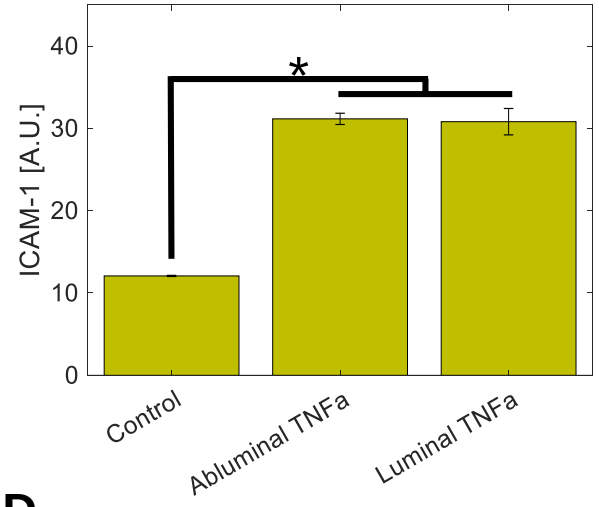

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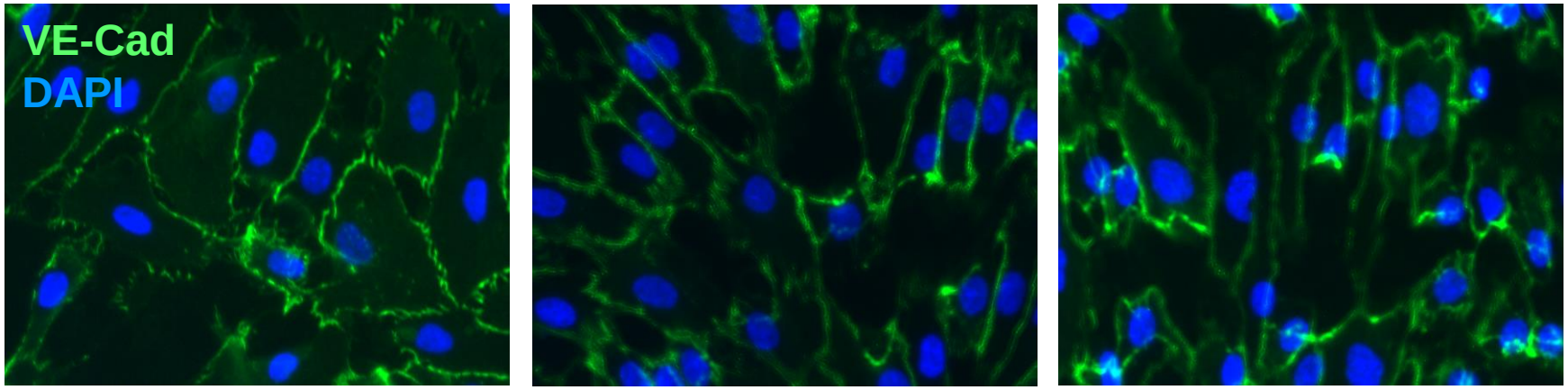
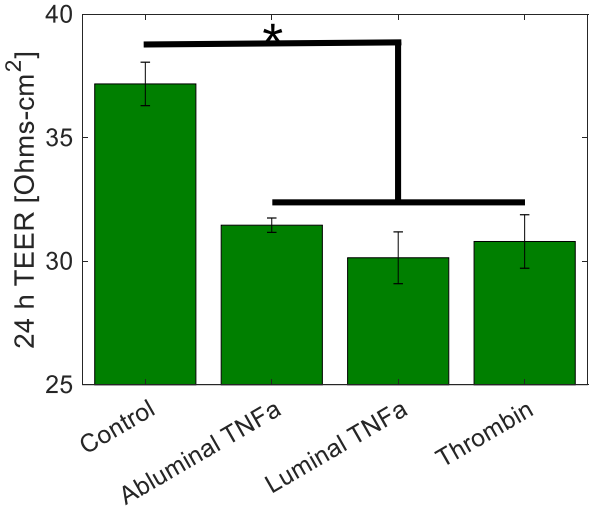


D



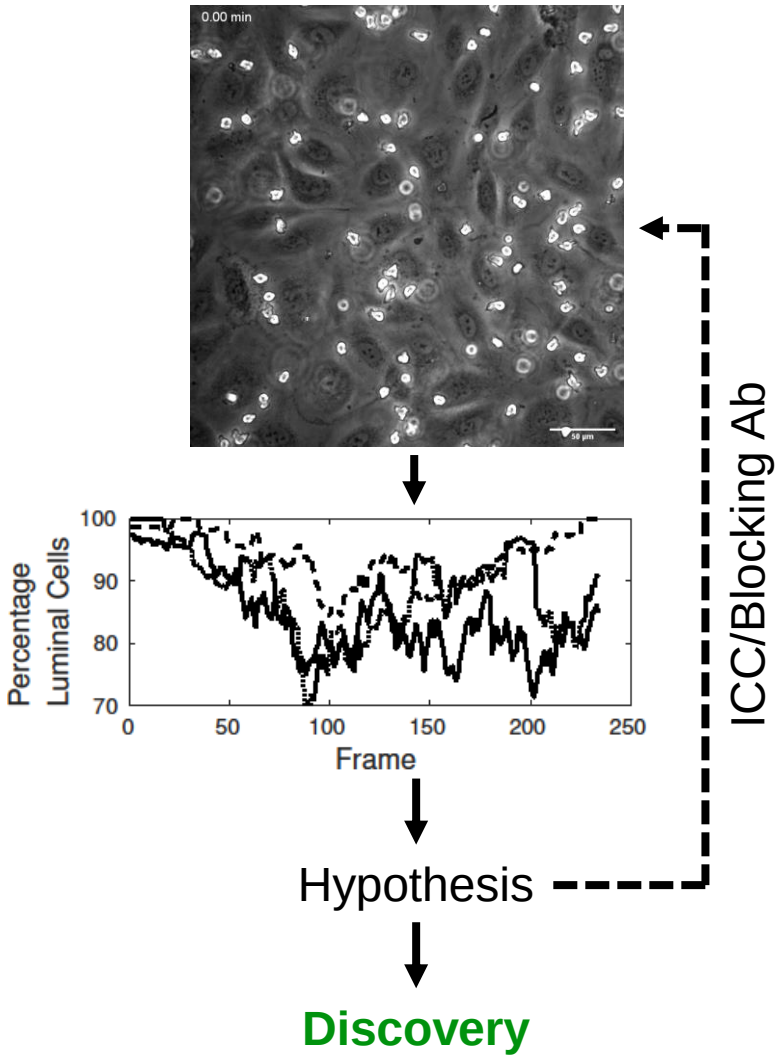
Aim 2 Summary

In Progress

- Improving tracking algorithm
- Head-to-head comparison of automated and hand-tracked data
- Elucidating mechanism of abluminal TNF-α induced PMN transmigration

Future Work

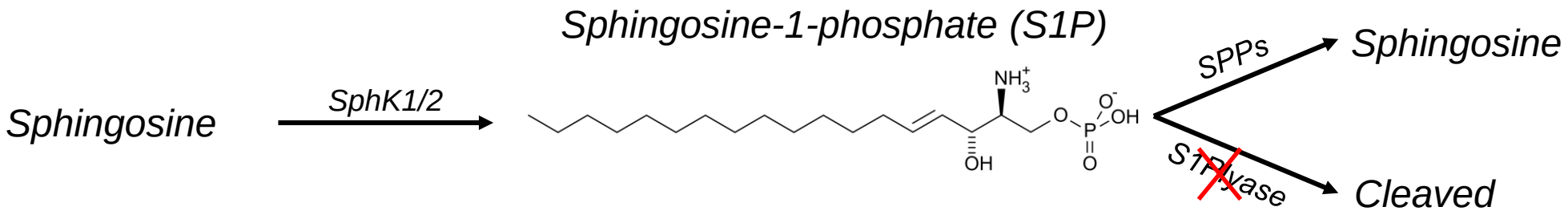
- Utilize primary human pulmonary microvasculature cell lines [PromoCell]
- Investigation endotoxin (LPS) induced immune responses

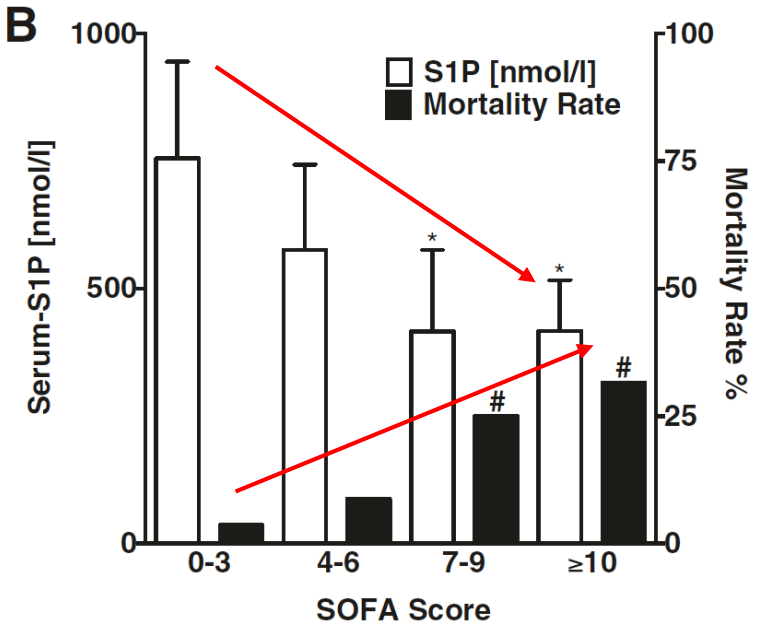


Specific Aims

1. Develop a facile microvasculature mimetic to quantify small molecule and neutrophil permeability in the presence of physiological shear
2. Elucidate the effects of polarized cytokine and endotoxin delivery on leukocyte dynamics and endothelial cell biology
3. Demonstrate the polarized dependence of S1P in regulating pulmonary vasculature permeability and neutrophil dynamics following septic insult *in vitro*

S1P, Sepsis, and ARDS





S1P and Sepsis Induced Lung Injury

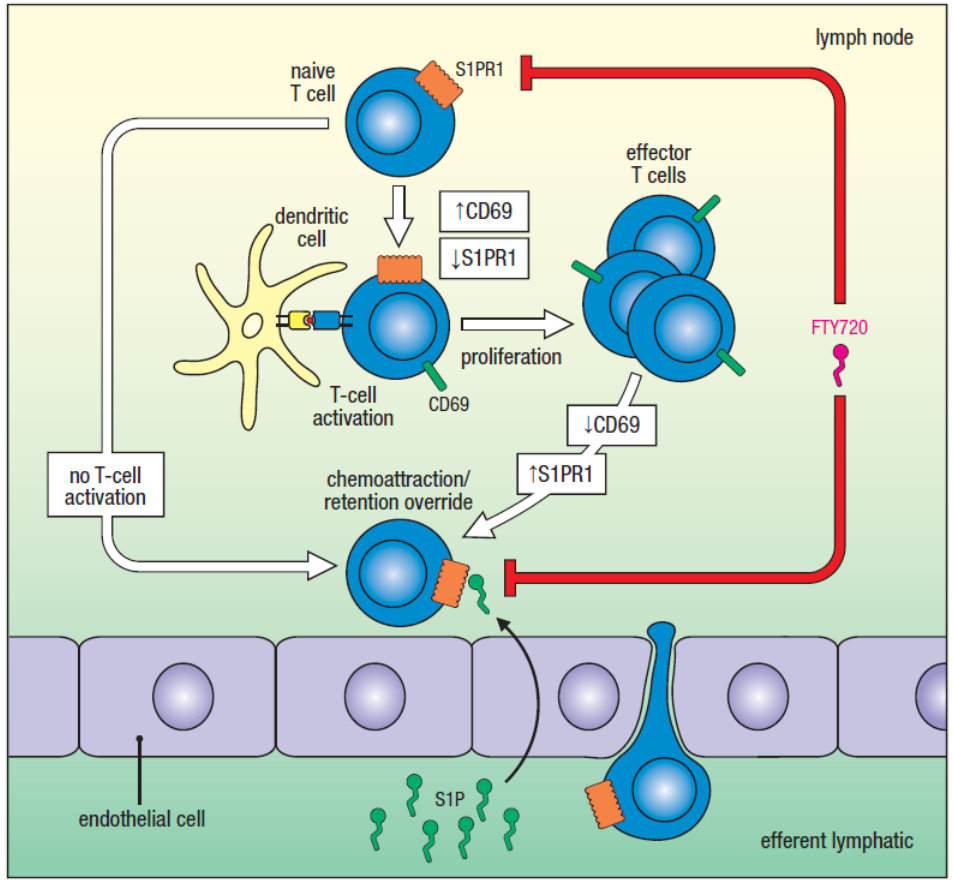
- S1P is barrier protective in LPS-induced lung injury
- S1PL deficiency protects against LPS-induced acute lung injury

Adapted from: Winkler et al., Critical Care 2015

Natarajan et al., Am J Respir Cell Mol Biol 2013

S1P = Sepsis and ARDS Cure-all

The S1P Paradox



- S1P/S1P$_1$ regulate lymphocyte egress

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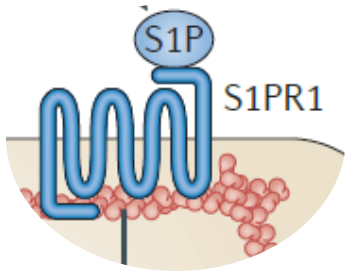
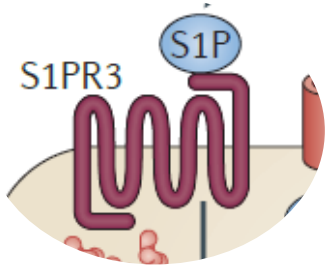
S1P signals through 5 GPCRs [S1P$_{1\text{-}5}$]



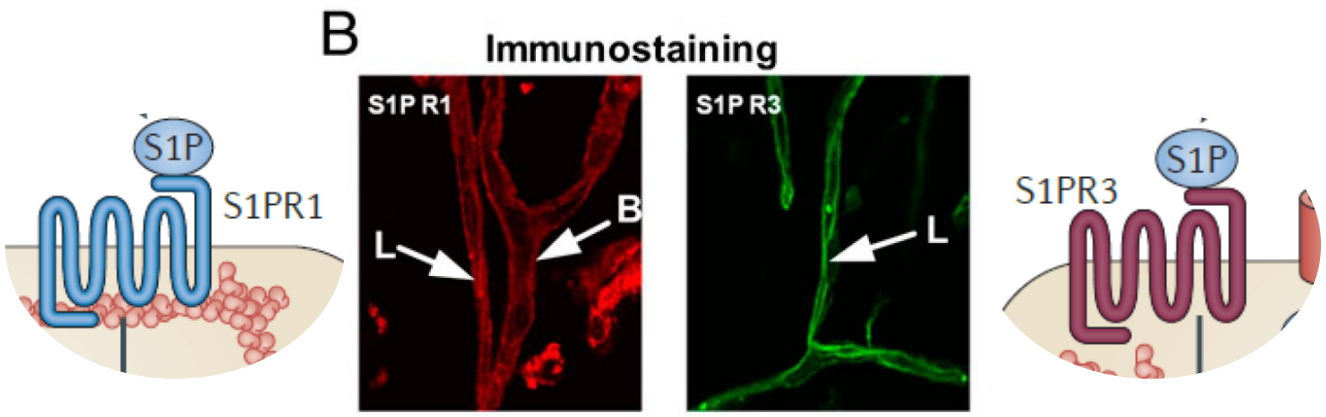
= ↓ EC Permeability



= ↑ EC Permeability, Leukocyte Recruitment

Natarajan et al., Am J Respir Cell Mol Biol 2013
Nussbaum et al., Nat Commun 2015
Graphic: Spiegel et al., Nat Rev Immunology 2011

EC Apicobasal Polarity and S1P



Cannon et al., PNAS 2012

Guiding Hypothesis: Abluminal delivery of S1P will preferentially target $S1P_1$ across a broad therapeutic window, without maladaptive immune effects.

Approach

1. Prime the μSiM-PVB with clinical septic serum
2. Measure leukocyte dynamics and EC permeability in response to serum challenge
3. Demonstrate S1P's polarized ability to alter EC permeability and neutrophil dynamics

Summary

Aim 1: Design and build a facile pulmonary microvasculature barrier mimetic to study EC permeability and PMN dynamics

Aim 2: Elucidate the distinguishing characteristics of the innate immune response to systemic inflammation versus primary infection

Aim 3: Demonstrate the potential of S1P in regulating the innate immune response when delivered abluminally

Acknowledgements

Proposal Exam Committee
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Co-advisor: Prof. Thomas Gaborski
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Prof. Lisa DeLouise
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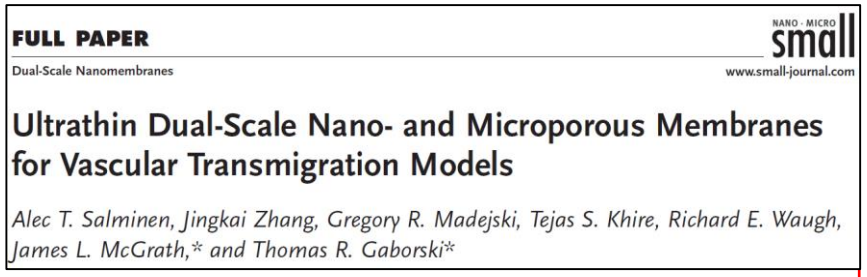
NIH R01HL125265 (JM, RW)
NIH P50 AR072000 (ES, JM)

Timeline



An *in vitro* platform for elucidating the molecular genetics of *S. aureus* invasion of the osteocyte lacuno-canalicular network during chronic osteomyelitis

	May-18	Aug-18	Nov-18	Feb-19	May-19	Aug-19	Nov-19	Feb-20	May-20	Aug-20	Nov-20	Feb-21	May-21
Aim 1: Dual-Scale Membranes	Aim 1-1												
Aim 1: Aline Inc/High-throughput Manufacturing	Aim 1-2												
Aim 1: In Situ Permeability		Aim 1-3											
Aim 2: Polarized TNF-α			Aim 2-1										
Aim 2: Polarized LPS				Aim 2-2									
Aim 3: Human/Septic Serum								Aim 3-1					
Aim 3: Polarized S1P											Aim 3-2		

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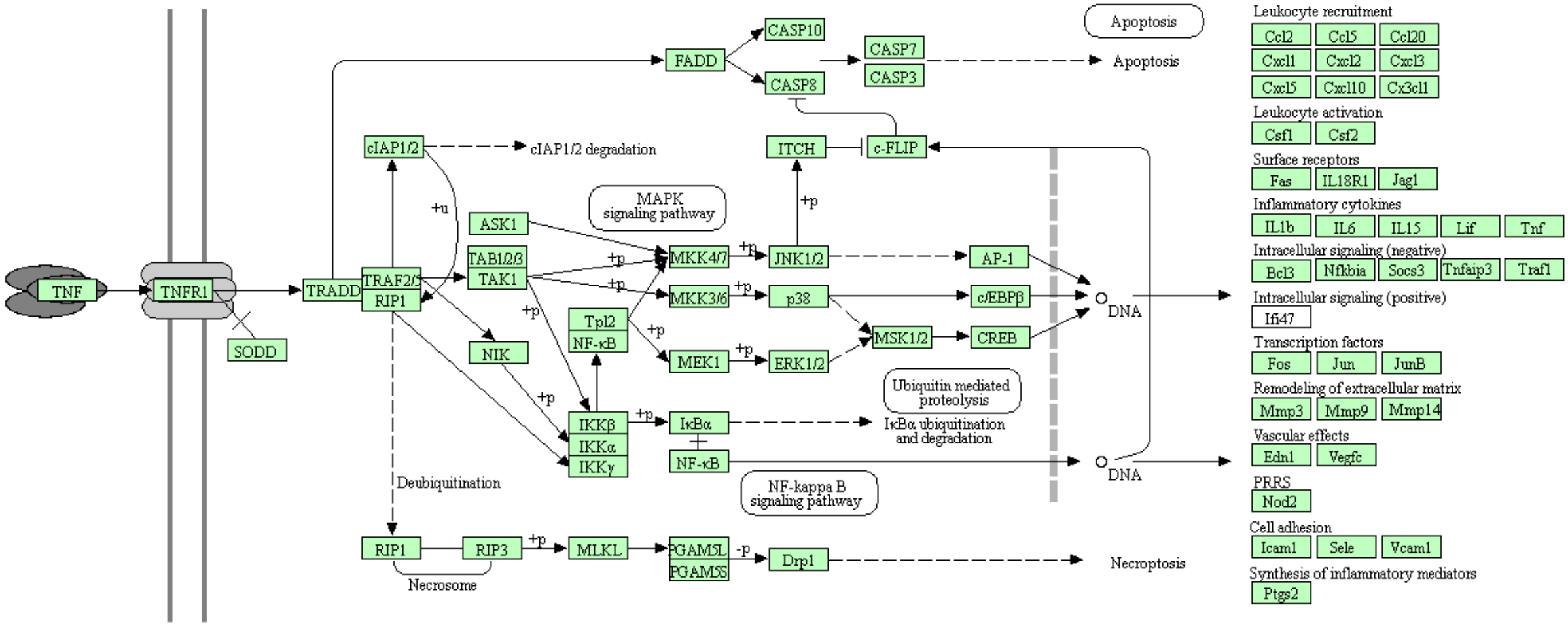
Supplemental Information

TNF SIGNALING PATHWAY



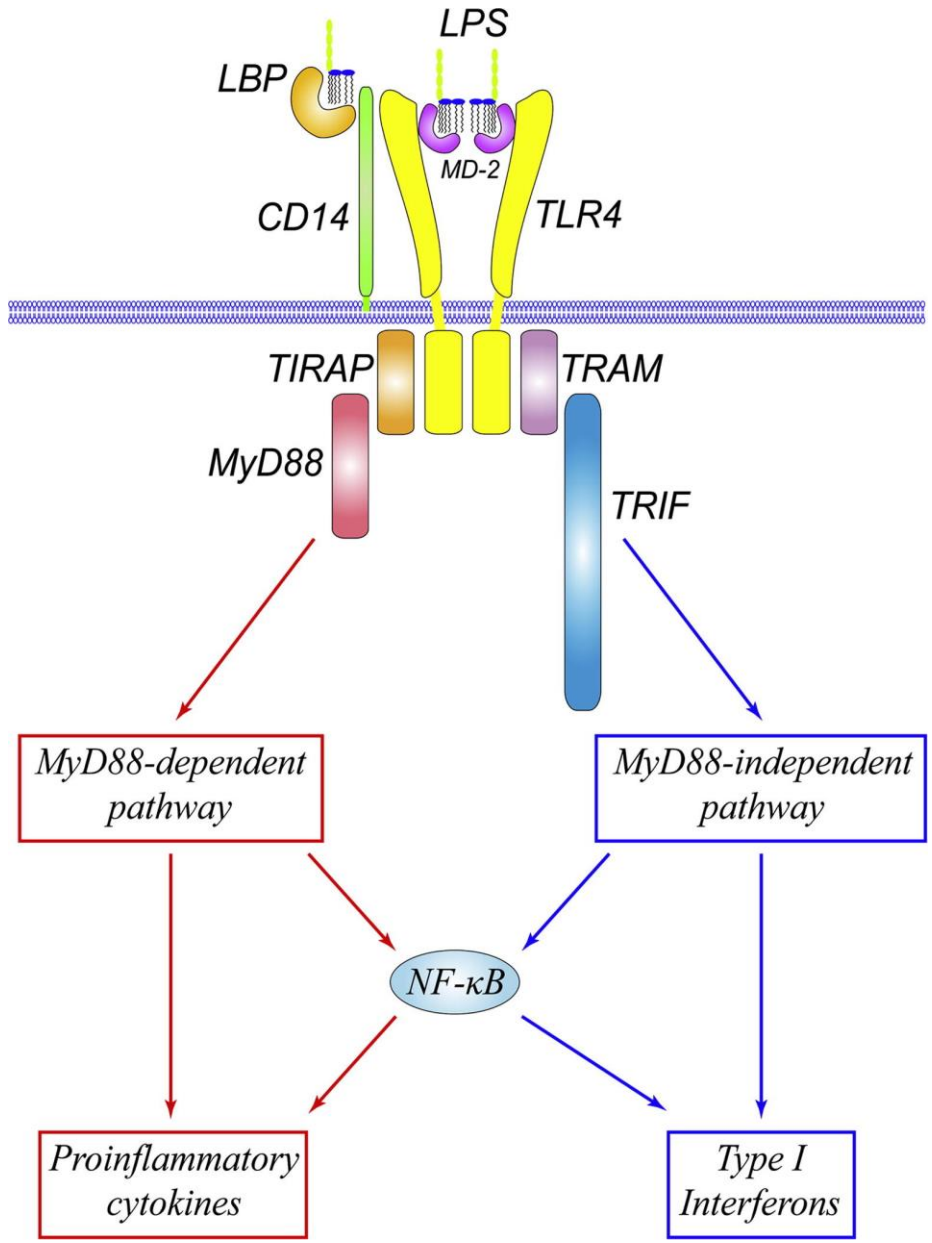

LPS
LBP
MD-2
CD14
TLR4
TIRAP
TRAM
MyD88
TRIF
MyD88-dependent pathway
MyD88-independent pathway
NF-κB
Proinflammatory cytokines
Type I Interferons

Table 1
The most important neutrophil receptors. See the text for further details.

G-protein-coupled receptors	Fc-receptors	Adhesion receptors	Cytokine receptors	Innate immune receptors
Formyl-peptide receptors	*Fcγ-receptors*	*Selectins and selectin ligands*	*Type I cytokine receptors*	*Toll-like receptors*
• FPR1 (FPR)	• FcγRI	• L-selectin	• IL-4R	• TLR1
• FPR2 (FPRL1)	• FcγRIIA (human)	• PSGL-1	• IL-6R	• TLR2
• FPR3 (FPRL2)	• FcγRIIB (inhibitory)	*Integrins*	• IL-12R	• TLR4
Classical chemoattractant receptors	• FcγRIII (mouse)	• LFA-1 ($\alpha_L\beta_2$)	• IL-15R	• TLR5
• BLT1 (LTB_4-rec.)	• FcγRIIIB (human)	• Mac-1 ($\alpha_M\beta_2$)	• G-CSFR	• TLR6
• BLT2 (LTB_4-rec.)	• FcγRIV (mouse)	• VLA-4 ($\alpha_4\beta_1$)	• GM-CSFR	• TLR7 (?)
• PAFR	*Fcα-receptors*		*Type II cytokine receptors*	• TLR8
• C5aR	• FcαRI (human)		• IFNAR (IFNα/β-rec.)	• TLR9
Chemokine receptors	*Fcε-receptors*		• IFNGR	*C-type lectins*
• CXCR1 (human)	• FcεRI		• IL-10R	• Dectin-1
• CXCR2	• FcεRII		*IL-1R family*	• Mincle
• CCR1			• IL-1RI	• MDL-1
• CCR2			• IL1RII (decoy)	• Mcl
			• IL-18R	• CLEC-2
			TNFR family	*NOD-like receptors*
			• TNFR1 (p55)	• NOD2
			• TNFR2 (p75)	• NLRP3
			• Fas	*RIG-like receptors*
			• LTβR	• RIG-I
			• RANK	• MDA5
			• TRAIL-R2	
			• TRAIL-R3	

Futosi et al., International Immunopharmacology 2013

Shear Stress (τ)

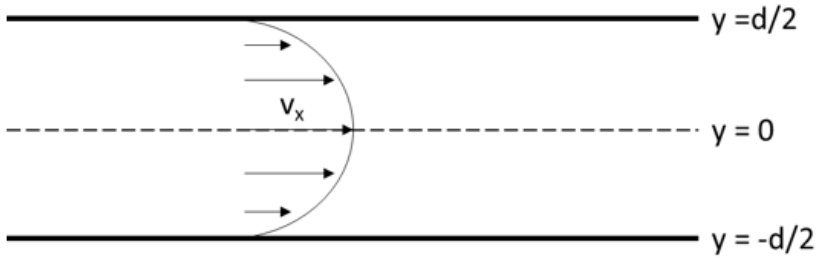
$$\rho\left(\frac{\partial v_x}{\partial t}+v_x\frac{\partial v_x}{\partial x}+v_y\frac{\partial v_x}{\partial y}+v_z\frac{\partial v_x}{\partial z}\right)=-\frac{\partial p}{\partial x}+\mu\left[\frac{\partial^2 v_x}{\partial x^2}+\frac{\partial^2 v_x}{\partial y^2}+\frac{\partial^2 v_x}{\partial z^2}\right]+\rho g_x$$

Assumptions: Steady, fully developed, laminar flow, negligible gravity, smooth surfaces, symmetry, parallel plates

$$\frac{\partial p}{\partial t}=\mu\left[\frac{\partial^2 v_x}{\partial y^2}\right]$$

Integrate both sides

$$\frac{\Delta p}{2L}y^2+C_2=\mu v_x+C_1 y$$



B.C.

$$v_x \; at \; y=\mp\frac{d}{2} \; is \; 0, no \; slip$$

$$v_x=\frac{-\Delta p}{2\mu L}\left[y^2-\left(\frac{d}{2}\right)^2\right] \longrightarrow \tau=\mu\frac{dv_x}{dy} \longrightarrow \tau_{wall}=\frac{6Q\mu}{d^2 w}$$

μSiM-PVB

τ = 4.5 dyn/cm^2
μ = 8.9 x 10^{-3} dyn. s/cm^2
d = 0.043 cm
w = 0.2 cm

Q = 1.87 mL/min

Entrance Length (L)

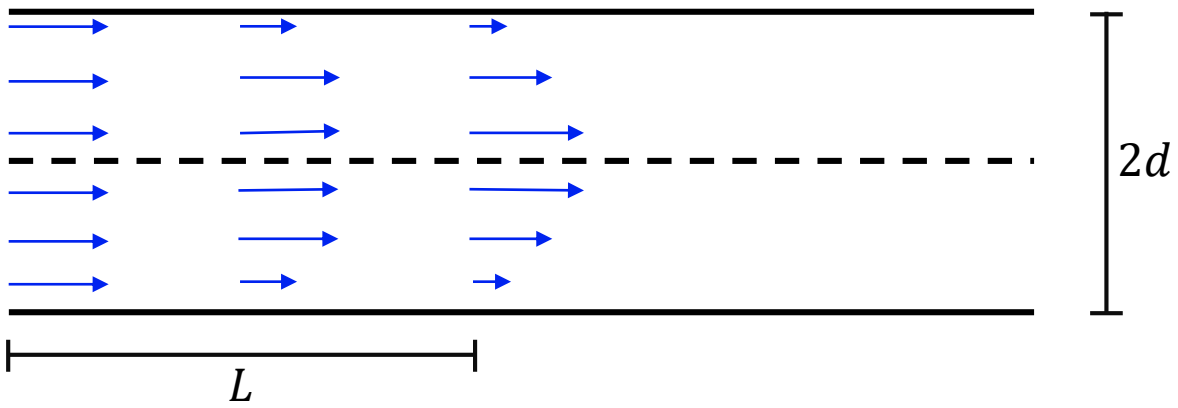
$$L = 0.1048\frac{\rho u d^2}{\mu}$$

ρ = density of the fluid

μ = viscosity of the fluid

d = half plate separation

u = velocity of the fluid



μSiM-PVB

ρ = 997 kg/m^3
μ = 8.9 x 10^{-4} Pa. s
d = 0.0215 cm
V = 0.036 m/s

L = 0.195 mm

Reynolds number (Re): μSiM-PVB

$$Re = \frac{inertial\ forces}{viscous\ forces} = \frac{\rho u^2}{(\mu u/D)} = \frac{\rho u D}{\mu}$$

ρ = density of the fluid

μ = viscosity of the fluid

D = diameter of the channel

u = velocity of the fluid

$$Hydraulic\ mean\ diameter\ (D_e) = \frac{4A}{P}$$

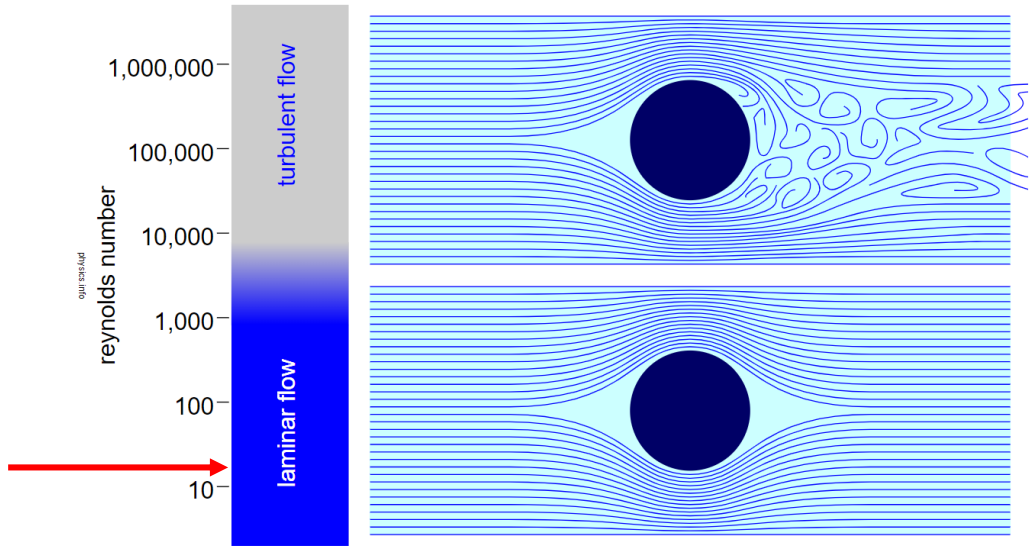
A = cross-sectional area

P = wetted perimeter

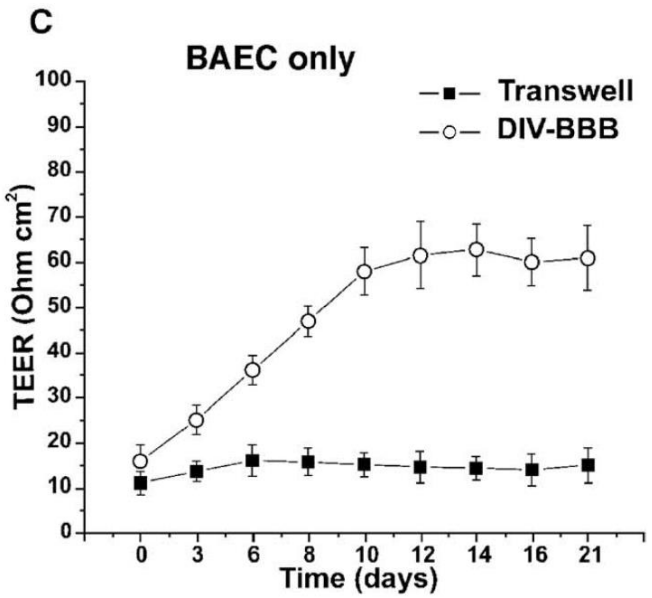
μSiM-PVB

ρ = 997 kg/m^3
μ = 8.9 x 10^{-4} Pa. s
D_e = 7.08 x 10^{-4} m
u = 0.036 m/s

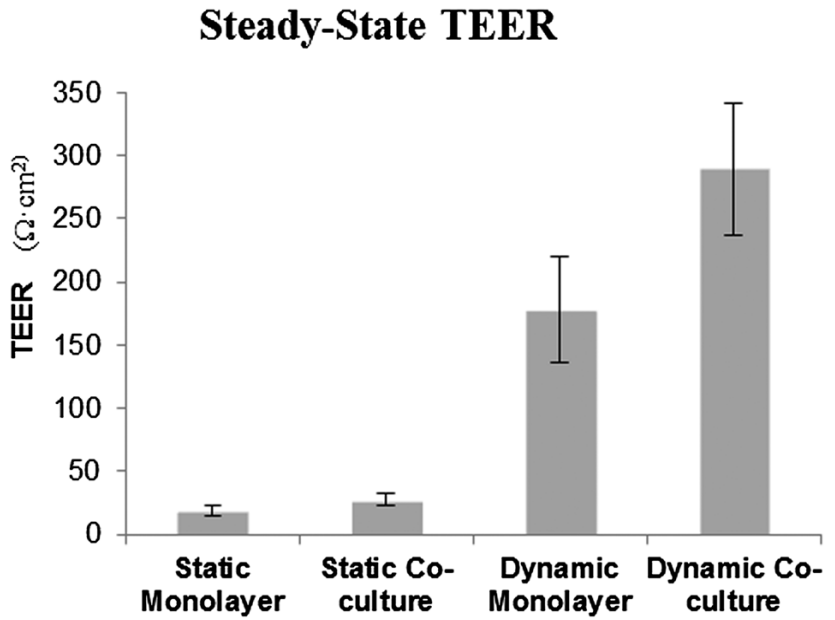
Re ~ 29



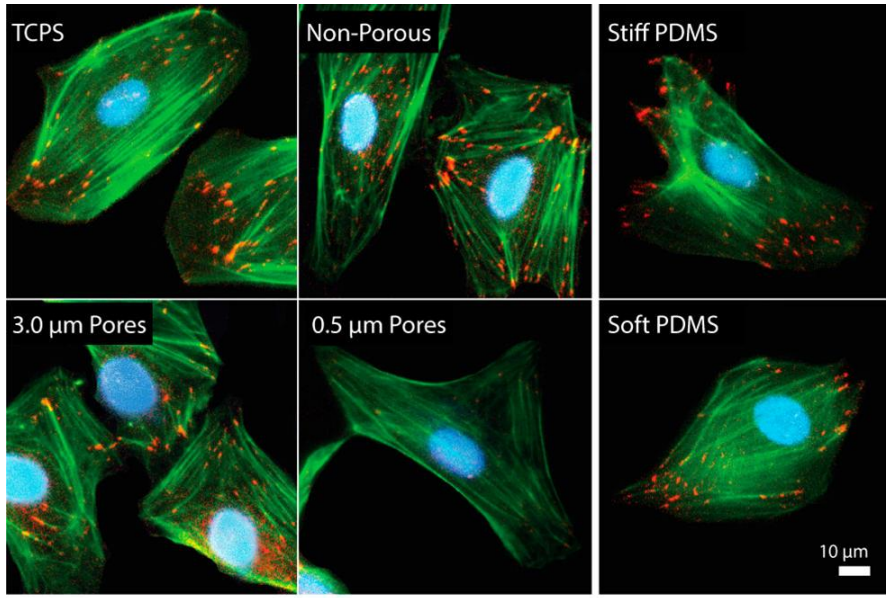
Shear Stress Improves EC Barrier *In Vitro*



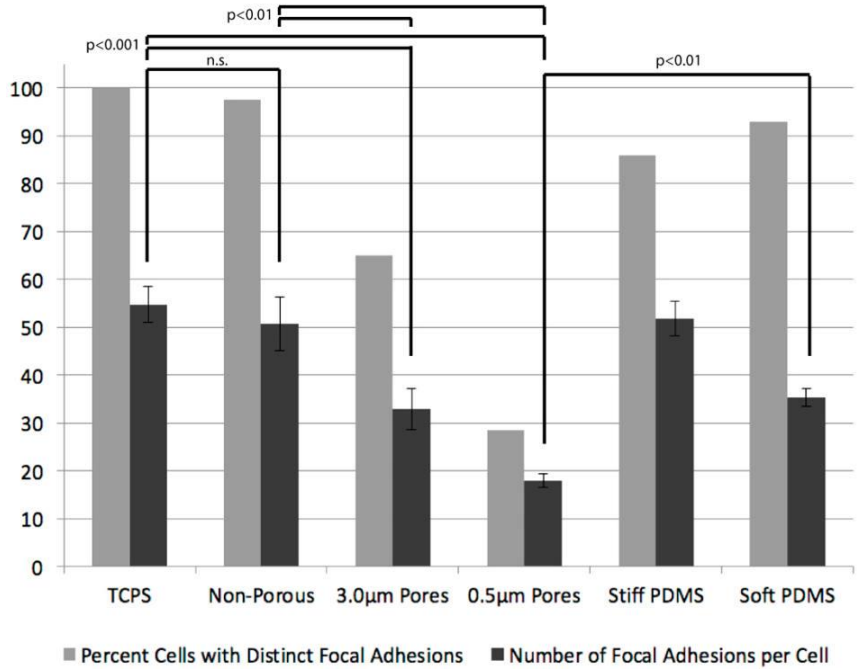
Santaguida et al., Brain Res. 2006

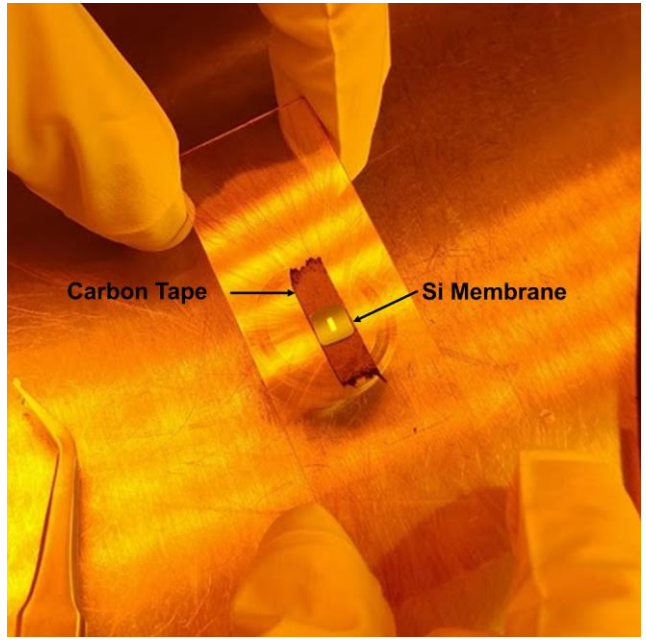


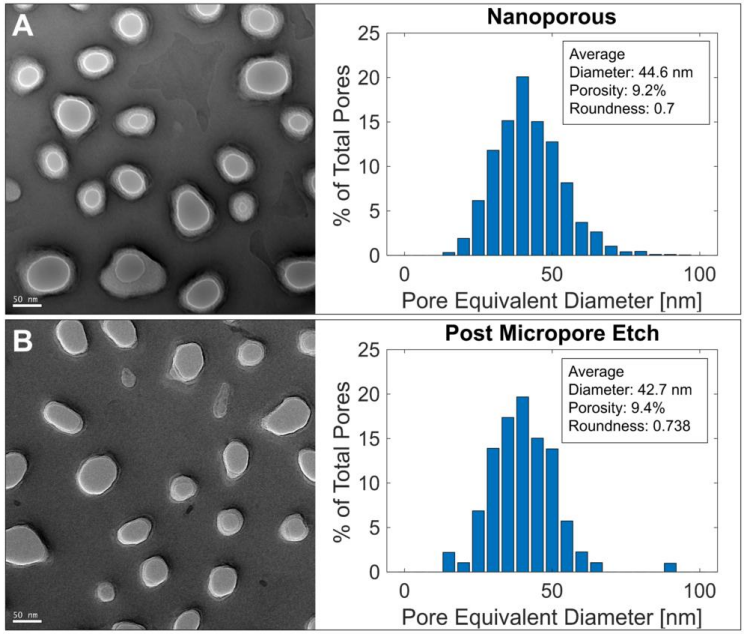
Booth et al., Lab Chip 2006

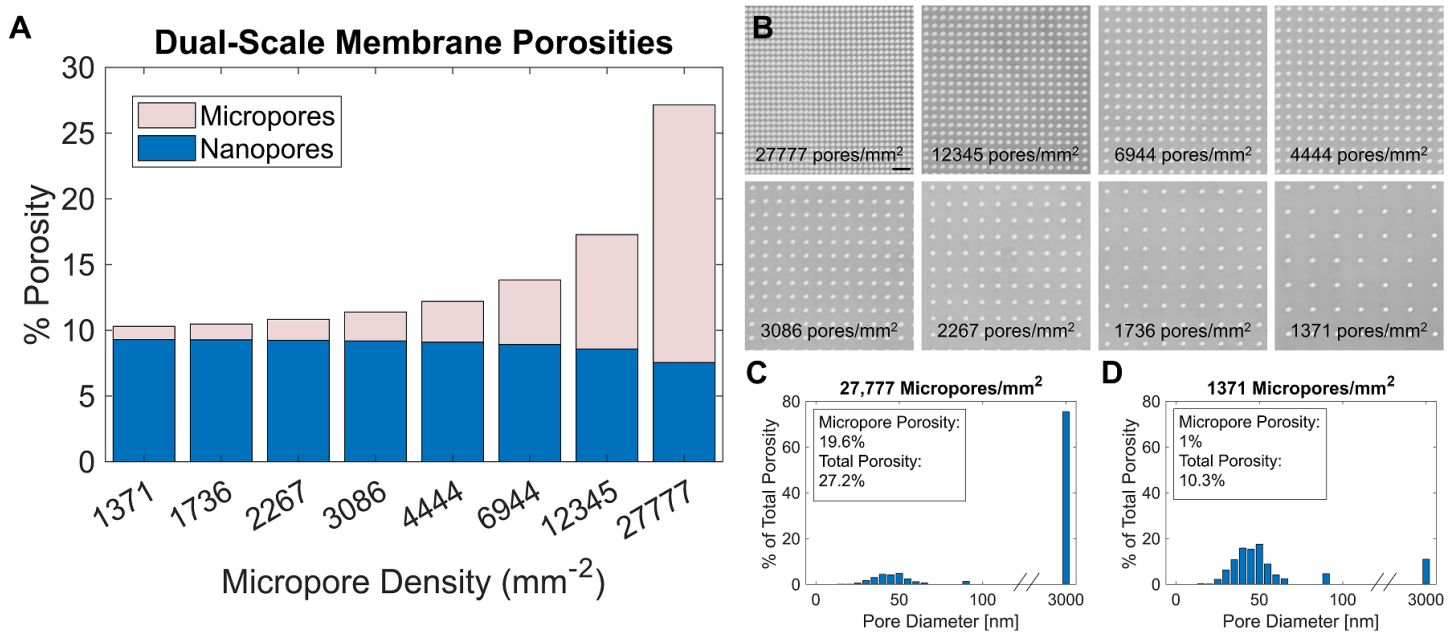


Casillo et al., ACS Biomater. Sci. Eng. 2017

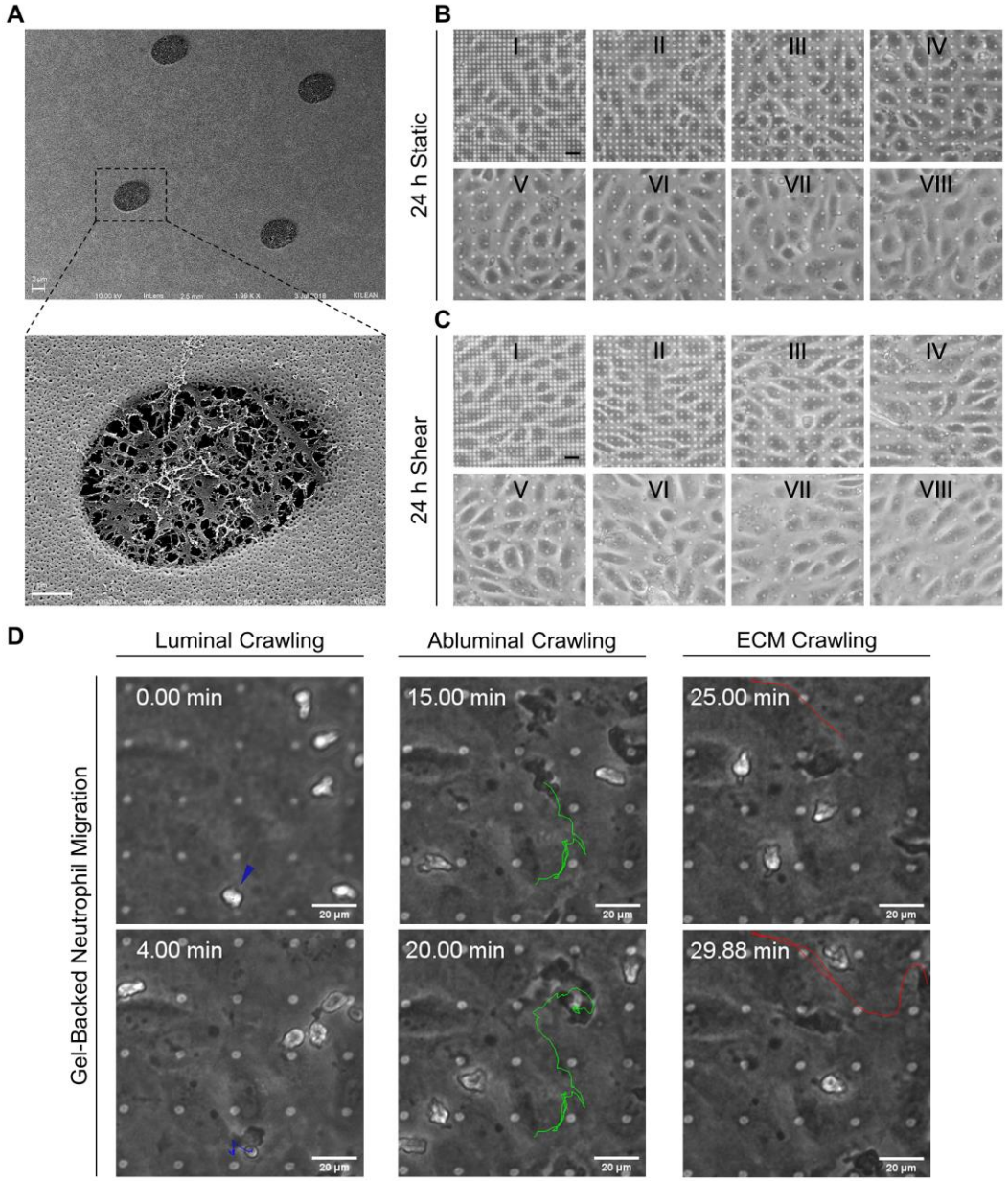


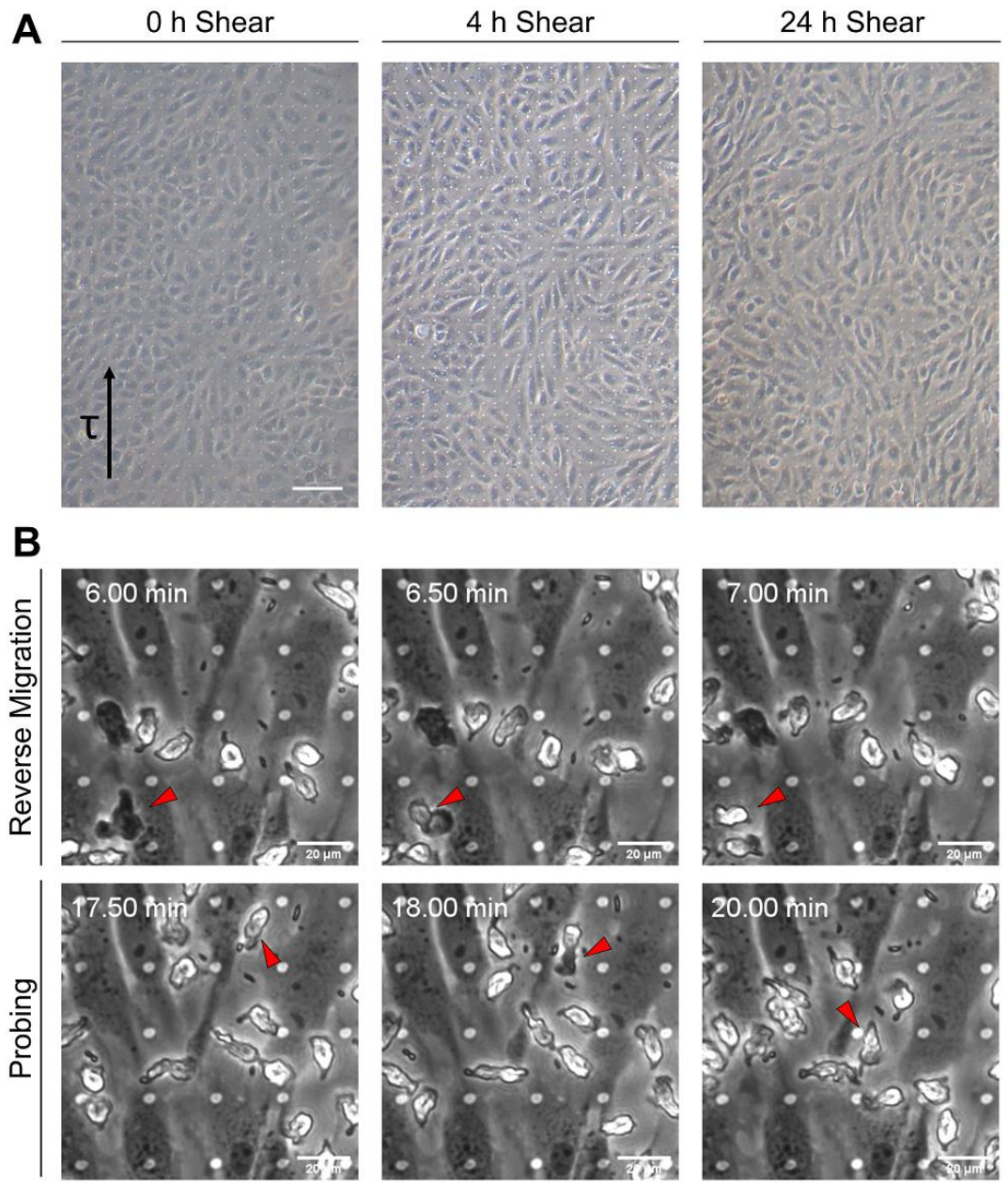






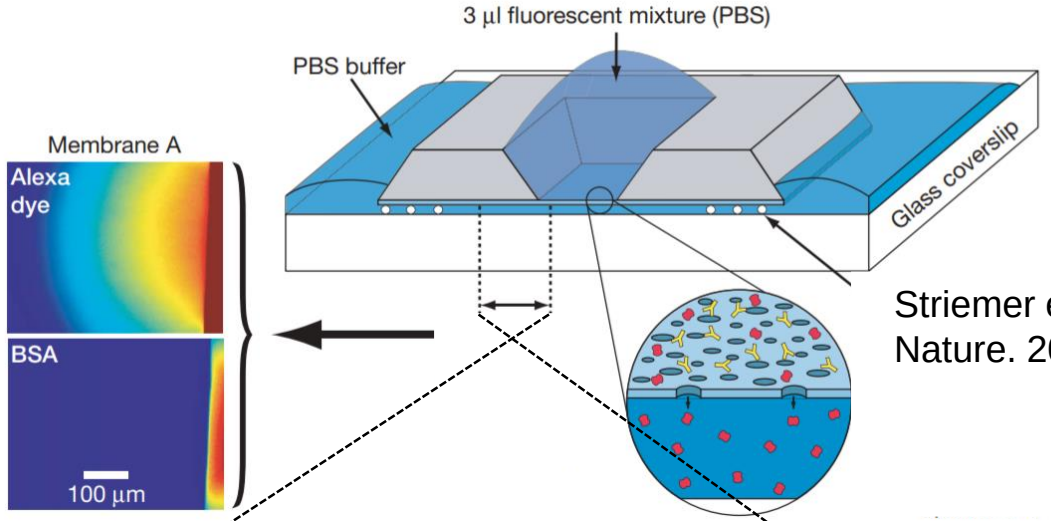
Salminen et al., SMALL 2019



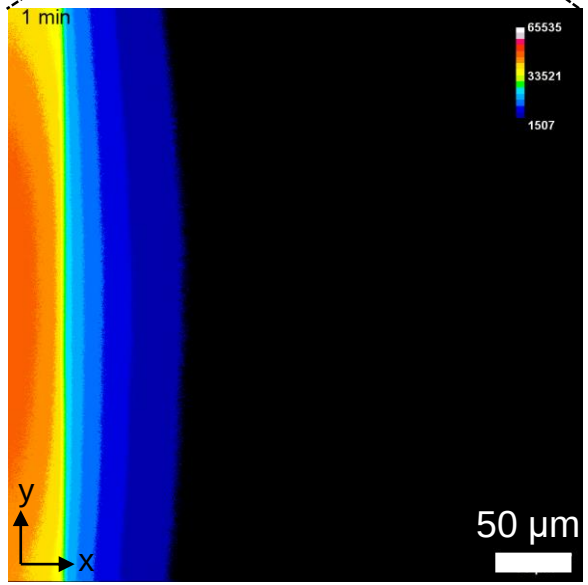


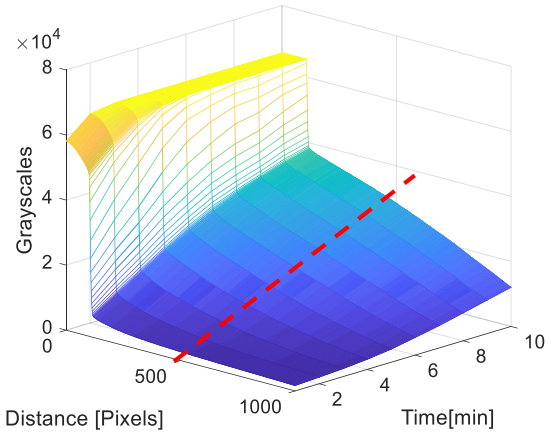
Salminen et al., SMALL 2019

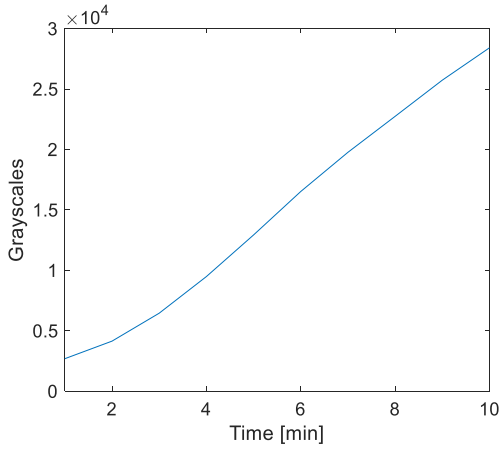
In Situ Permeability Measurements



Striemer et al., Nature. 2007







Question: How do Fick's laws of diffusion govern concentration change through a point over time?

$$C_x = C_o erfc\left(\frac{x}{2\sqrt{Dt}}\right) \quad \propto \quad I_x = I_o erfc\left(\frac{x}{2\sqrt{Dt}}\right)$$

Crank., The Mathematics of Diffusion

Diffusion Function Derivation

Solution of Fick's Second Law in Semi-Infinite Medium with a Constant Source

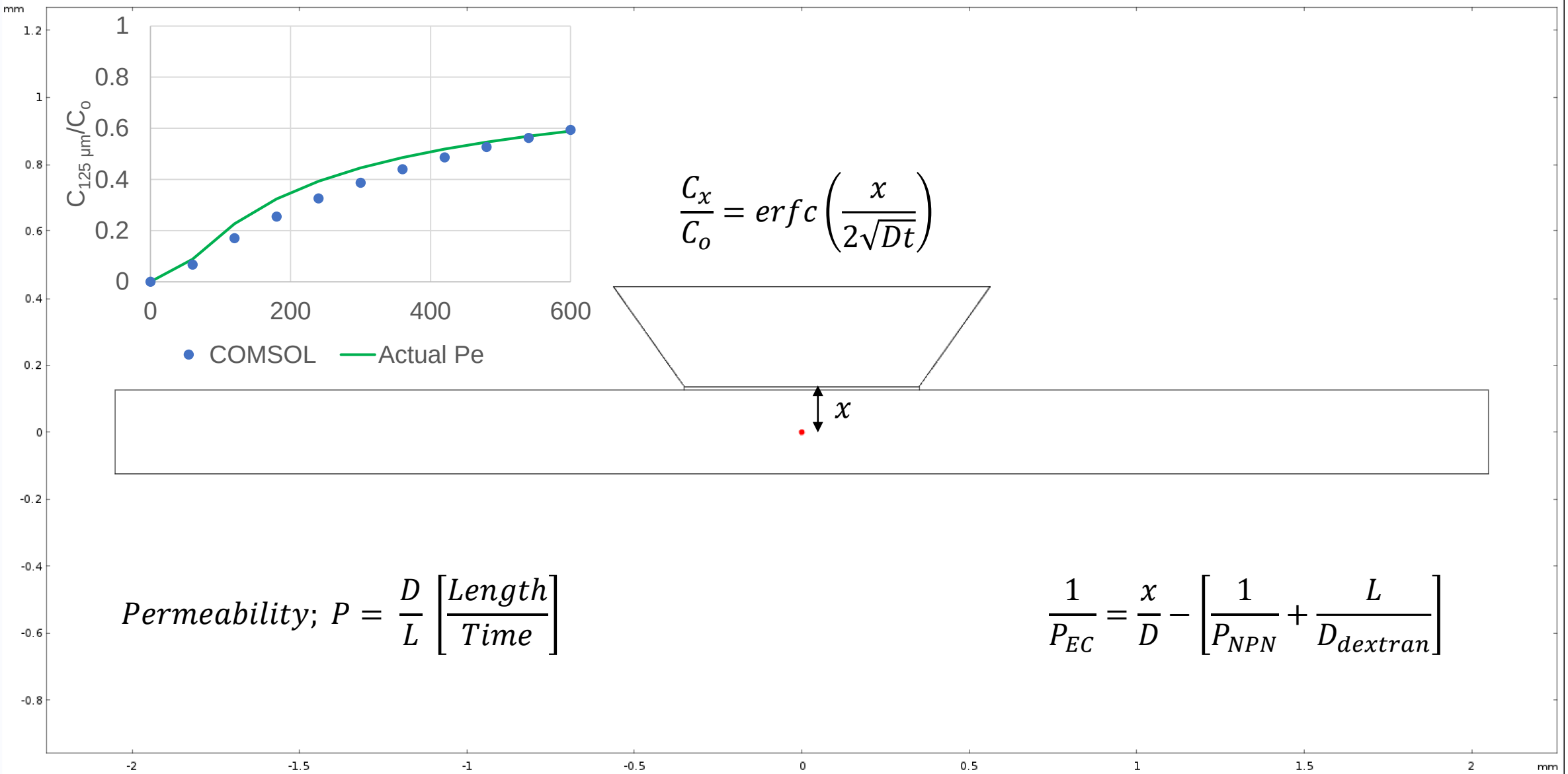
$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2}$$

B.C. $C = C_o, x = 0, t > 0$

$C = 0, x > 0, t = 0$

$$C_x = C_o erfc\left(\frac{x}{2\sqrt{Dt}}\right) \quad \propto \quad I_x = I_o erfc\left(\frac{x}{2\sqrt{Dt}}\right)$$

Ideal COMSOL Configuration

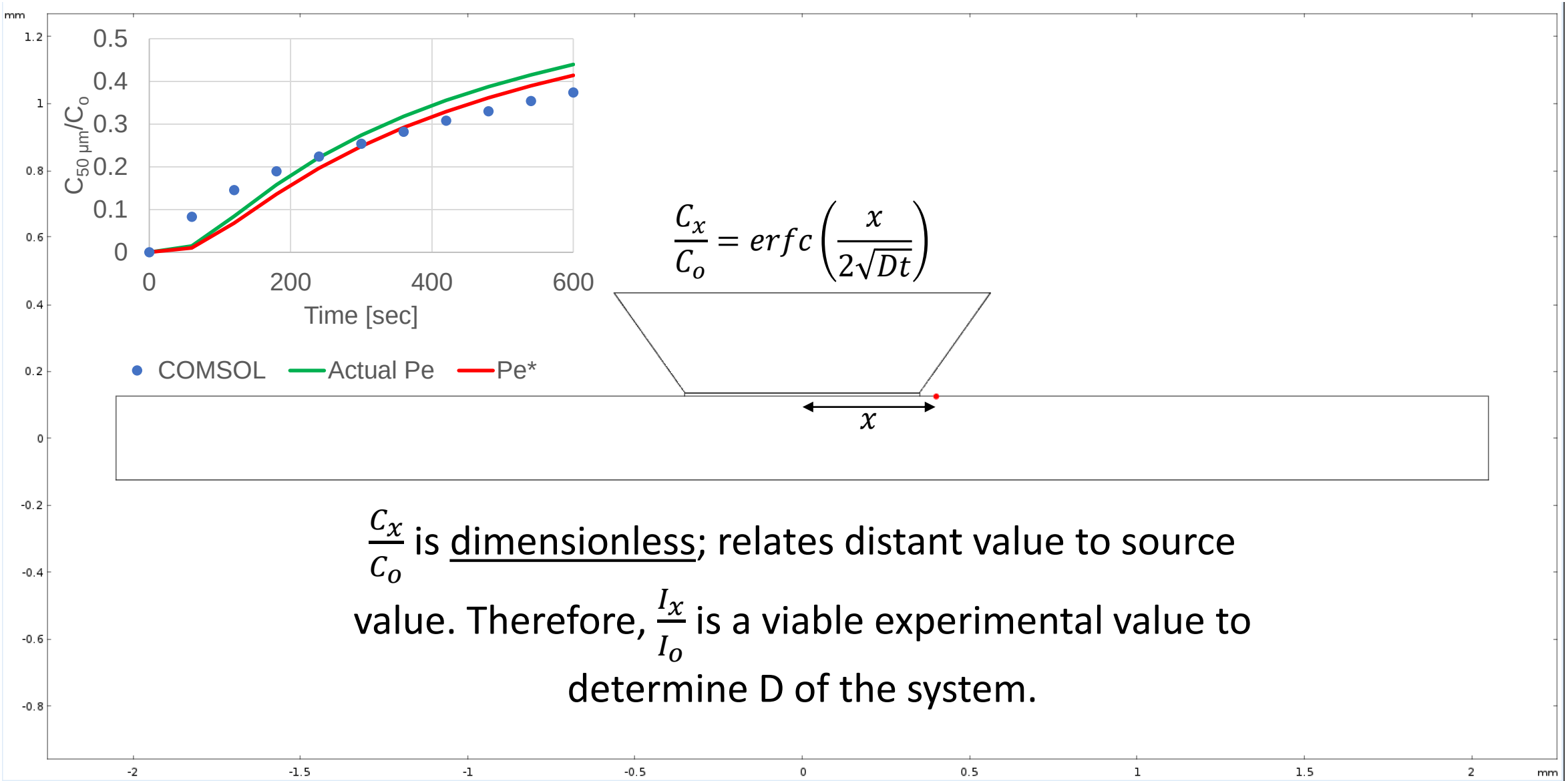


$$\frac{C_x}{C_o} = erfc\left(\frac{x}{2\sqrt{Dt}}\right)$$

$$Permeability;\ P = \frac{D}{L}\left[\frac{Length}{Time}\right]$$

$$\frac{1}{P_{EC}} = \frac{x}{D} - \left[\frac{1}{P_{NPN}} + \frac{L}{D_{dextran}}\right]$$

Experimental COMSOL Configuration

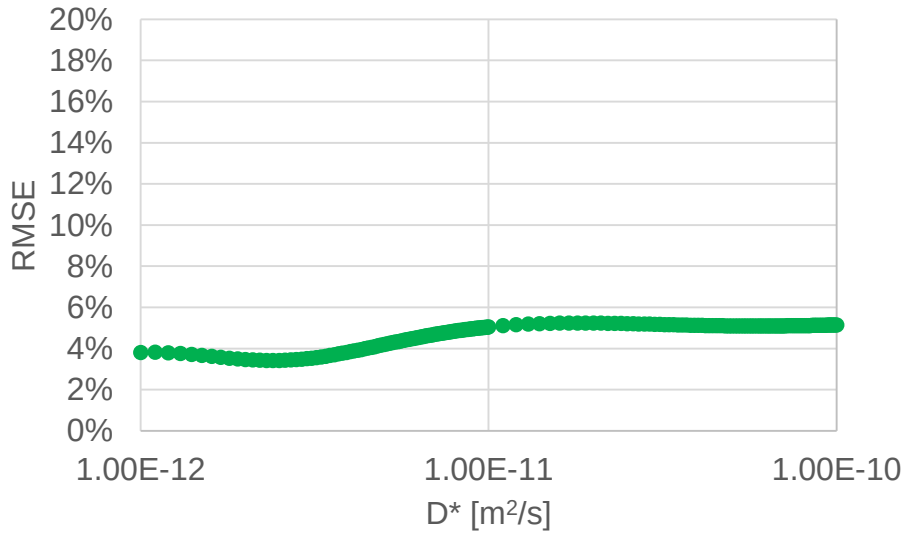


$\frac{C_x}{C_o}$ is <u>dimensionless</u>; relates distant value to source value. Therefore, $\frac{I_x}{I_o}$ is a viable experimental value to determine D of the system.

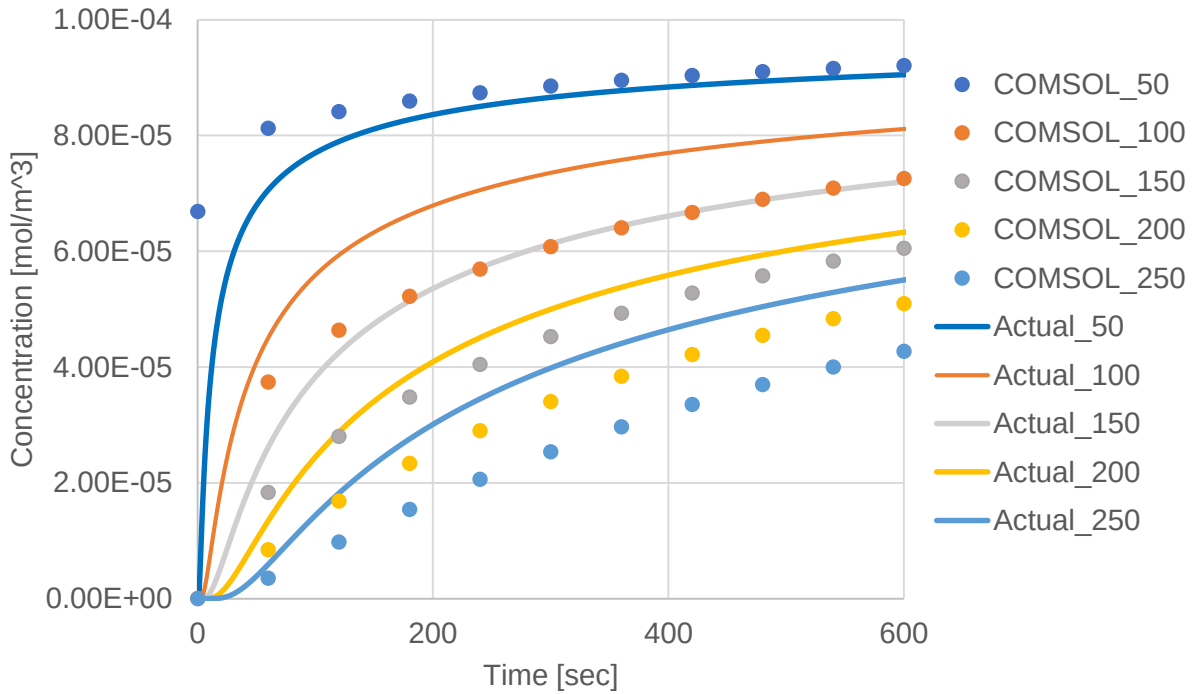
RMSE: COMSOL vs Analytical

Validating Our Model

- Parametric sweep of 200 ‘Pe’ values
- RMSE compares COMSOL data for concentration to analytical data
- RMSE is consistent around 5%
- Lowest RMSE through iterative check was comparable

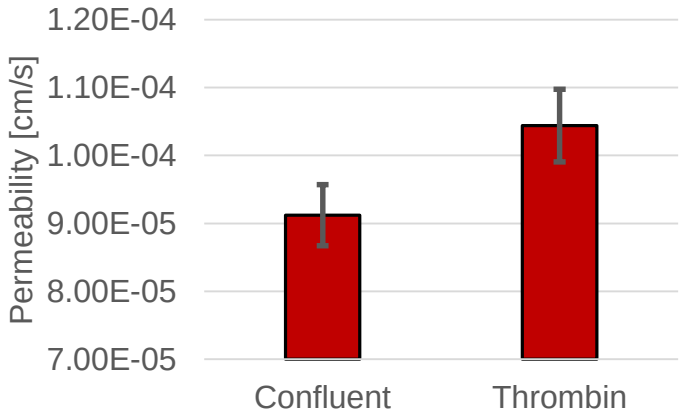


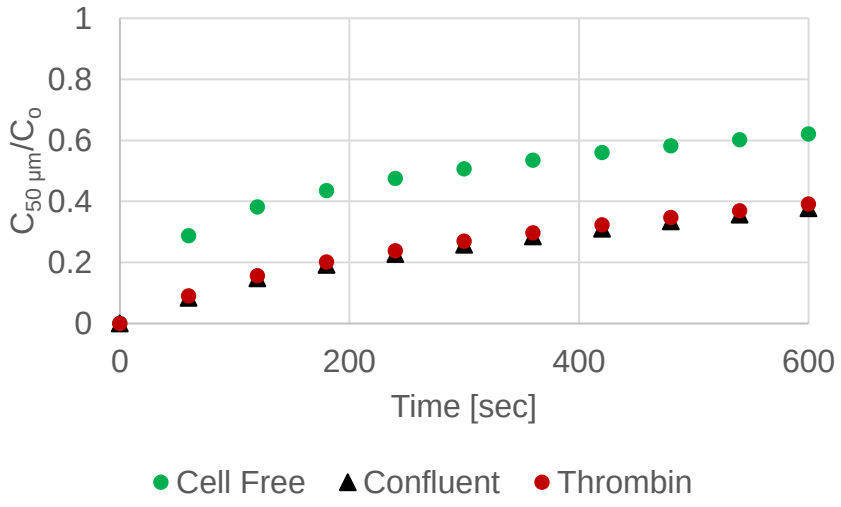
Solution Falls Apart as x Increases

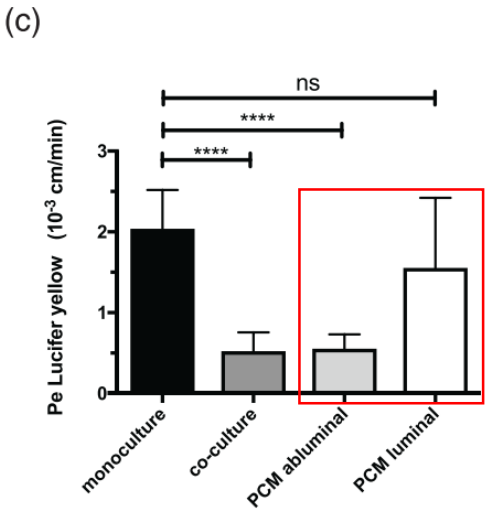


$$\uparrow x = \uparrow Root\ Mean\ Square\ Error$$

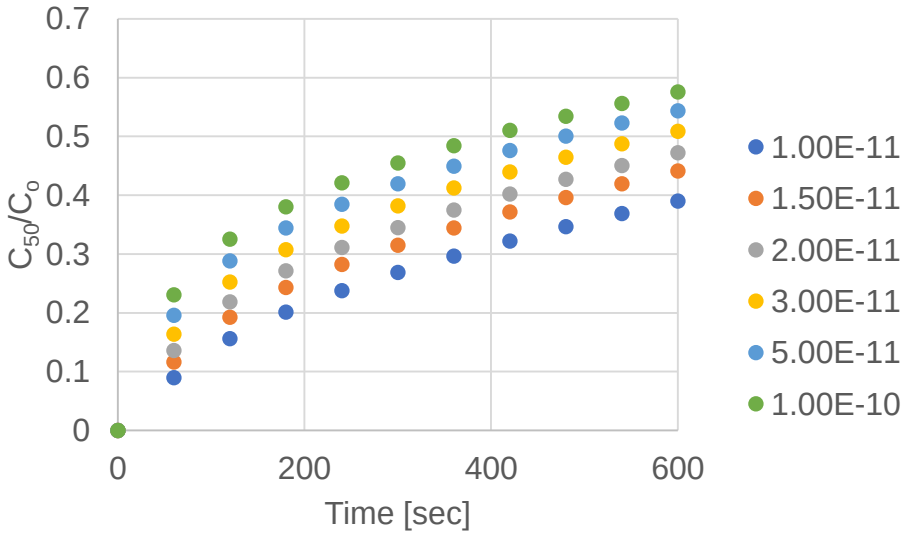
Transwell Data



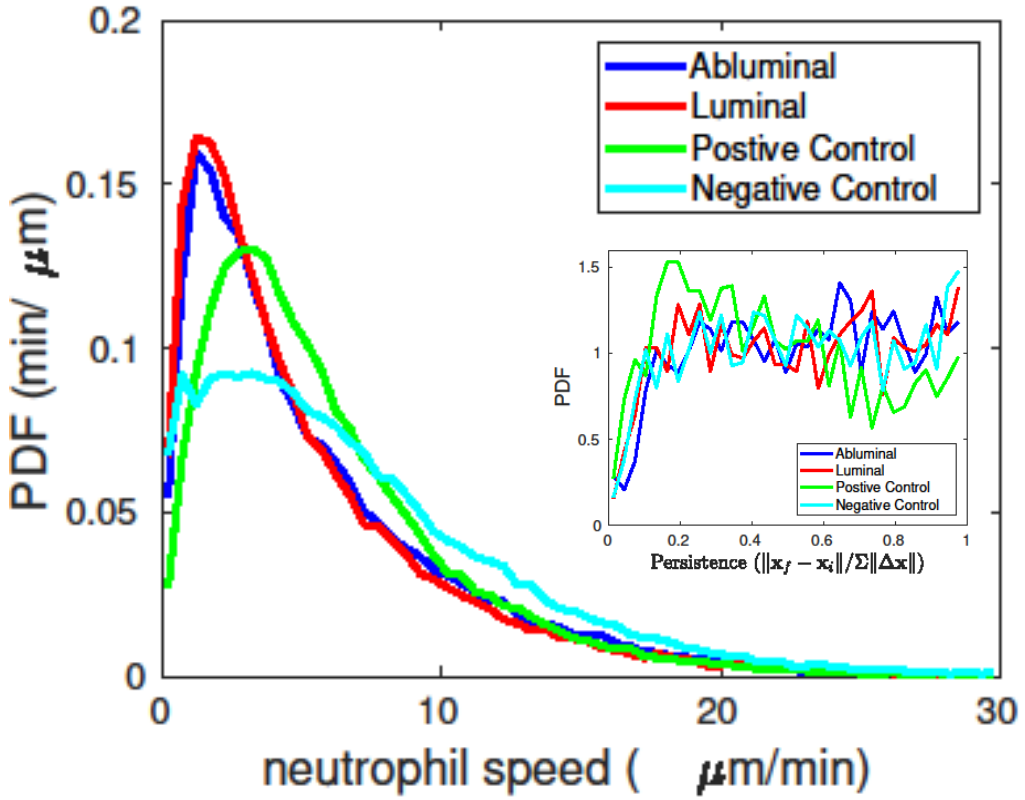


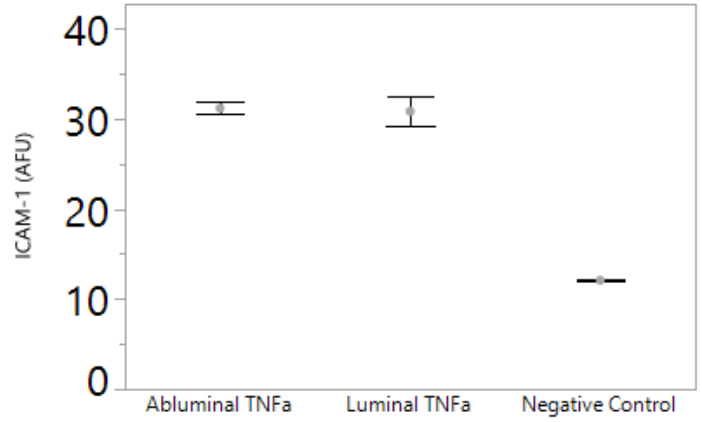


Apicobasal Polarity

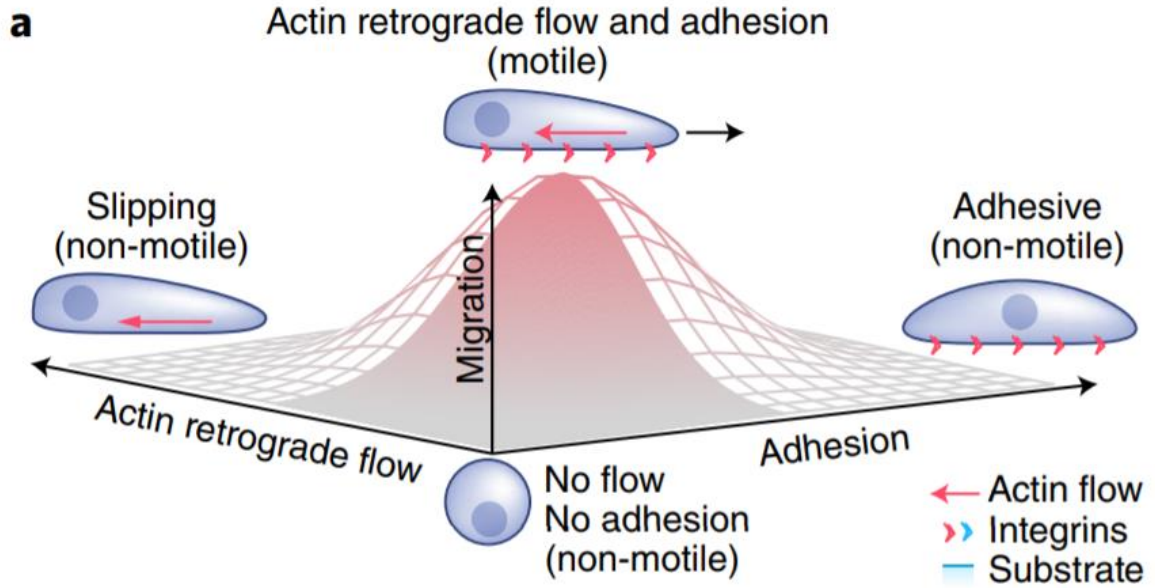


Increased ICAM-1 Decreases PMN Velocity





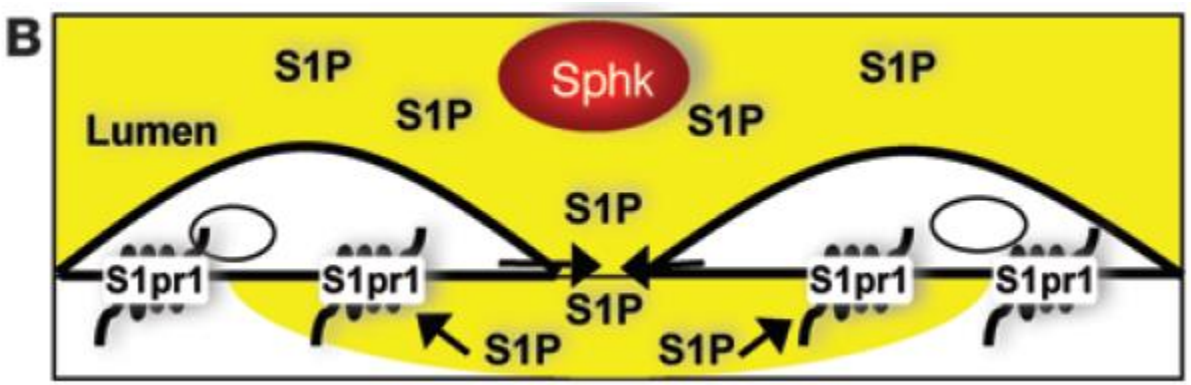
Each error bar is constructed using 1 standard error from the mean.



Sphingosine-1-phosphate in the plasma compartment regulates basal and inflammation-induced vascular leak in mice

Eric Camerer,[1,2,3] Jean B. Regard,[1] Ivo Cornelissen,[1] Yoga Srinivasan,[1] Daniel N. Duong,[1] Daniel Palmer,[1] Trung H. Pham,[4] Jinny S. Wong,[5] Rajita Pappu,[1] and Shaun R. Coughlin[1,2]

[1]Cardiovascular Research Institute and [2]Department of Medicine, UCSF, San Francisco, California, USA. [3]Equipe Avenir, INSERM U970, Paris Cardiovascular Research Center (PARCC), Paris, France. [4]Immunology Graduate Program, UCSF, San Francisco, California, USA. [5]Gladstone Institutes, San Francisco, California, USA.



Level of receptor versus downstream effectors?