Seeing is believing? High speed imaging of cell cultures in ultrasonic fields.

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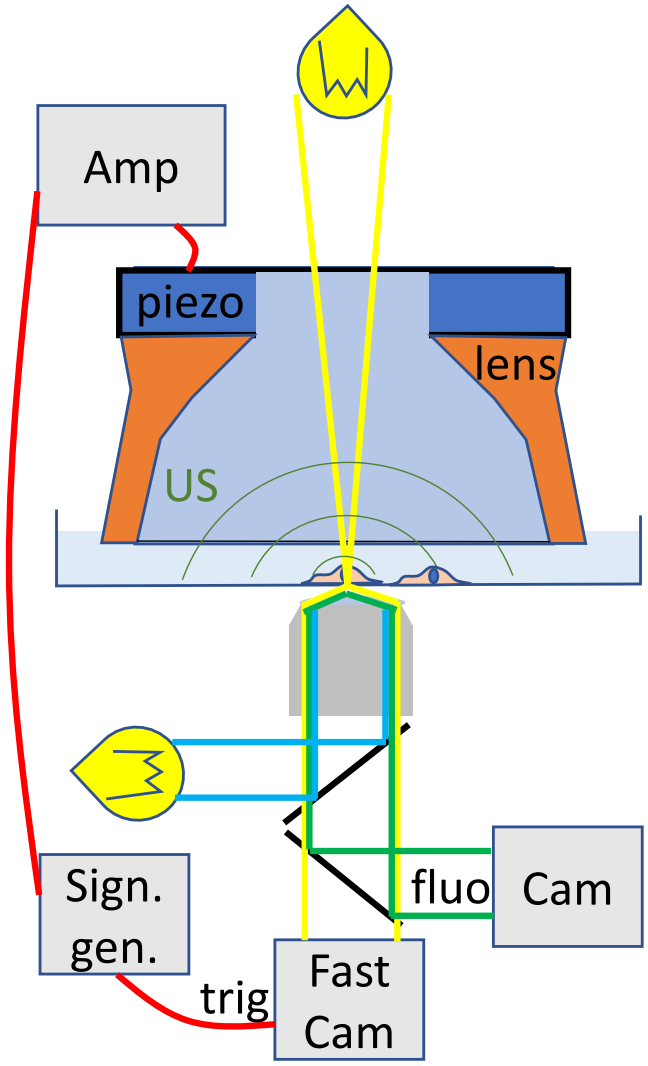
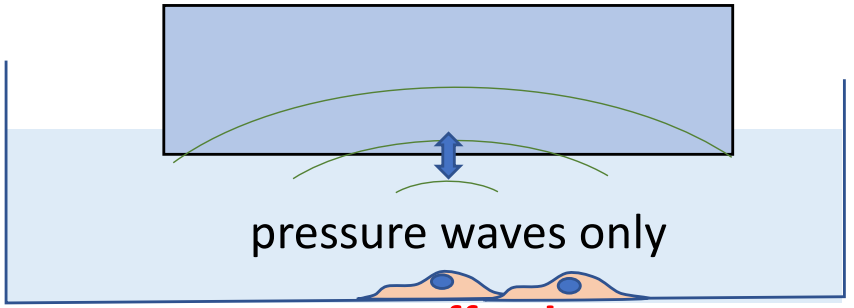
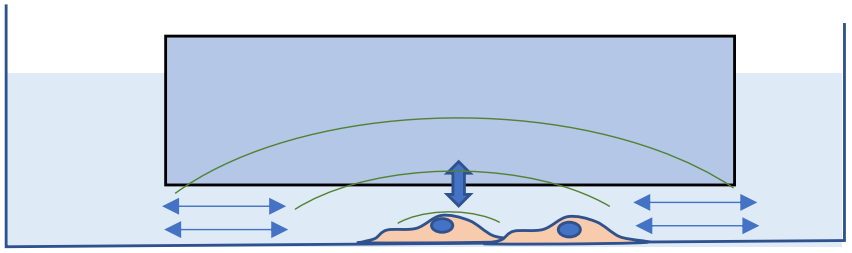
Summary

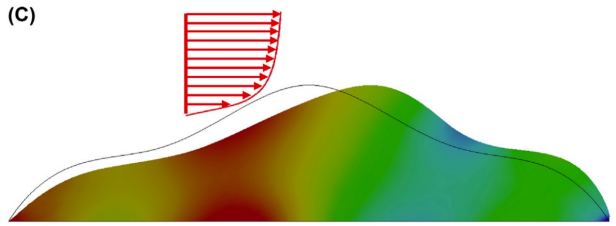


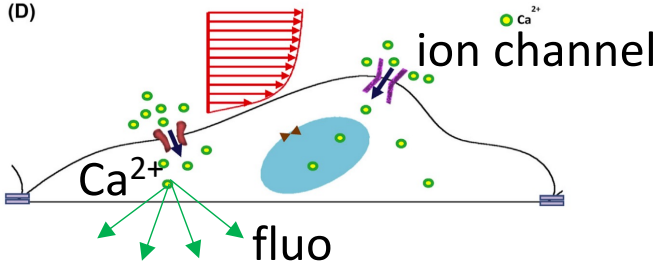


no effect!

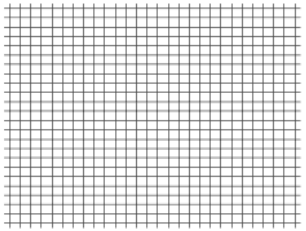


shear flow





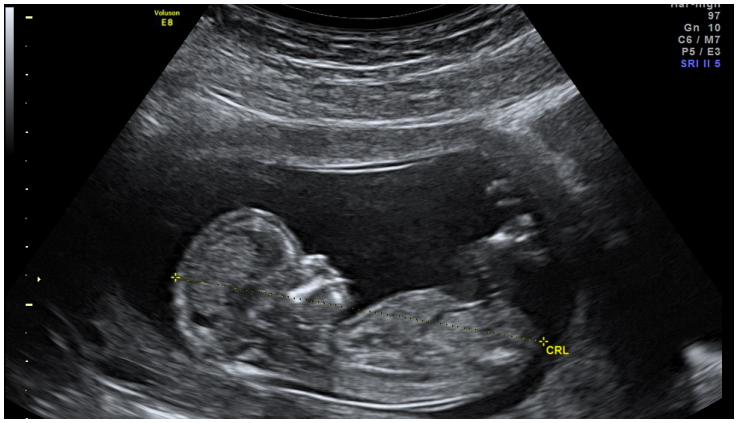
Ultrasound effects on cells and tissue



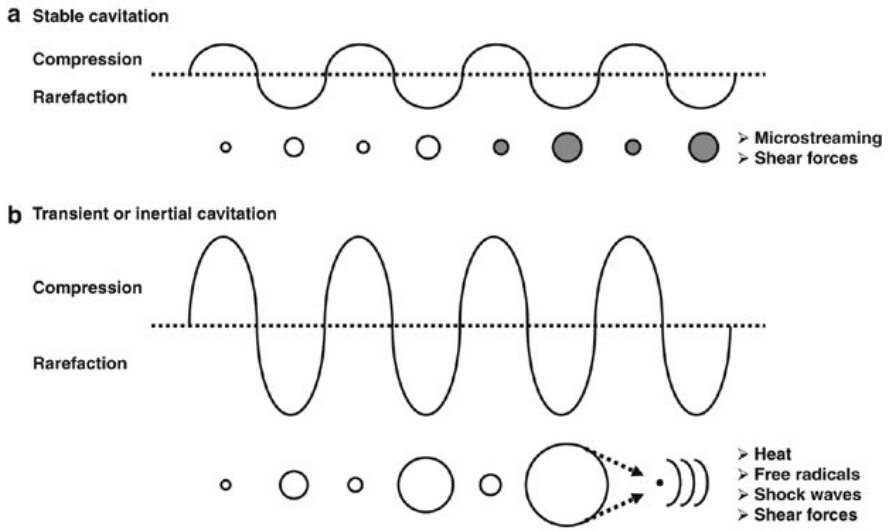
Pressure wave generates forces

$F = P/A$

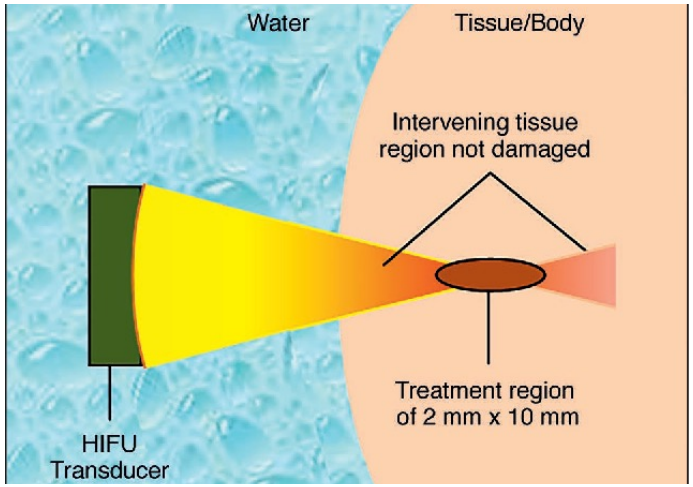
Imaging



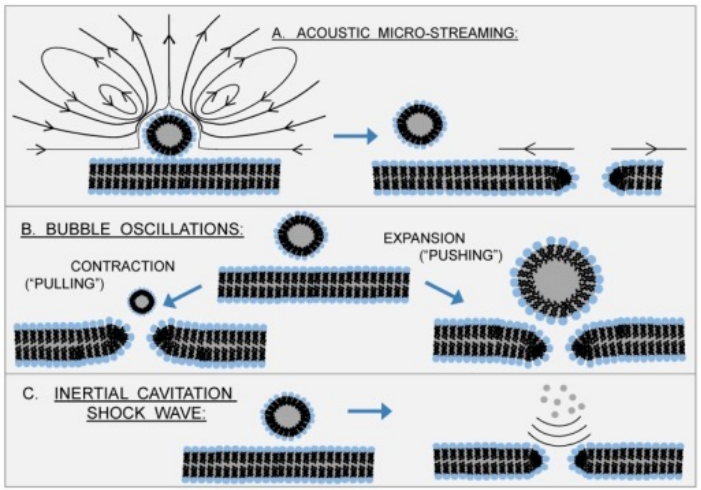
Cavitation



Heating



Microbubbles

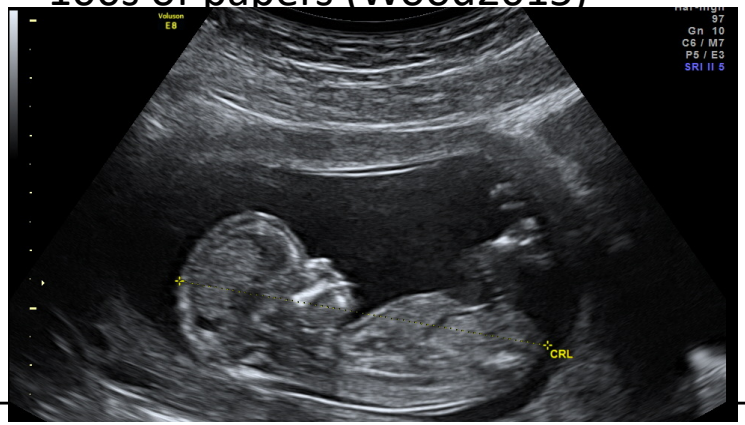


Low intensity pulsed US (LIPUS): No heating, no cavitation, no microbubbles

Some reported biological effects of LIPUS

Killing cancer cells selectively:

- "Oncotripsy": 4 publ. 2016-2020 (J.Appl.Phys.++)
- US+hyperthermia ⇒ Apoptosis (Feril 2002)
- Mike Sheetz group: Piezo ⇒ Ca^{2+} ⇒ apoptosis
- Apoptosis while other cells increased proliferation (Schuster2013)
- Microbubbles, cavitation or thermal ⇒ 100s of papers (Wood2015)

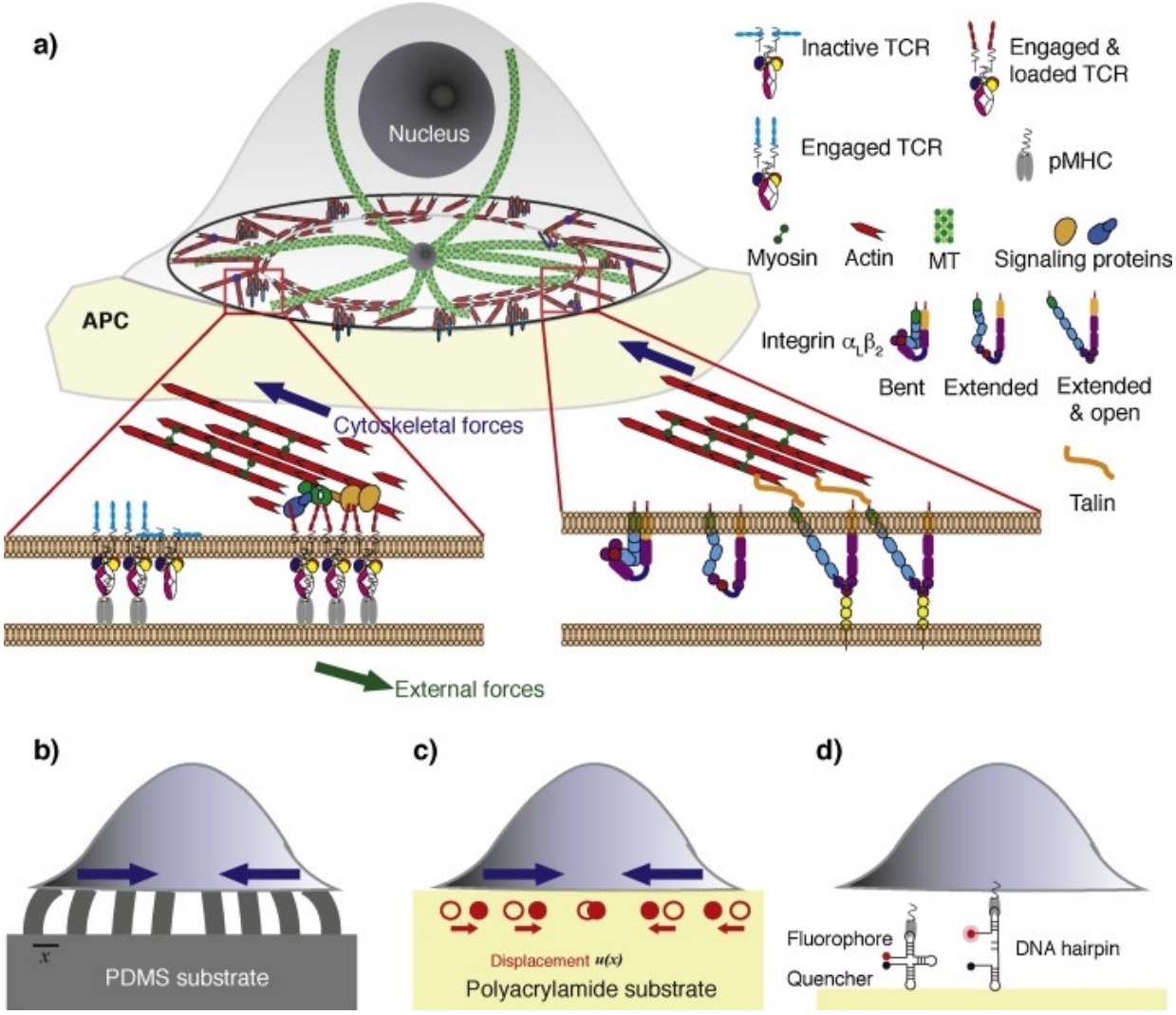


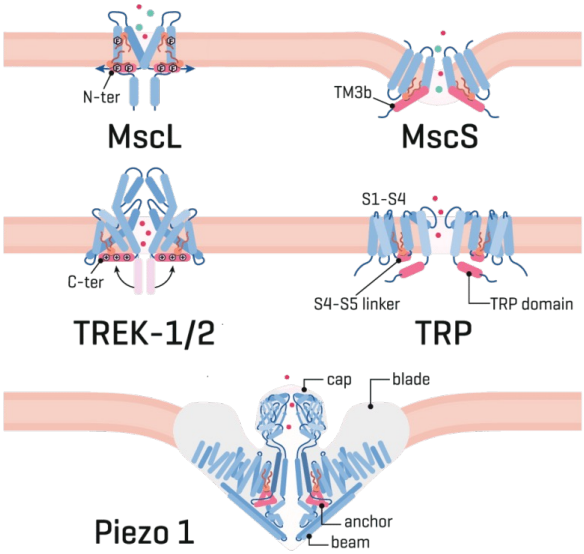
No effect: One single study (Lucas 2021)!

- Increased insulin release from pancreatic beta cells (Castellanos 2017)
- Enhanced diverse transcription factors, increased proliferation (Puts 2016,2018,2018b)
- Improved osteogenic comittment & differentiation (Costa 2018)
- Enhanced viability & proliferation of iPSC (Lv 2013)
- Increased expression of chondrogenic markers (Subramanian group 2012, 2013, 2017)
- Perturbs cytoskeleton dynamics (Misrahi 2012, incl. Dave Weitz!)
- Increased growth & proliferation of stem cells (Gao 2016)
- Modulates ion channel currents (Kubanek 2016)
- Interleaflet cavitation (Kimmel group, PNAS 2011)
- +++++

Mechanosensing

how can cells be affected by US pressure?

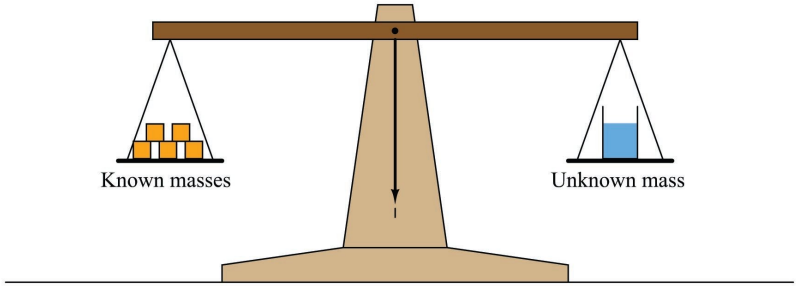




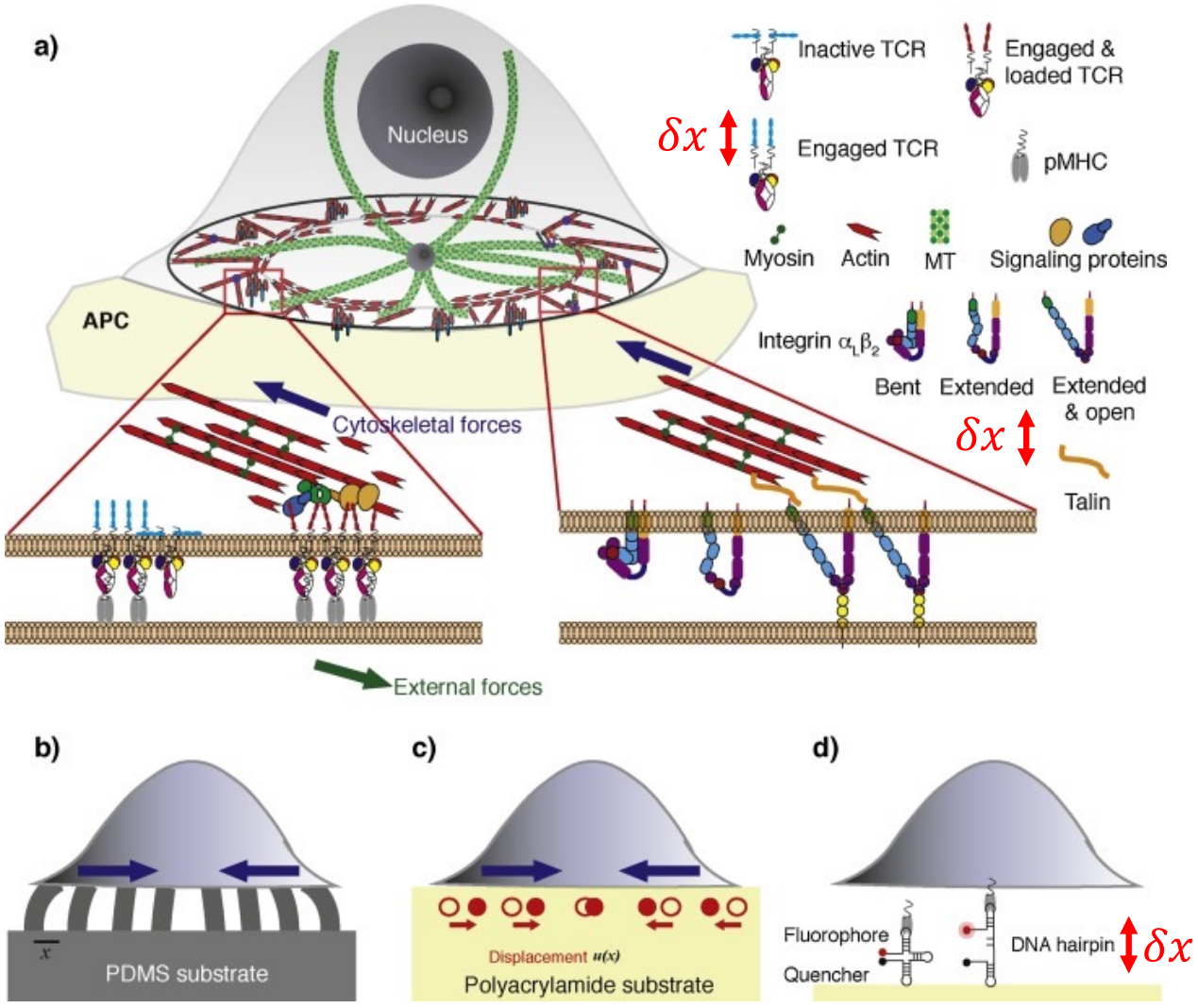
How do you measure a force?

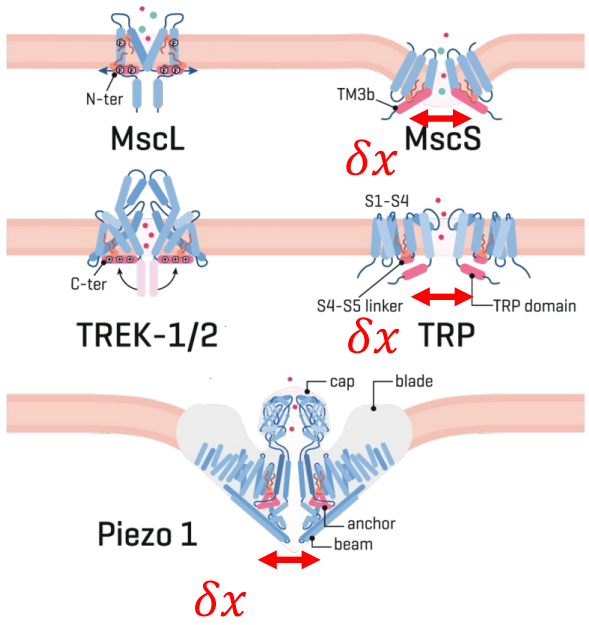
- Newtons law: $f = ma$
- Force balance: $f_1 = f_2$
- Elasticity: $f = \mathrm{k}\delta x$

That's how cells do it



Mechanosensing





2-states: open/closed
open probability:

$$P(f) = \frac{1}{1 + e^{-(\Delta F - f\delta x)/kT}}$$

displacement δx is necessary!

Stress, strain and waves in homogeneous, isotropic solids

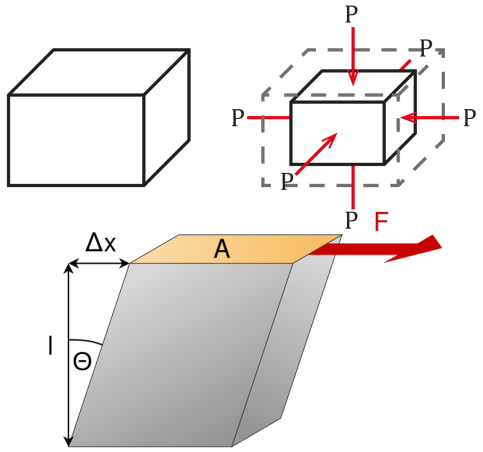
Bulk modulus: $K = -V\frac{dP}{dV} = \frac{\text{normal stress}}{\text{normal strain}}$

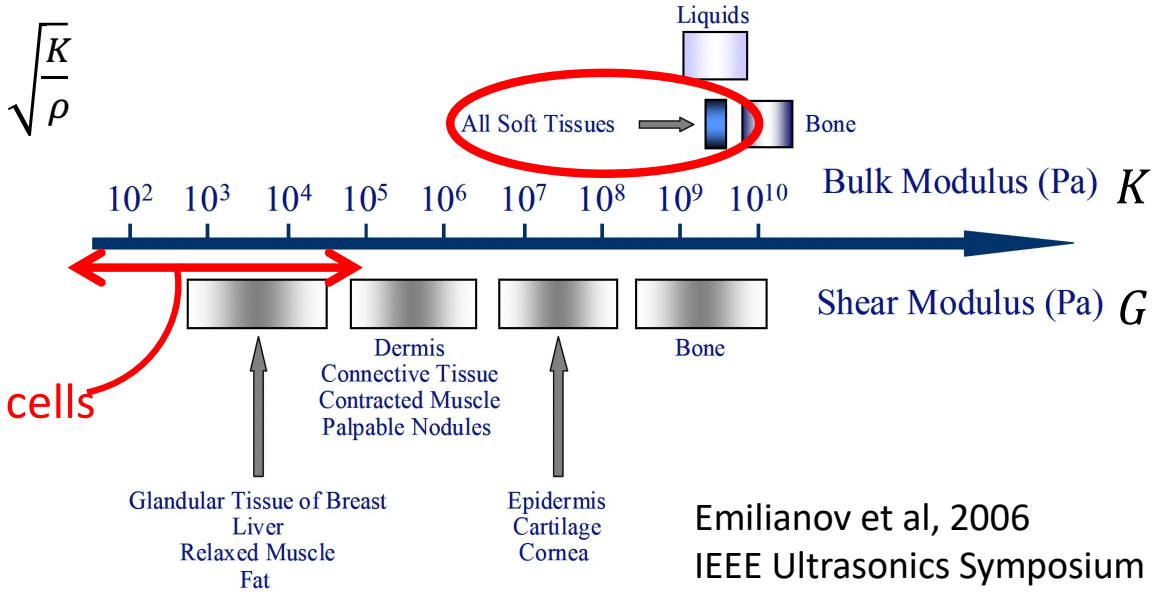
Shear modulus: $G = \frac{\sigma_{xy}}{\gamma_{xy}} = \frac{F/A}{\Delta x/l} = \frac{\text{shear stress}}{\text{shear strain}}$

In soft tissues $K \gg G$

Pressure waves: $v_p = \sqrt{\frac{K+{}^3/_4 G}{\rho}} \approx \sqrt{\frac{K}{\rho}}$

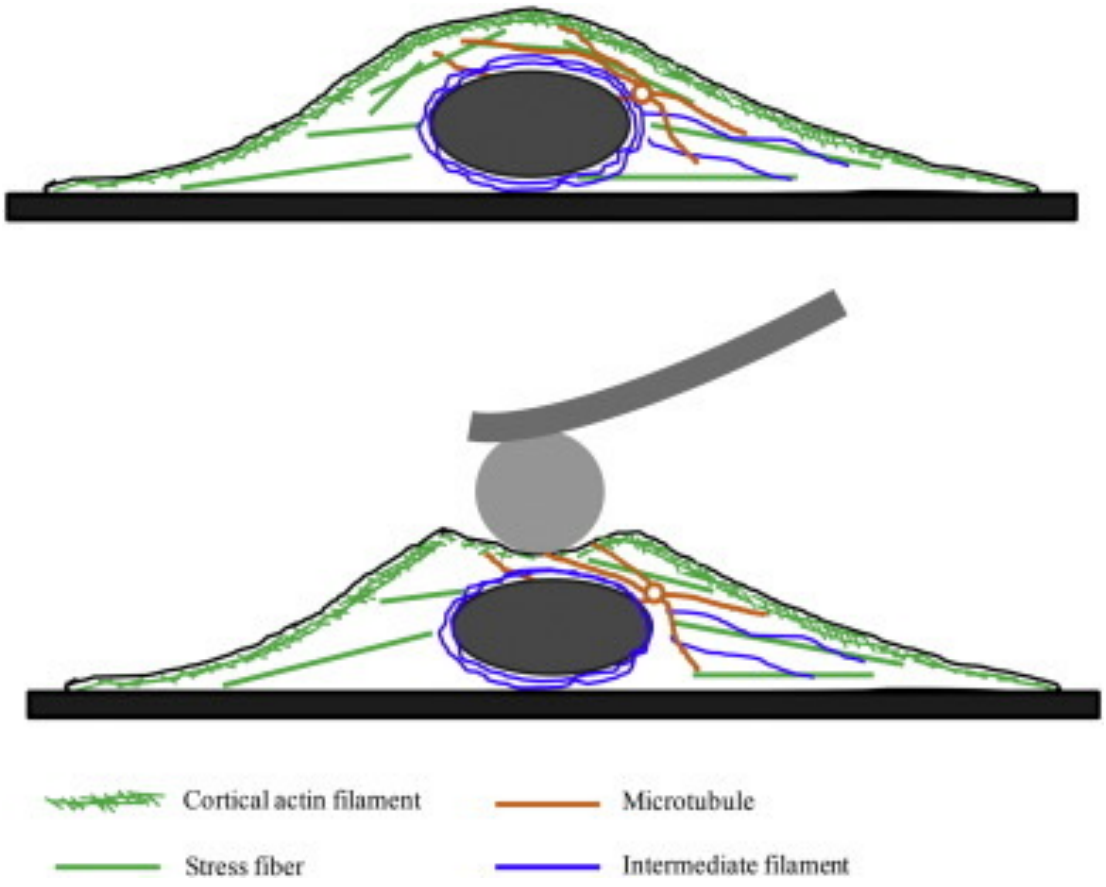
Shear waves: $v_s = \sqrt{{}^G/_\rho}$





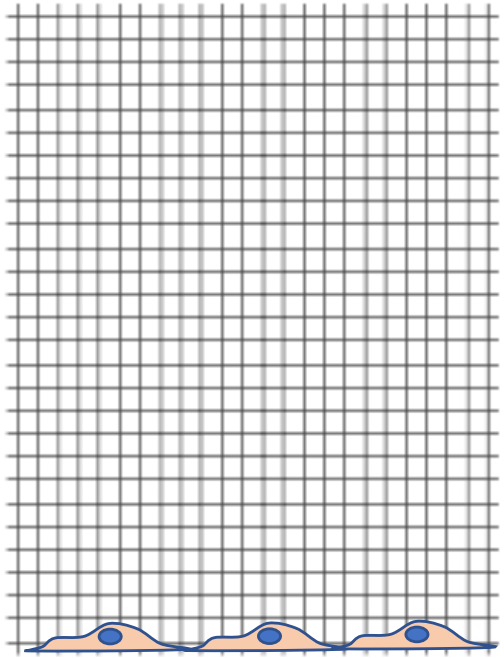
Emilianov et al, 2006
IEEE Ultrasonics Symposium

Soft tissue, soft cells



- "stiffness"
- $= G$: shear modulus
- $\approx \frac{E}{3}$: Youngs modulus
- $\approx 1 - 100$ kPa

Sound wave



water, cells: $K \approx 2$ GPa

$$v_p \approx \sqrt{\frac{K}{\rho}} \approx 1500 \text{ m/s}$$

$$\lambda = {}^{v_p}/_{\nu}$$

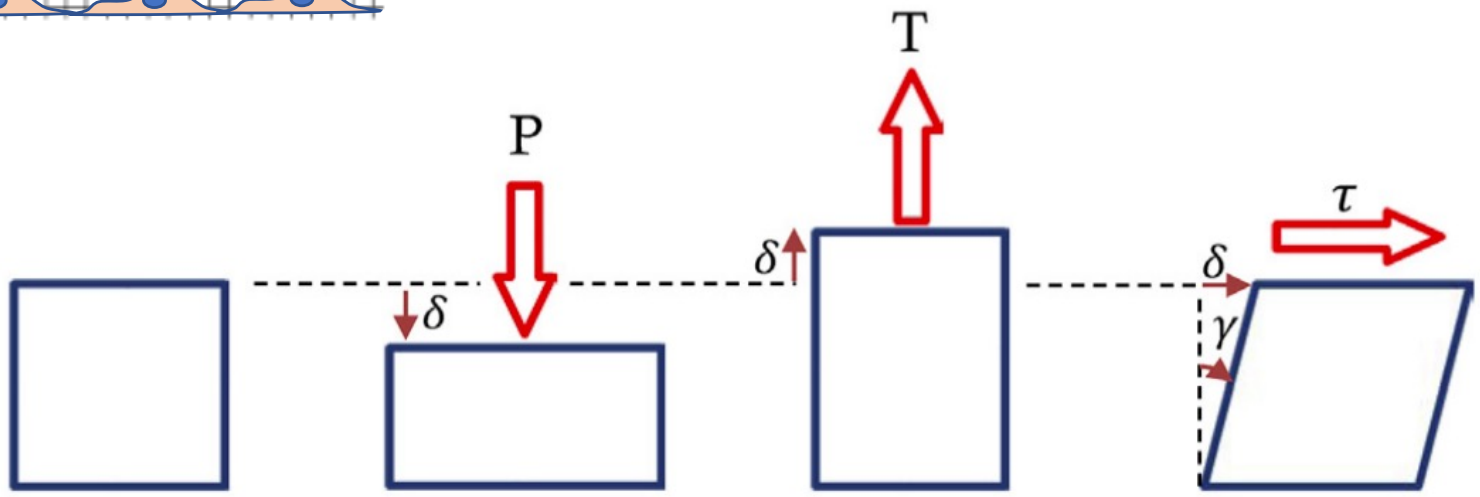
Tissue or Material	Density (g/cm^3)	Speed of Sound (m/sec)
Water	1	1480
Blood	1.055	1575
Fat	0.95	1450
Liver	1.06	1590
Kidney	1.05	1570
Brain	1.03	1550
Heart	1.045	1570
Muscle (along the fibers)	1.065	1575
Muscle (across the fibers)	1.065	1590
Skin	1.15	1730
Eye (lens)	1.04	1650
Eye (vitreous humor)	1.01	1525

20k	100k	1M	10M	1G
7.5cm	1.5cm	1.5mm	150µm	15µm

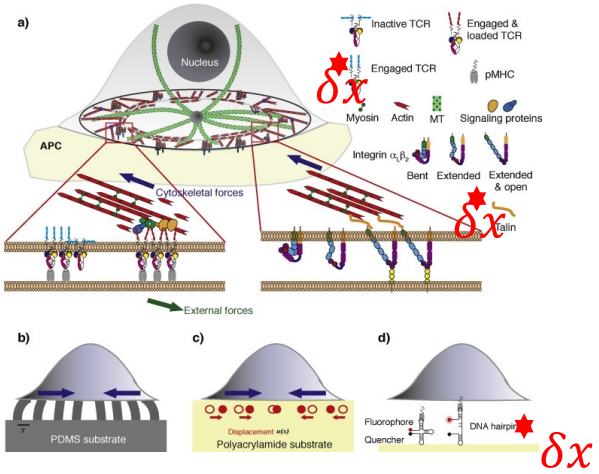
low intensity amplitude: $p_{max} < 10^5$ Pa

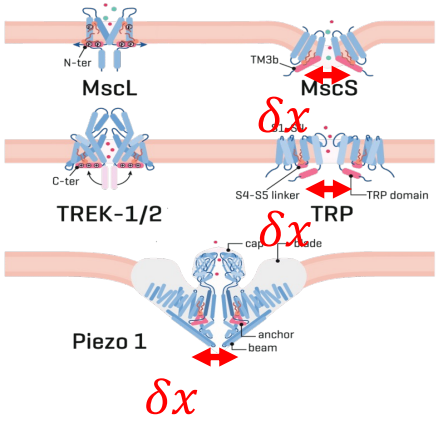
For a cell $l \sim 10\ \mu m$:

- hydrostatic pressure variation
- $\delta = \varepsilon l = \frac{pl}{K} < \frac{10^5 10^{-5}}{2 \cdot 10^9} = \mathbf{5\ Å}, \quad (\varepsilon < 5 \cdot 10^{-5})$



I will not believe it unless I see it!





2-states: open/closed
open probability:

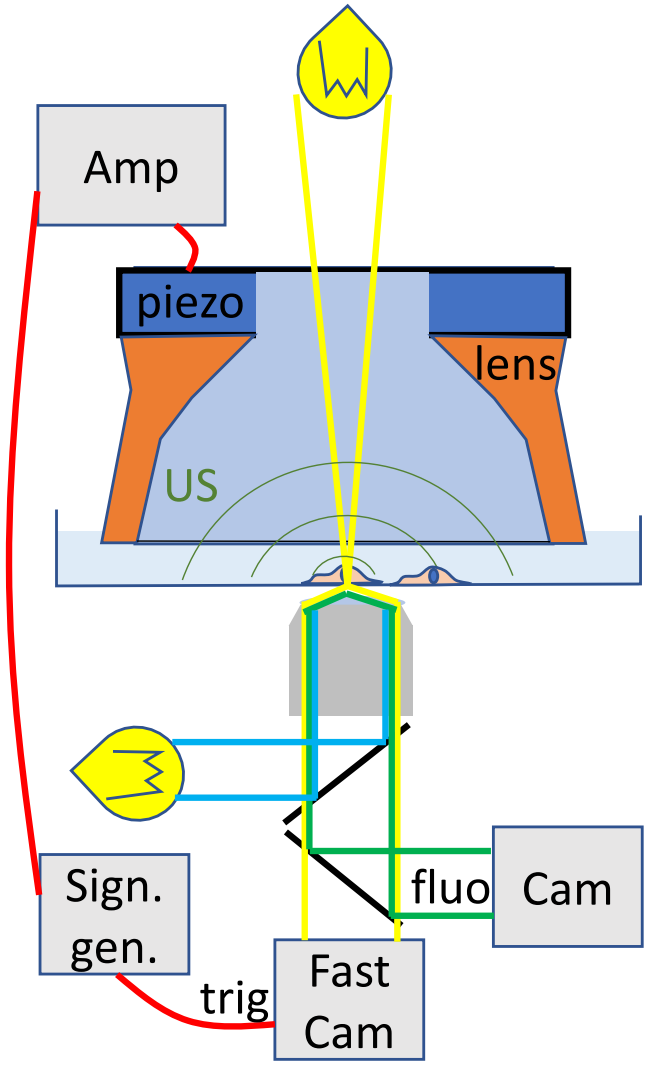
$$P(f) = \frac{1}{1 + e^{-(\Delta F - f\,\delta x)/kT}}$$

$\delta x < 5$ Å

⇒ **Attempt direct measurement of**

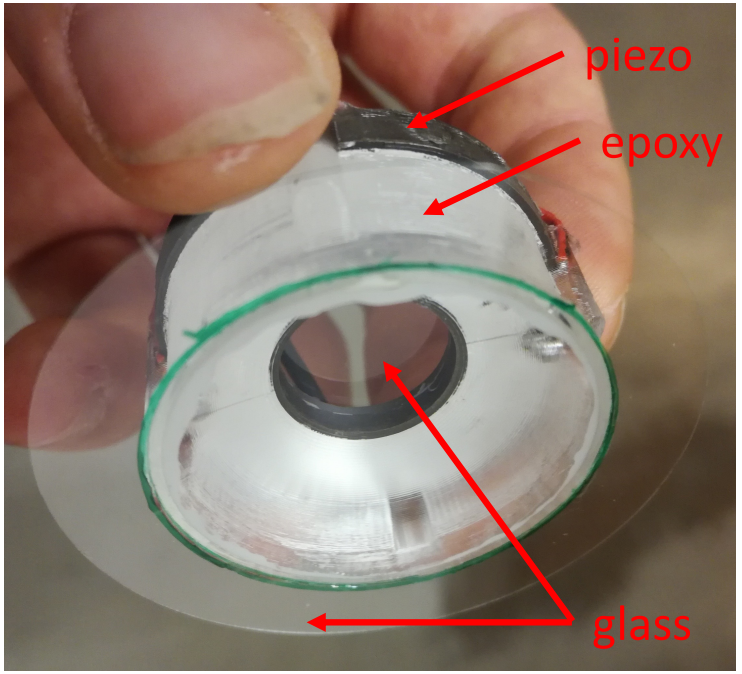
- pressure field
  - calibrate transducer
  - high speed (interferometric) imaging of substrate displacements
  - simulate wave propagation
- displacement field
  - high speed imaging of cells & tracers

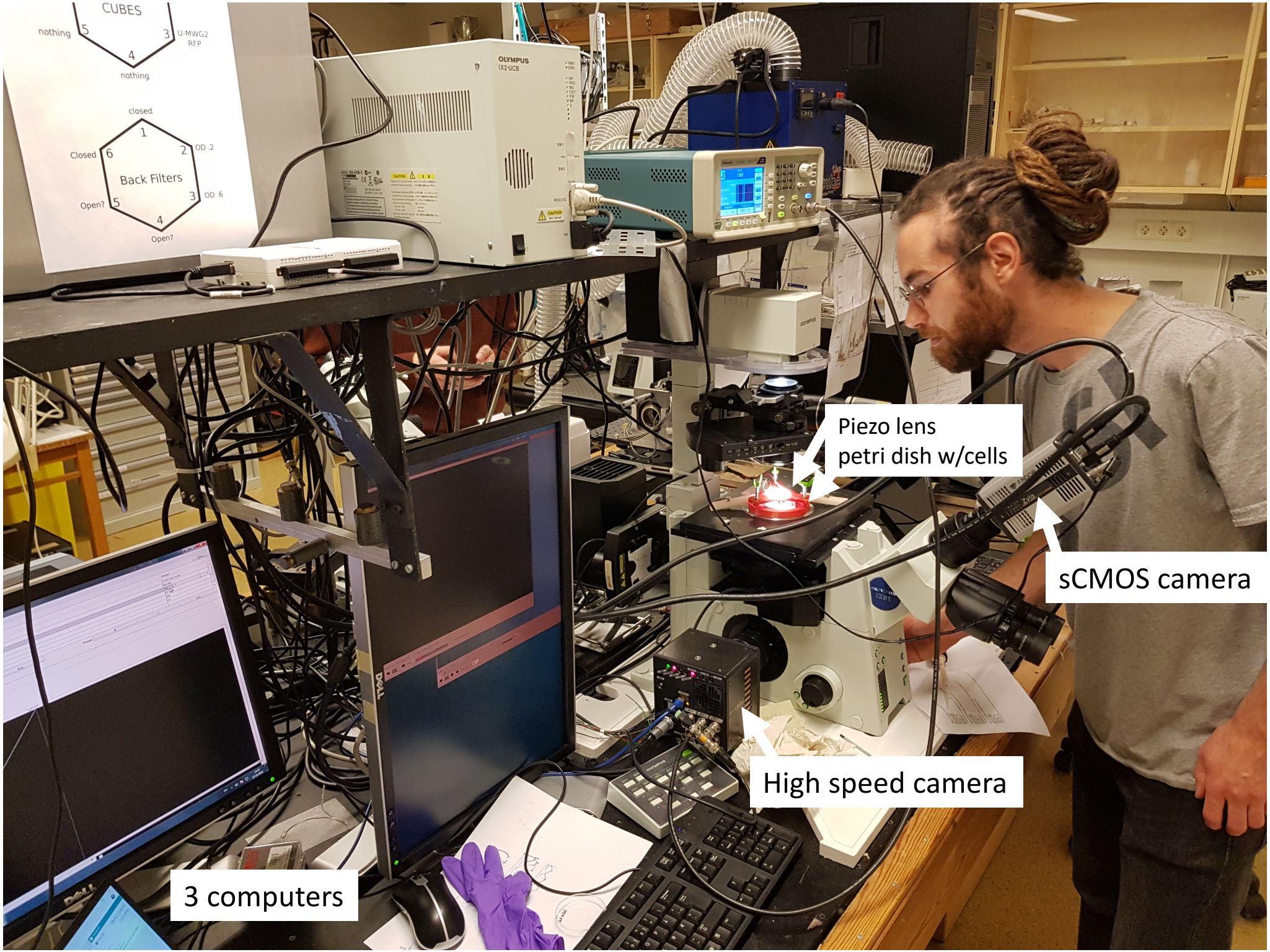
Current instrument



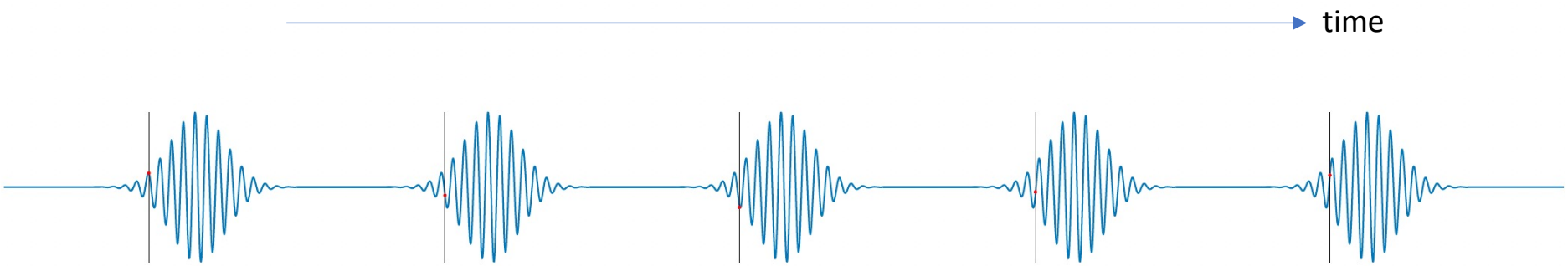
US transducer

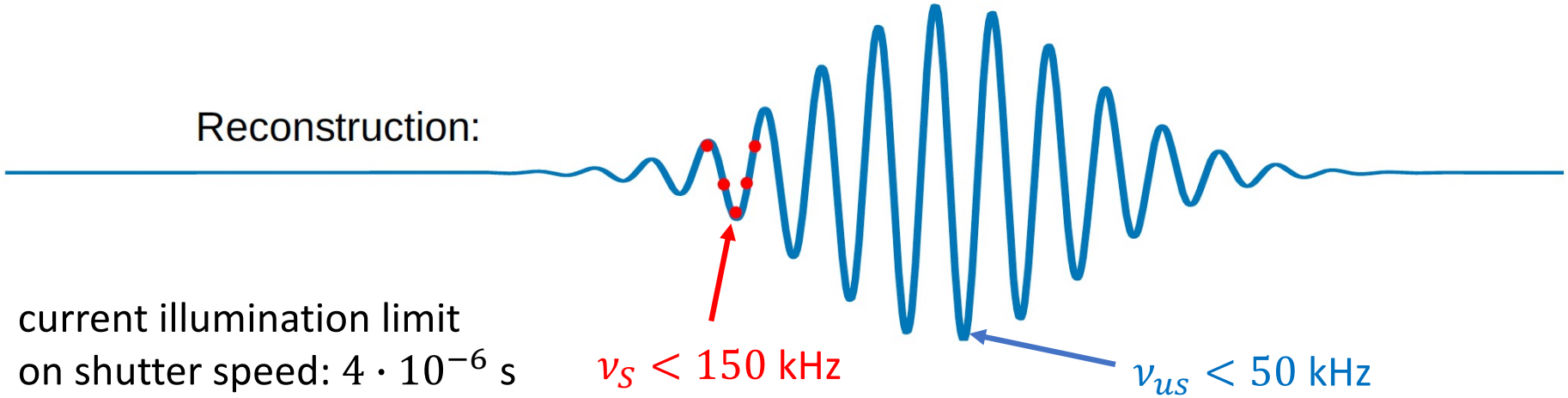
- annular for illumination
- lens focuses US
- water filled for impedance matching
- submersible in petri dish




CUBES
nothing
5
3
U-MWG2
RFP
4
nothing
closed
1
Closed
6
2
OD .2
Back Filters
Open?
5
3
OD .6
4
Open?
OLYMPUS
IX2-UCB
Piezo lens
petri dish w/cells
sCMOS camera
High speed camera
3 computers

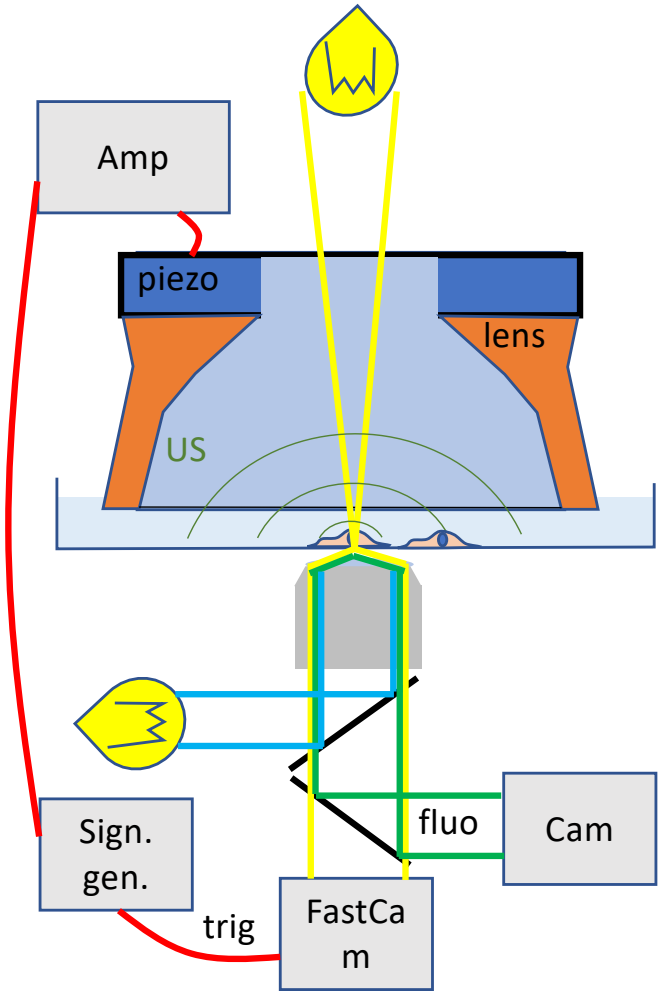
STROBOSCOPIC IMAGING - PRINCIPLE

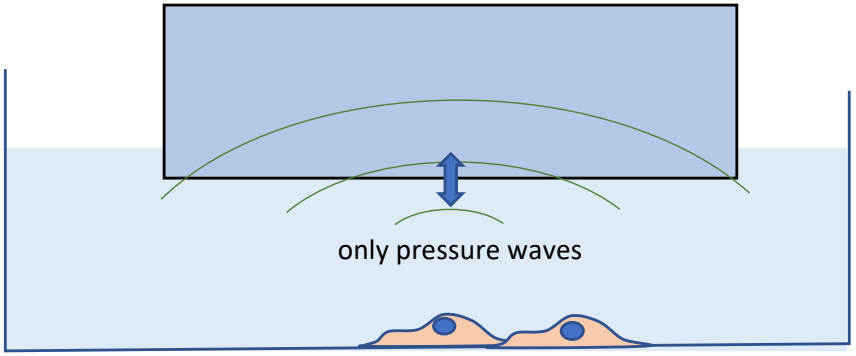




current illumination limit
on shutter speed: $4 \cdot 10^{-6}$ s

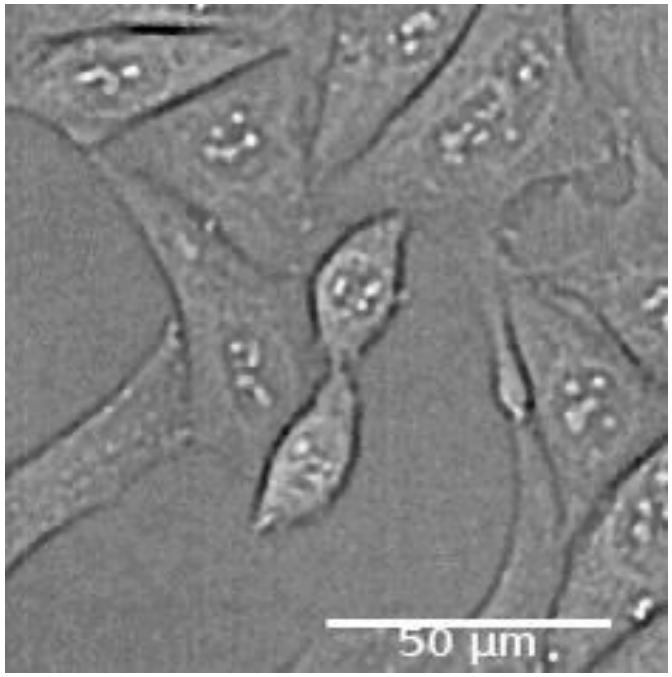
First result: pressure waves do nothing

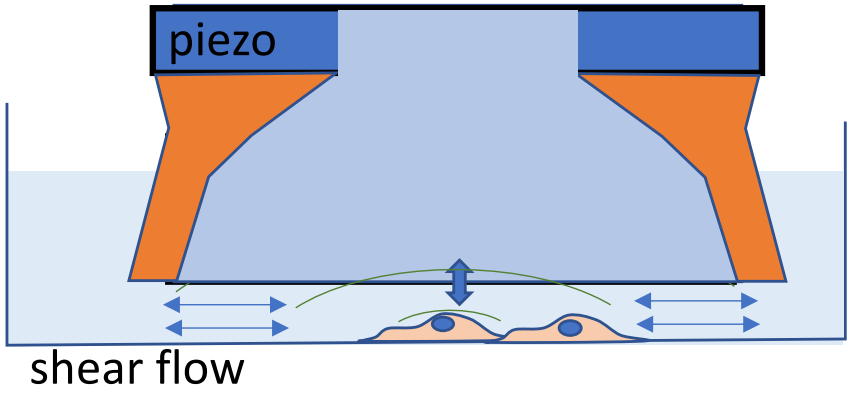




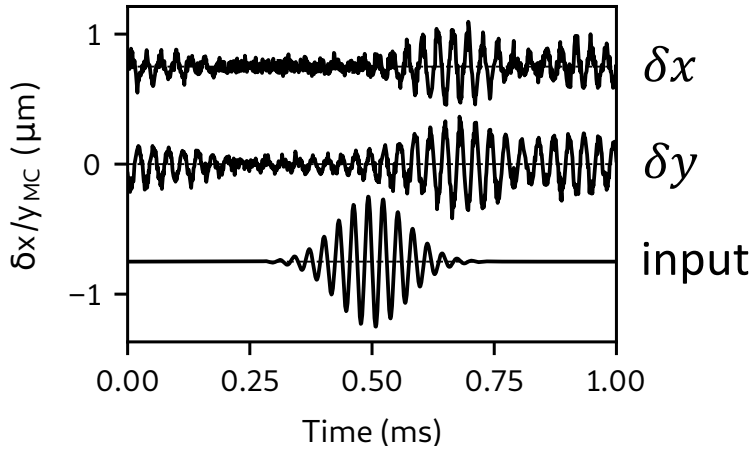
- No motion
- No fluorescense

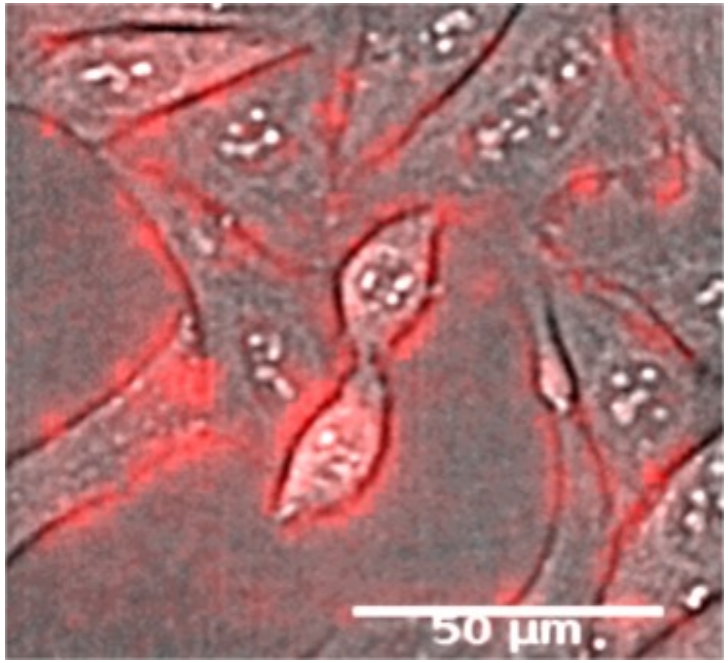
Second result: Shear waves give biological response



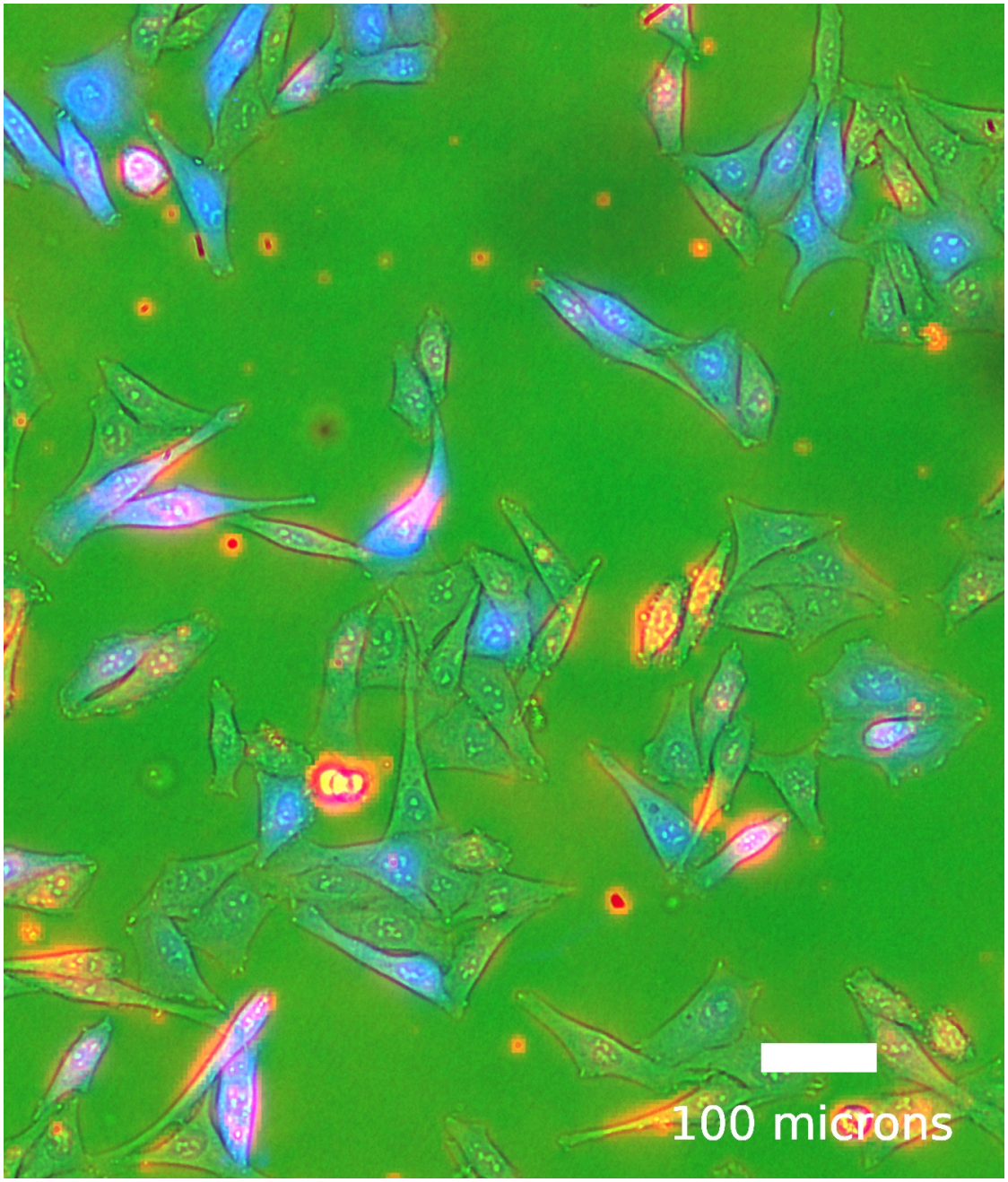


Digital image correlation (DIC)





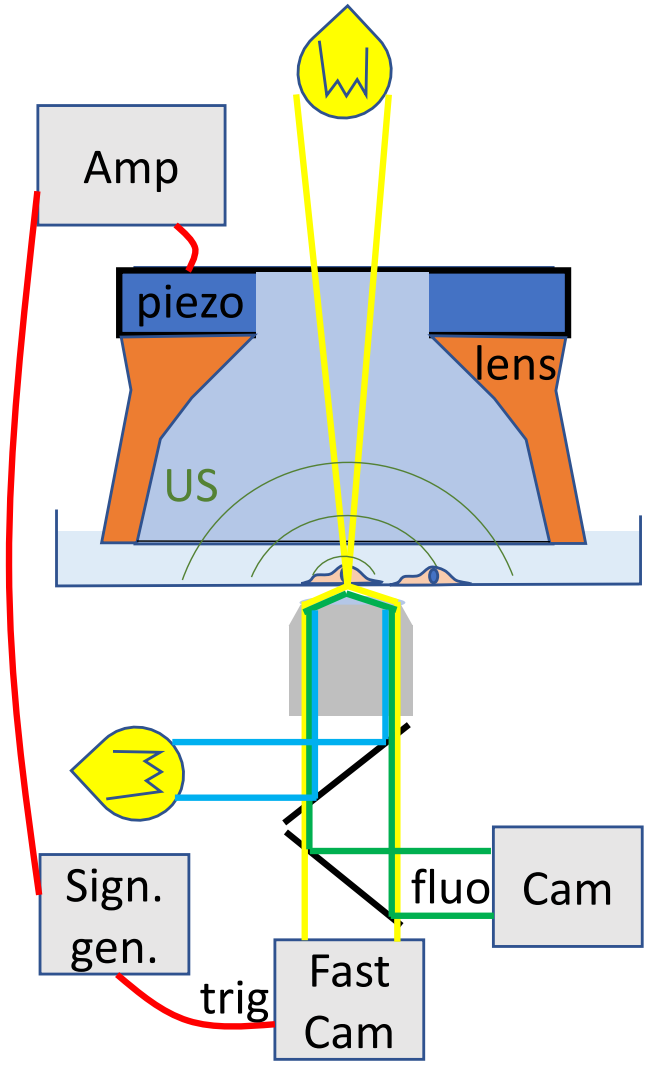
Correlative imaging

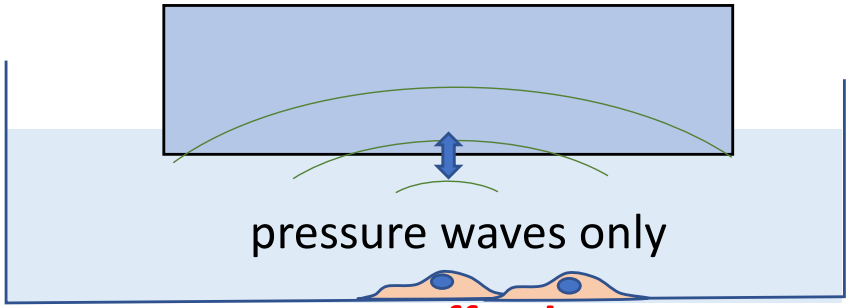


- phase contrast
- **mechanical stimulus:**
  mean displacement
  33 kHz
- **biological response:**
  Ca^{2+} fluorescence
  2s - 2h

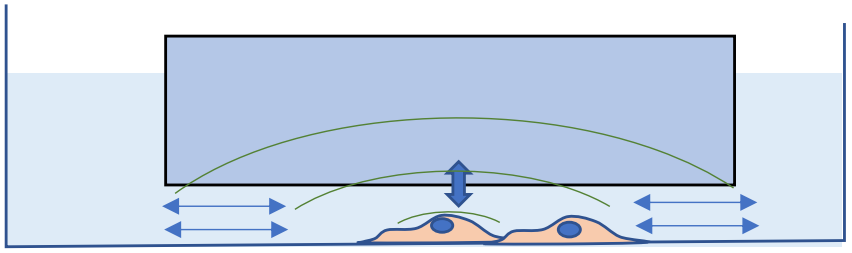
frequencies, amplitudes and fluorescent reporters to be varied

Summary

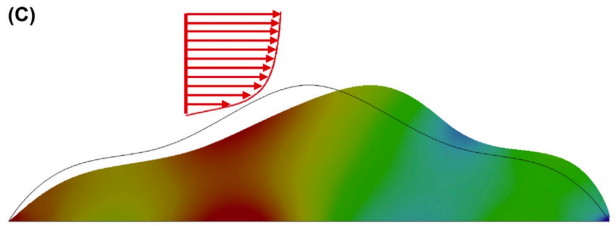


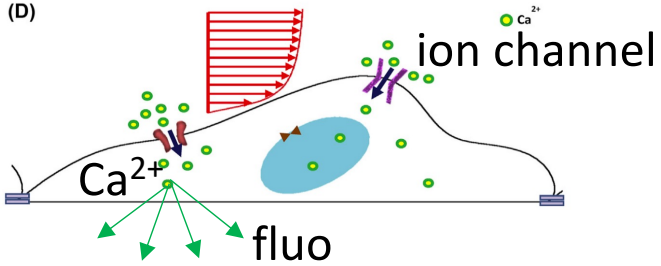


no effect!

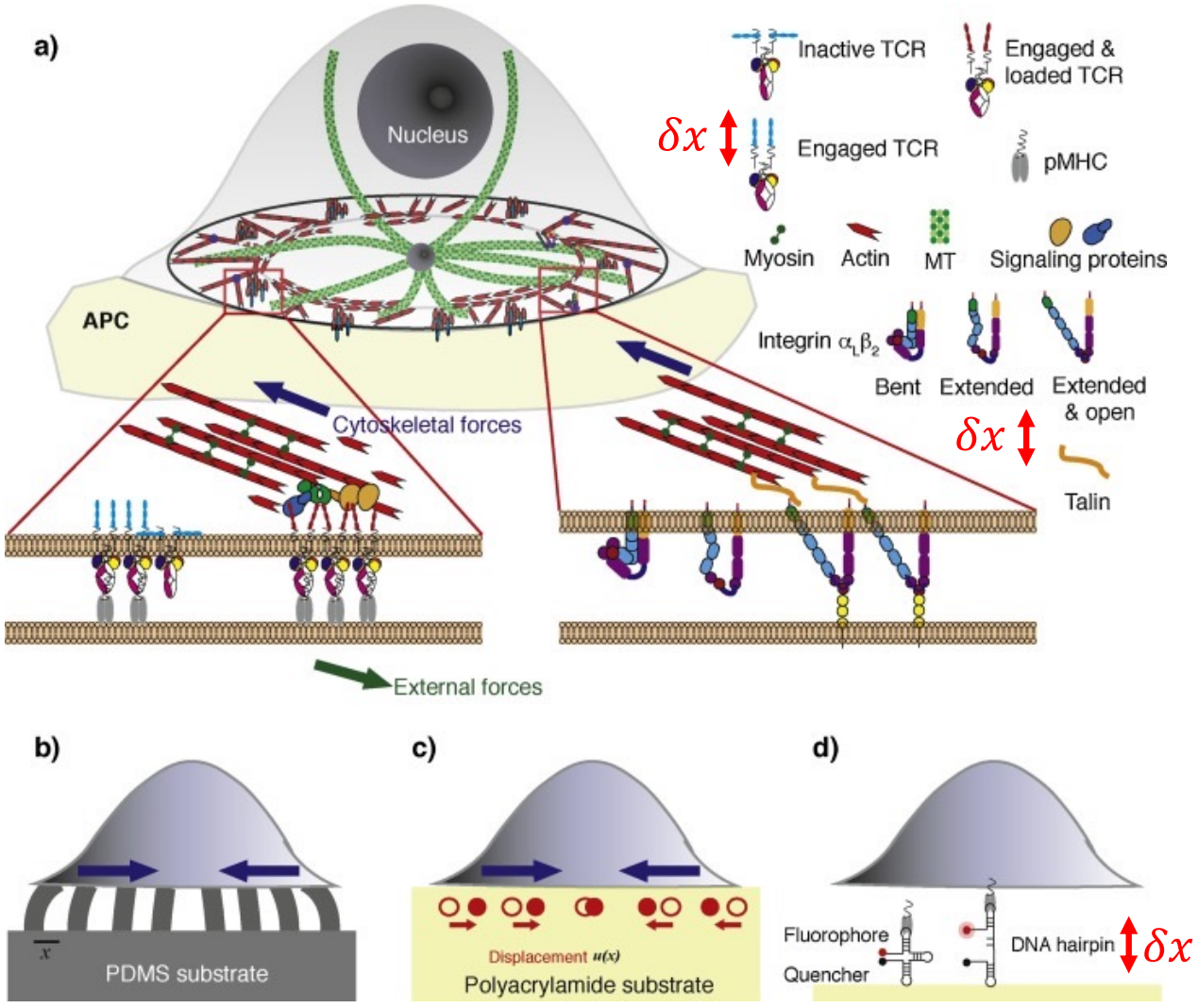


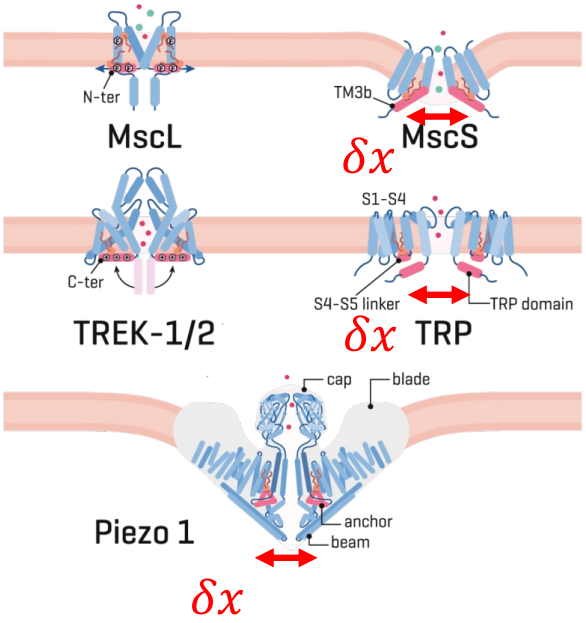
shear flow





Mechanosensing: strain, not stress





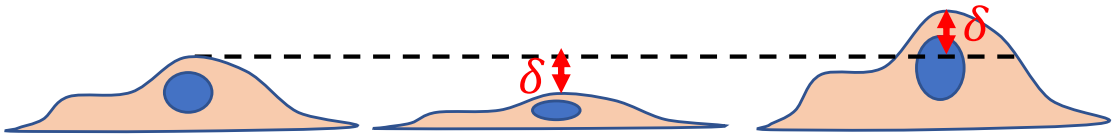
2-states: open/closed
open probability:

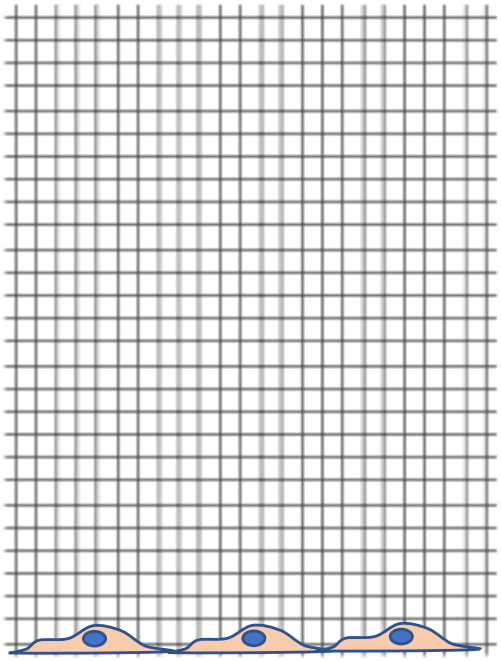
$$P(f) = \frac{1}{1 + e^{-(\Delta F - f\,\delta x)/kT}}$$

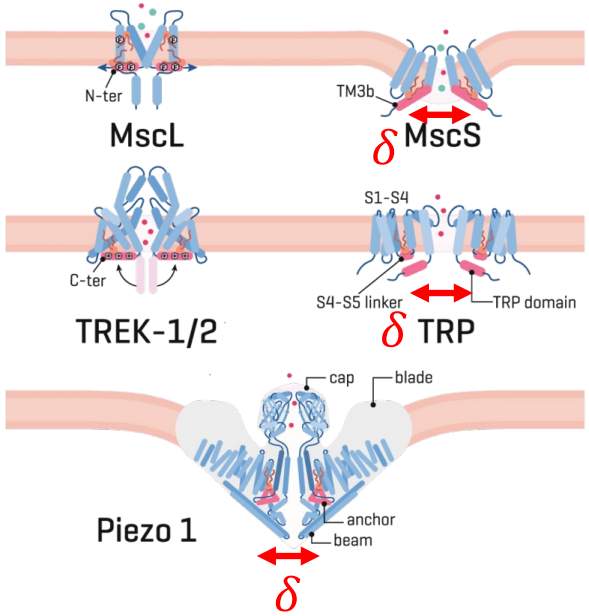
displacement δx is necessary!

Bulk modulus: K=2 GPa

$$\delta = \varepsilon l = \frac{Pl}{K} \approx 5\text{Å}$$

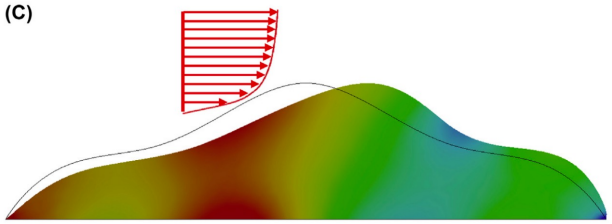






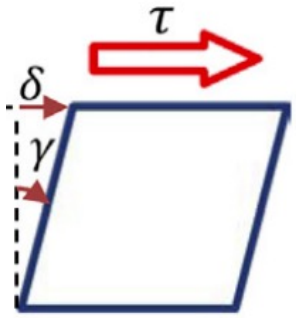
Large forces do nothing

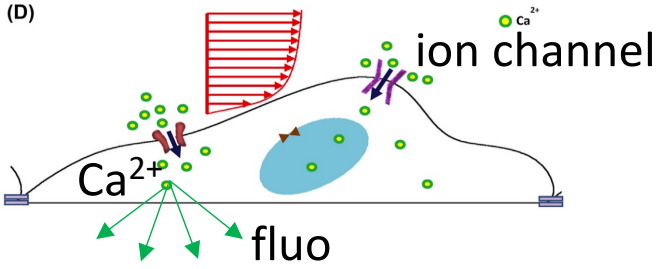
$\delta = 5\text{Å}$ can not open ion channels



Shear modulus: G=0.01-10 kPa

$$\delta = \varepsilon l = \frac{\tau l}{G} \approx 1\mu m$$





Biological effects of LIUS: sound wave -> shear wave transformation

Fysikkens universalitet vs. biologiens spesialisering

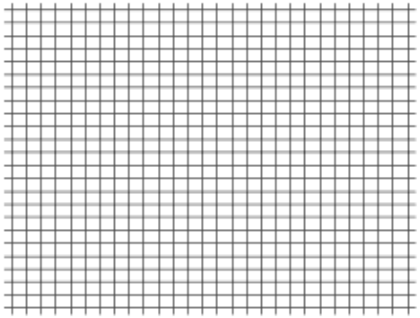
- Våre resultater (en celletype, lite utvalg frekvenser, rytmer, pulser) viser at skjær trigger respons, trykkbølger gjør ingenting
- Fysiske argumenter indikerer at resultatene er universelt gyldige
- Men andre forfattere fant at trykkbølger gir biologisk virkning
- => våre resultater gjelder kun for den celletypen ved nøyaktig de tilstandene
- => forferdelig utilfredsstillende for en fysiker
- Mer universell fysikk: trykkbølger kan omformes til skjærbølger
- Kanskje skyldes alle biologiske virkninger av LIPUS skjærbølger ingen tenkte på?
- Hvordan bevise det?

Ultralyd er både trykkbølger og skjærbølger!

Trykkbølger

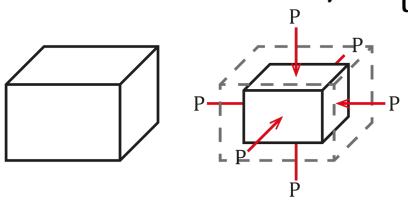
$$\partial_t^2 P = c_p^2 \nabla^2 P$$

$$c_p = \sqrt{K/\rho}$$



Volum-tøyning (strain): $\varepsilon_V = \frac{dV}{V}$

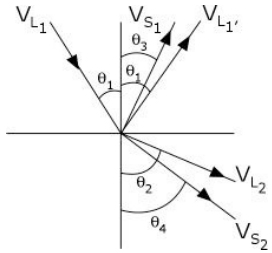
Bulk-modul: $K = P/\varepsilon_V = \frac{\text{trykk}}{\text{tøyning}}$



Bølger i materialer

Snells brytningslov

$$\frac{\sin\theta_1}{V_{L_1}} = \frac{\sin\theta_1}{V_{L_1'}} = \frac{\sin\theta_2}{V_{L_2}} = \frac{\sin\theta_3}{V_{S_1}} = \frac{\sin\theta_4}{V_{S_2}}$$



Impedans:

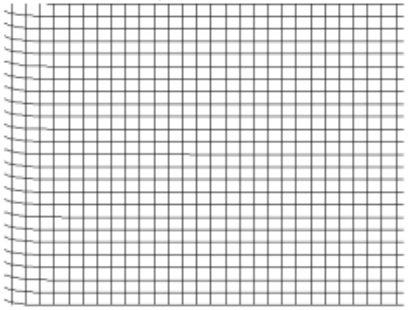
$$Z_P = \left|\frac{P}{v}\right| = \sqrt{\rho c_p}, \quad v = \text{partikkel}$$

$$Z_S = \left|\frac{\sigma}{v}\right| = \sqrt{\rho c_s} \quad \text{-hastighet}$$

Skjærbølger

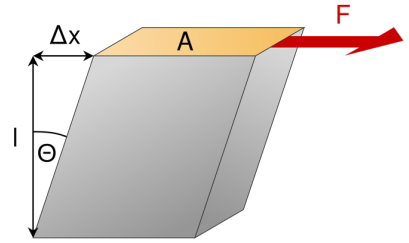
$$\partial_t^2 \boldsymbol{\varepsilon} = c_s^2 \nabla^2 \boldsymbol{\varepsilon}$$

$$c_s = \sqrt{G/\rho}$$



Skjær-tøyning: $\varepsilon_{xy} = \frac{dx}{l}$

Skjær-modul: $G = F/A\varepsilon_S = \frac{\text{spenning}}{\text{tøyning}}$



Trykkbølger kan omformes til skjærbølger
Kanskje skyldes alle biologiske virkninger av LIPUS skjærbølger ingen tenkte på?

Oncotripsy: finlesing av artikler kan avsløre feil

- Heyden & Ortiz 2016, 2017: theoretical
- Mittelstein et al 2020, J. Appl. Phys: ”Moreover, our experiments revealed that the formation of standing waves and the emergence of cavitation were necessary to disrupt cancer cells.”

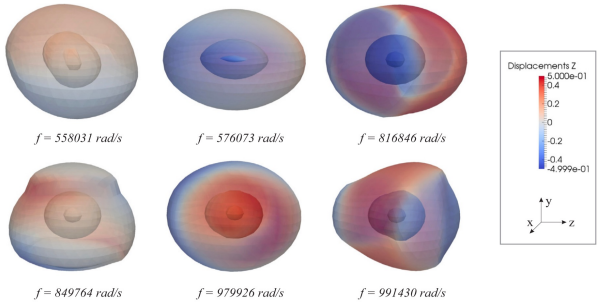


Table 1
Set of constitutive parameters (bulk modulus κ and shear moduli μ_1 and μ_2) used in the eigenfrequency analyses.

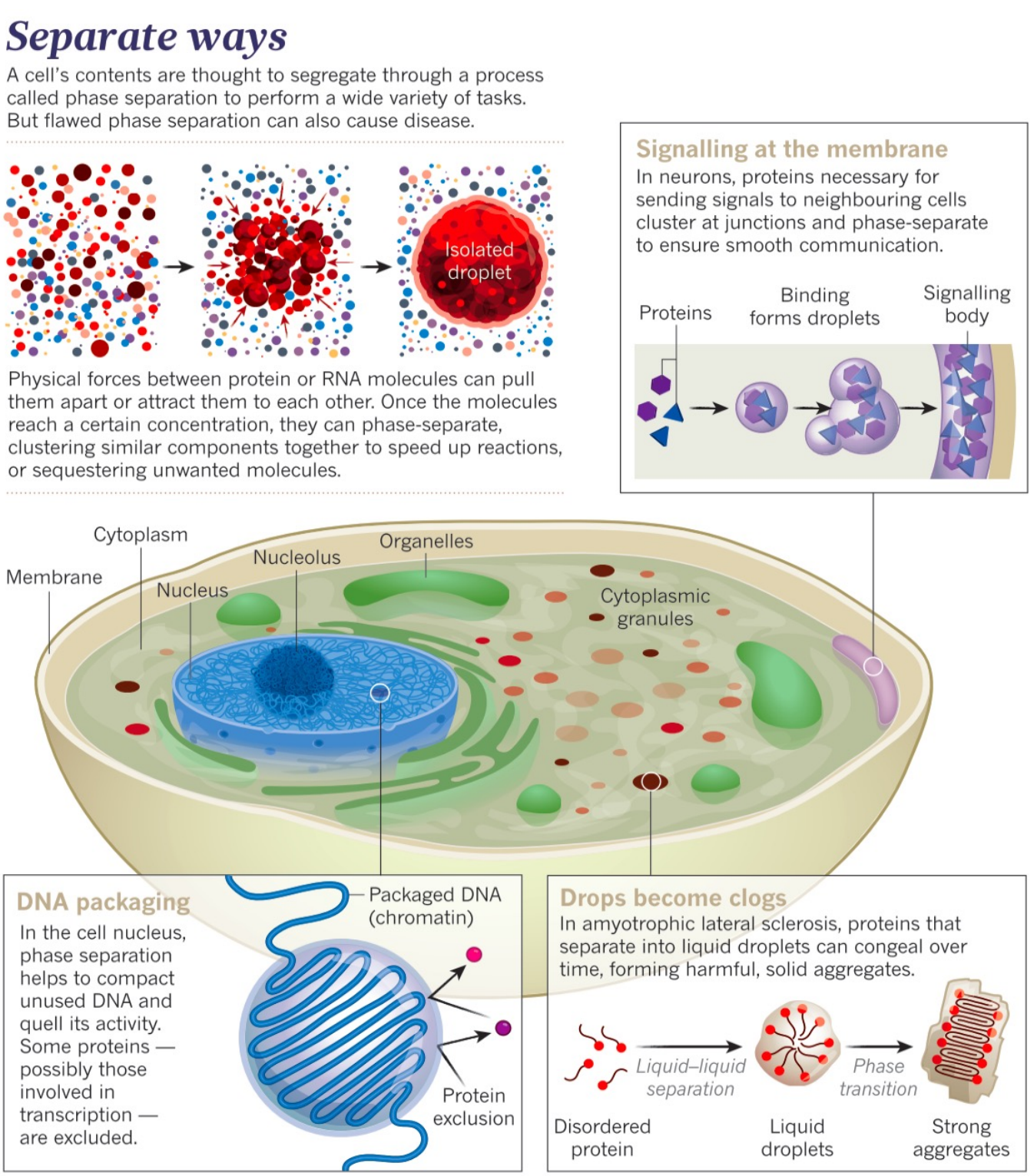
	κ [kPa]	μ_1 [kPa]	μ_2 [kPa]
Plasma membrane	39.7333	0.41	0.422
Cytoplasm	39.7333	0.41	0.422
Nuclear envelope	239.989	2.41	2.422
Nucleoplasm	239.989	2.41	2.422
Nucleolus	719.967	7.23	7.266
ECM	248.333	5.0	5.0

faktisk: 2 GPa!

- Data based on data fitting of AFM indentation by Kim et al Med Biol Eng Comput (2011) 49:453–462 assuming Poisson ratio ν= 0.499, Heyden & Ortiz assume ν= 0.49!

Other mechanisms are possible

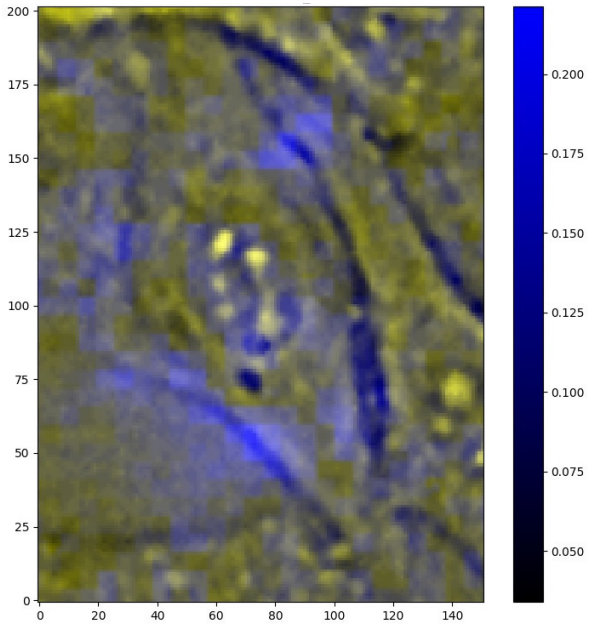
Pressure waves trigger phase separation?



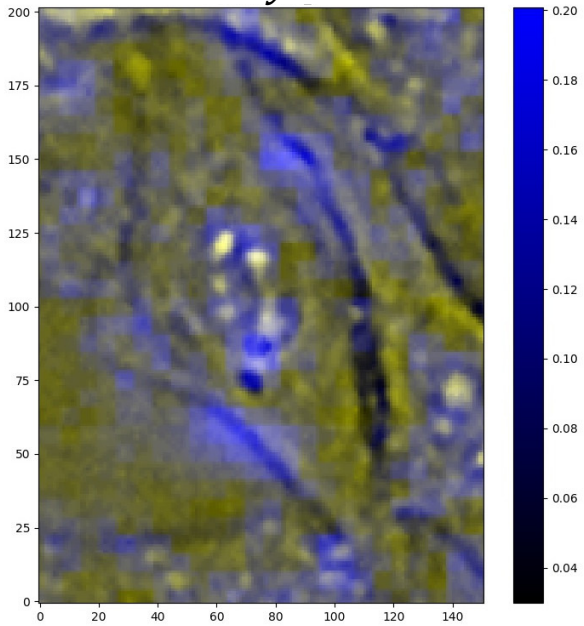
Dolgin, *Nature* 2018

US rheology of cell cultures?

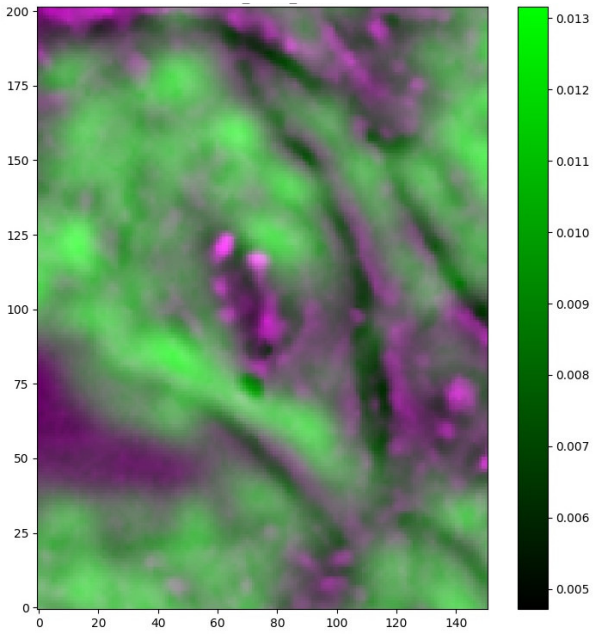
u_x



u_y



$$\varepsilon_{xy} = \frac{\partial u_x}{\partial y} + \frac{\partial u_y}{\partial x}$$



20X objective, 33 kHz US

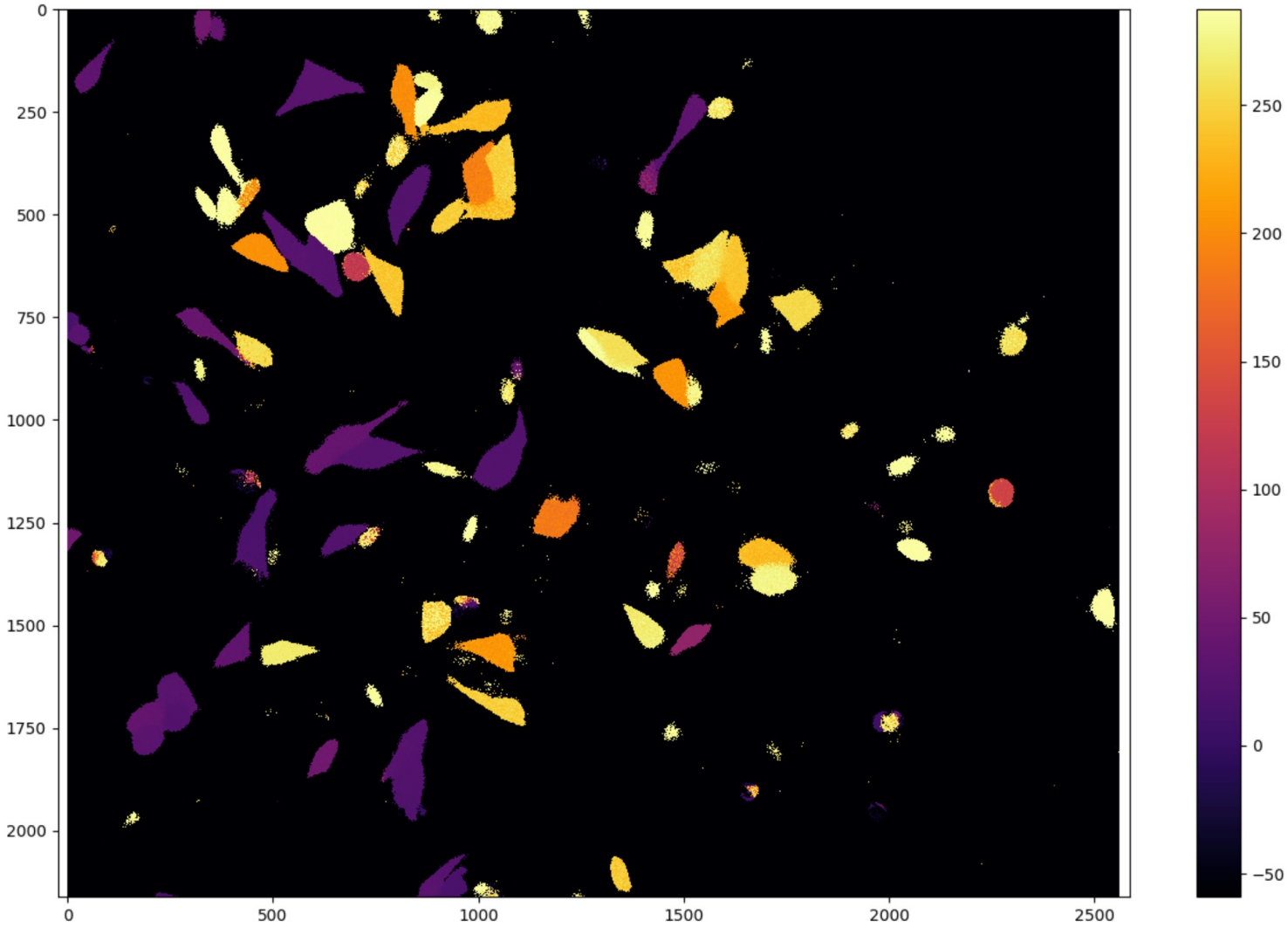
shear strain:

- smaller in nucleus
- noisy

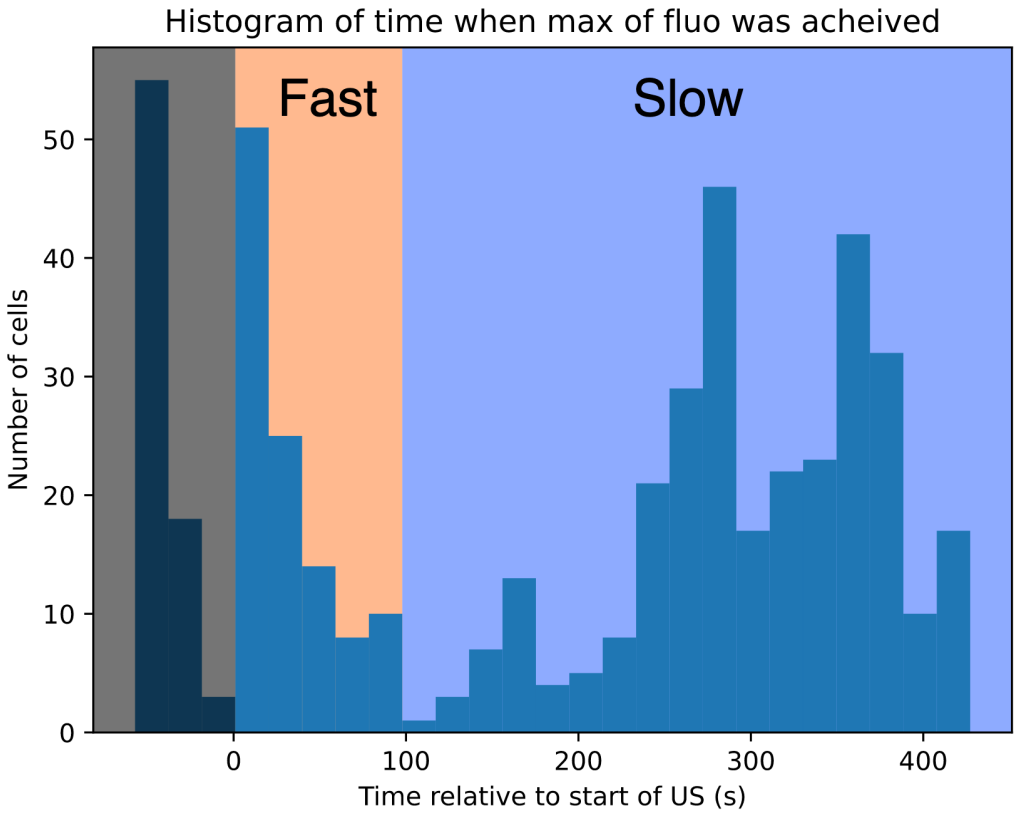
Conclusions

+ Shear, not pressure waves triggers Ca^{2+} influx in adherent HeLa cells
– Biological effects of LIPUS are probably always due to induced shear, not pressure waves
– That is impossible to prove (and brands lots of papers “rubbish”)
– Finding and proving a pressure wave mechanism is very high risk and (moderately?) high gain.
+ US driven shear flow opens a huge frequency domain for rheology studies.

ALL CELLS DO NOT REACH MAXIMUM OF FLUORESCENCE AT THE SAME TIME

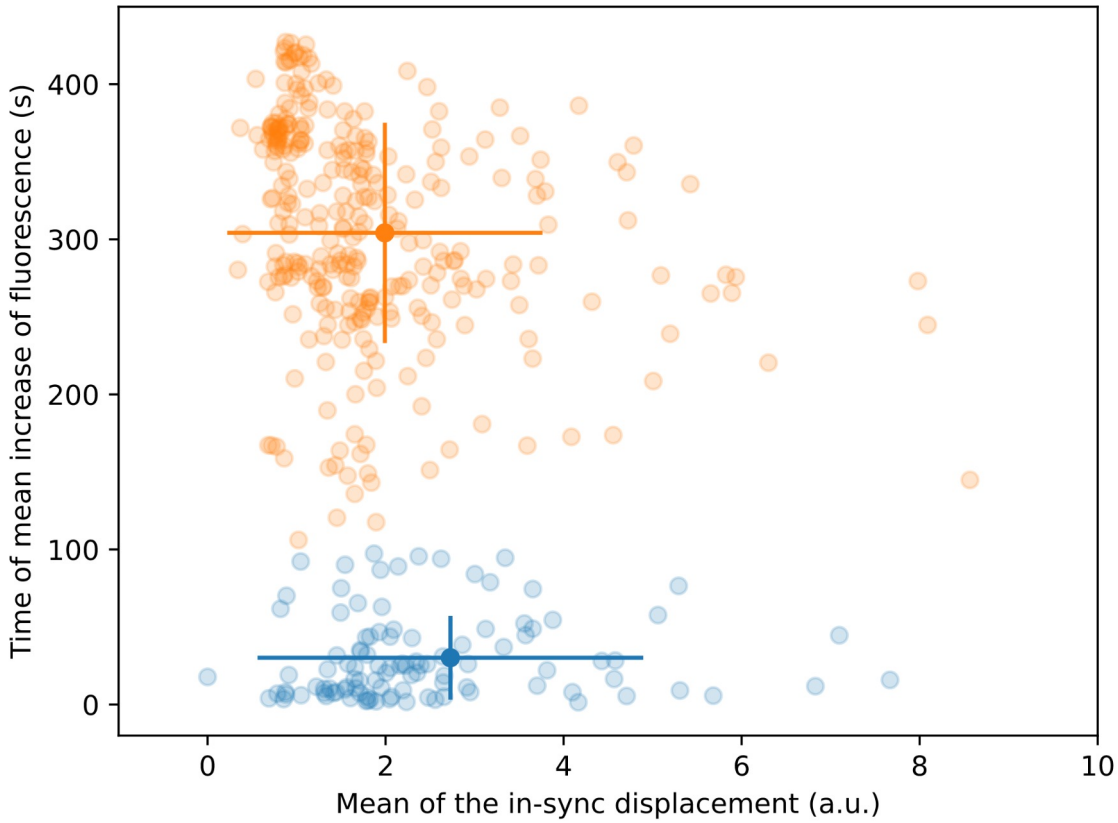


TWO MAIN GROUPS OF FLUORESCENCE BEHAVIOR

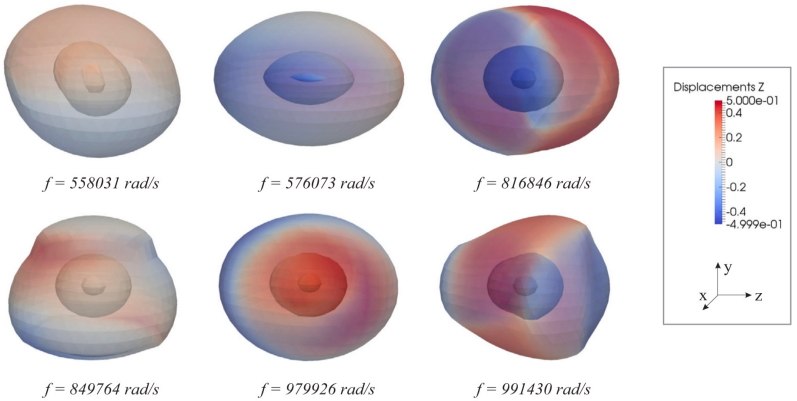


- Fast responding cells
- Slow responding cells

"IN-SYNC" DISPLACEMENT FOR THE TWO GROUPS



Oncotripsy



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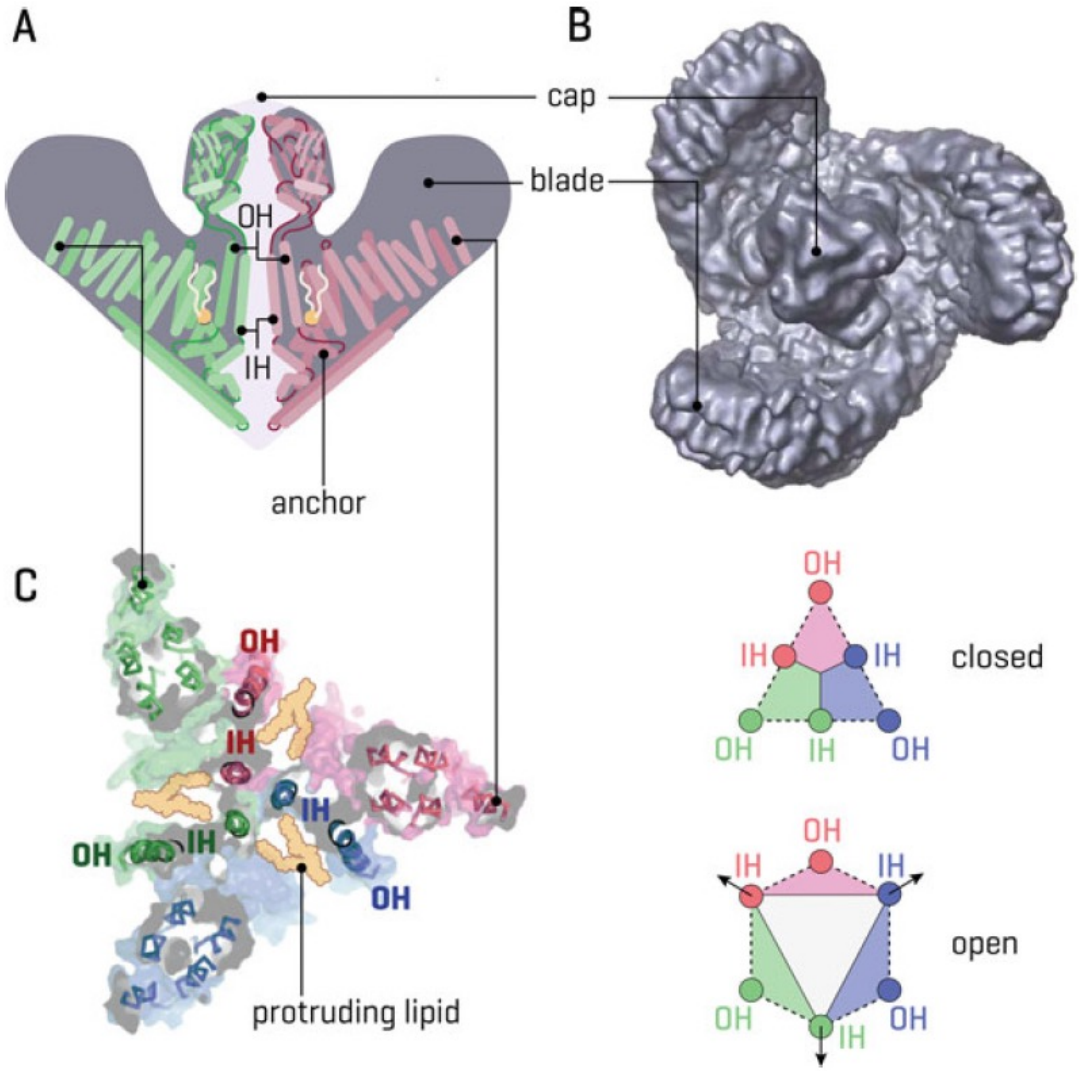
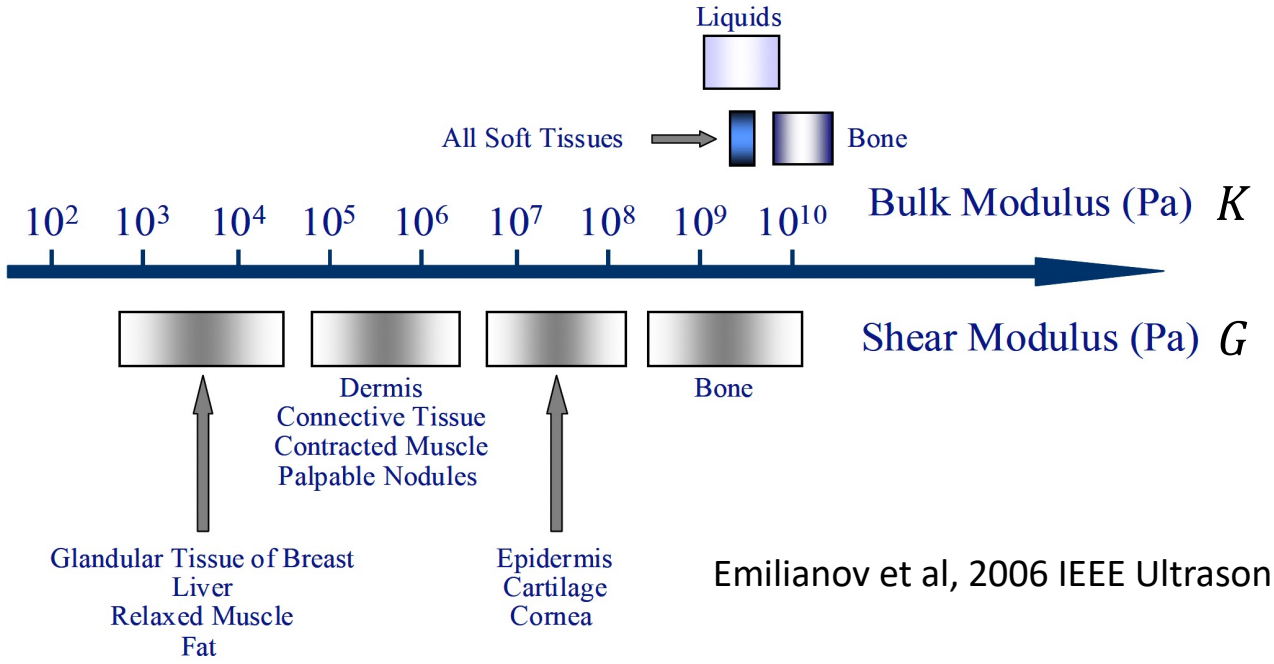


Fig. 4.5 Mammalian mechanosensitive Piezo1 architecture and a putative membrane-mediated gating mechanism. (**a**) Schematic of the side view of Piezo1 structure. (**b**) *Top view* of Cryo-EM structure of mouse Piezo1 as shown in *shaded grey* surface (PDB: 3JAC) [72]. (**c**) View from the *top* of the human Piezo1 (homology model based on mouse Piezo1) shows the interlocked arrangement of its 3 subunits at the level of the hydrophobic core of the lipid bilayer. An increase in lateral bilayer tension is thought to result in a clockwise or counter-clockwise deflection of the 'Blade' domains around the 'Anchor' and outer helix (OH) domains. This movement ultimately results in the displacement of the inner helices (IH) away from the center of the pore to allow ion conduction, as shown in the diagram. This hypothesis aims to explain the intrinsic mechanosensitivity of the channel different from the Blade-deflection model proposed by Ge et al. (2015) [72]

Bulk and shear modulus of tissues



Emilianov et al, 2006 IEEE Ultrasonics Symposium

$\Rightarrow$ soft tissue and cells can be considered incompressible and Poisson's ratio $\nu = 0.5$

$$\varepsilon_{ij} = \frac{1}{E}\left[\sigma_{ij}(1+\nu) - \nu\delta_{ij}\sum_{kk}\sigma_{kk}\right]$$

Young's modulus, E, shear modulus, G, and bulk modulus, K:

$$E = 2G(1+\nu) \approx 3G$$
$$= 3K(1-2\nu)$$