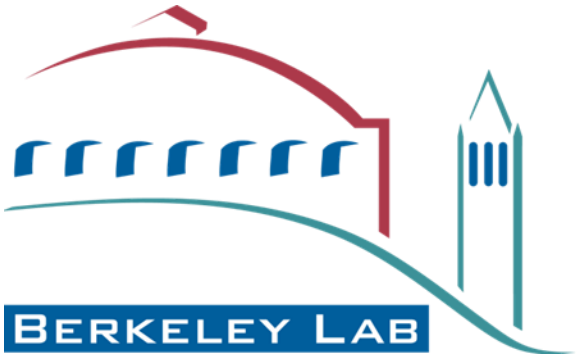
NRAMM Workshop
Oct. 29 – Nov 3, 2017

CHALLENGES REMAINING FOR SINGLE-PARTICLE CRYO-EM GRID PREPARATION



Bob Glaeser
Lawrence Berkeley National Lab

CURRENT ISSUES WITH "DIFFICULT" PARTICLES - AND SOME POSSIBLE CAUSES

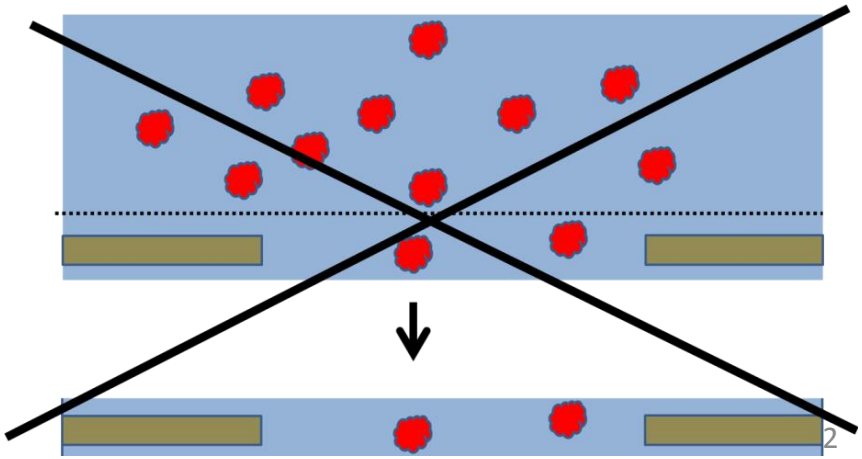
THINGS THAT SOMETIMES HAPPEN

- Preferential orientation of particles
- Too few particles seen within holes
- Particle disintegration occurs within thin aqueous films
- Unexpected aggregation of sample material

The standard picture has been too naïve, and very misleading

POSSIBLE REASONS

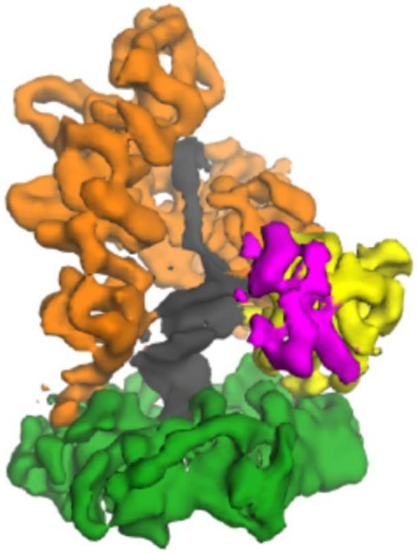
- ~~Bad biochemical preparation~~
- Interaction with the air-water & carbon-water interfaces
- Fluid shearing forces during wicking



NUMEROUS RECIPES ARE USED TO OPTIMIZE SPECIMEN PREPARATION

- Optimize the buffer
  - Salt, pH, additives
- Chemical crosslinking
  - Glutaraldehyde, BS3
- Add a surfactant
  - Detergent, amphipol, nanodisks
- Apply sample 2 or more times
- Ultrafast thinning and quenching
  - Spotiton + self-wicking grids
- Adsorption to a support film
  - Carbon, graphene oxide
  - Biochemical-affinity grids

Galej et al. (2016)
Nature 537:197-201
3.8 Å structure of the spliceasome immediately after lariat formation
Crosslinked with BS3



Fernandez-Leiro et al. (2017) Nat Struct Mol Biol 24:140-143
E.coli Pol IIIa, ~6 Å Resolution
Tween 20 kept particle intact and not oriented

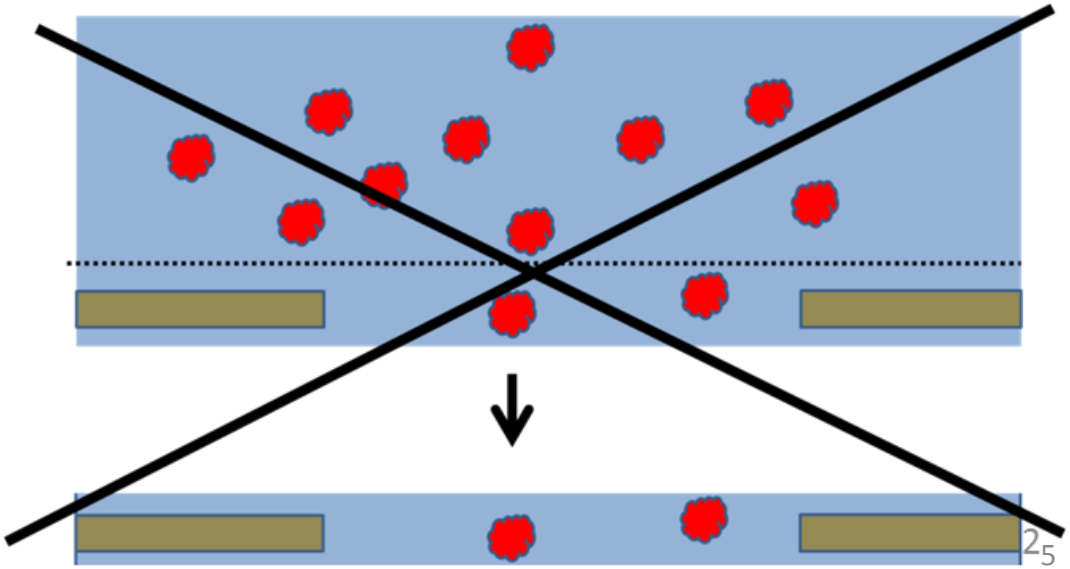
* HOW SUCCESSFUL HAVE THESE RECIPES BEEN?

- **Wonderfully successful !**
  - **Many of the publications that are driving the field forward have relied on one or another of those recipes**
- **However, from this work we know that no one recipe yet works for everything**
  - **There is no way to predict which recipe is the most likely to work for *YOUR* "difficult" particle**
- **And, for some (many?) specimens, none of the recipes seem to work**
  - **In other words, current approaches are not yet as successful as we wish**

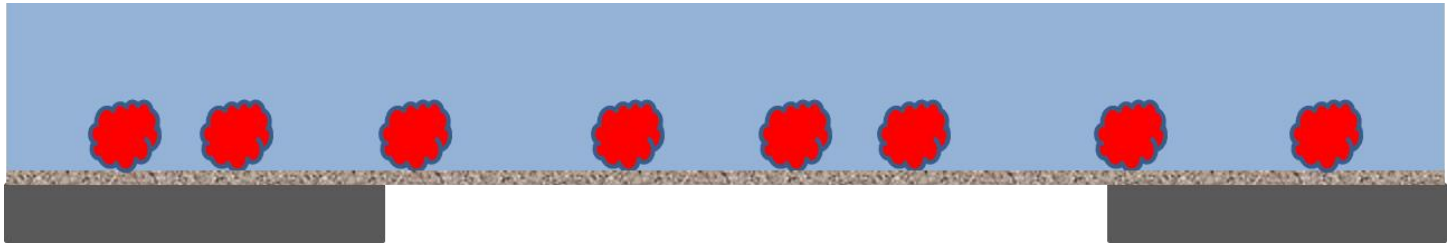
RETURNING TO THE CASES IN WHICH SAMPLE PREPARATION FAILS

POSSIBLE REASONS

- ~~Bad biochemical preparation~~
- Interaction with the air-water & carbon-water interfaces
- Fluid shearing forces during wicking



IMMOBILIZING PARTICLES ON A SUPPORT FILM IS EXPECTED TO PREVENT INTERACTION WITH THE AIR-WATER INTERFACE



- **INDEED, UNLESS A SUPPORT FILM IS USED**
  - **Particles diffuse freely within a 100 nm, thin film**
  - **Each particle will collide with the air-water interface about 1000 times per second**
  - **The same is true at the bottom of the hole**
- **BUT THERE STILL IS A CAUTION: THE ICE THICKNESS MUST NOT BE *TOO* THIN**

SOME CURRENT OPTIONS FOR SUPPORT FILMS

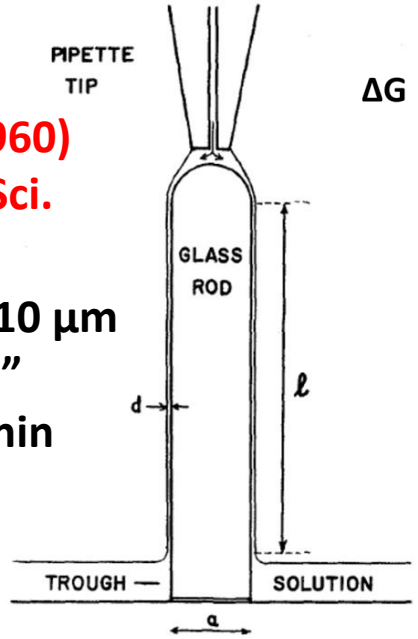
- **Glow-discharge treated, evaporated carbon films**
- **Chemically functionalized, evaporated carbon films** e.g. Llaguno et al. (2014) J Struct Biol 185:405-17
- **Graphene oxide** e.g. Boland et al. (2017) Nature Struc & Mol Biol 24:414-418
- **Biochemical-affinity support films**
  - **Ni-NTA lipid monolayers** e.g. Kelly et al. (2010) J Mol Biol 400:675-81
  - **Antibody-functionalized carbon films** e.g. Yu et al. (2016) Methods. 100:16-24
  - **Streptavidin monolayer-crystals** e.g. Wang et al. (2008) Journal of Structural Biology.164:190-8

IMMOBILIZATION ON ANY OF THESE SUPPORT FILMS CAN PREVENT CONTACT BETWEEN PARTICLES AND THE AIR-WATER INTERFACE

BUT WHY SHOULD THAT BE IMPORTANT?

THE AIR-WATER INTERFACE IS A DANGEROUS PLACE TO BE

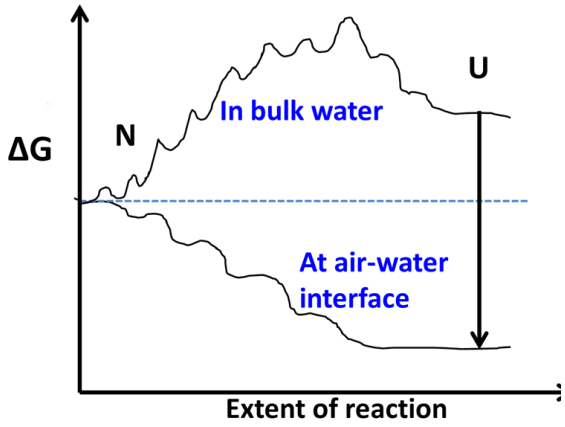
- Q: IF YOU WANTED TO QUANTITATIVELY DELIVER PROTEINS TO THE AIR-WATER INTERFACE, HOW WOLD YOU DO IT?



Trurnit (1960)
J. Colloid Sci.
15:1-13

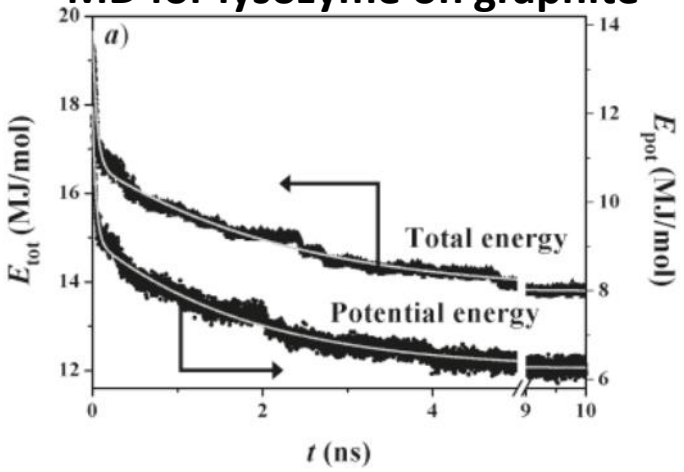
Proteins in a 10 µm thick "curtain" denature within seconds

- THE ENERGY LANDSCAPE FOR DENATURATION AT A HYDROPHOBIC INTERFACE IS VERY DIFFERENT THAN IN BULK



Glaeser & Han (2017)
Biophys Reports 3:1-7

MD for lysozyme on graphite

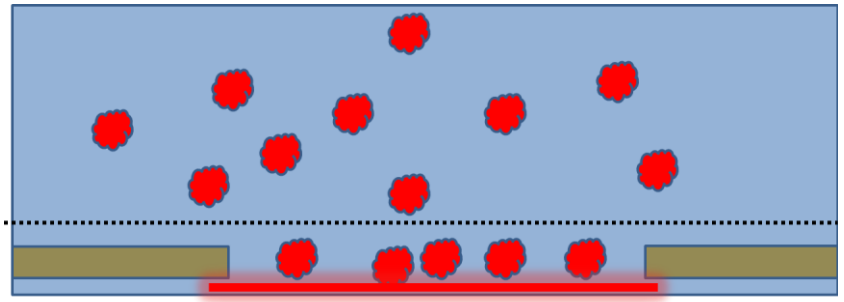


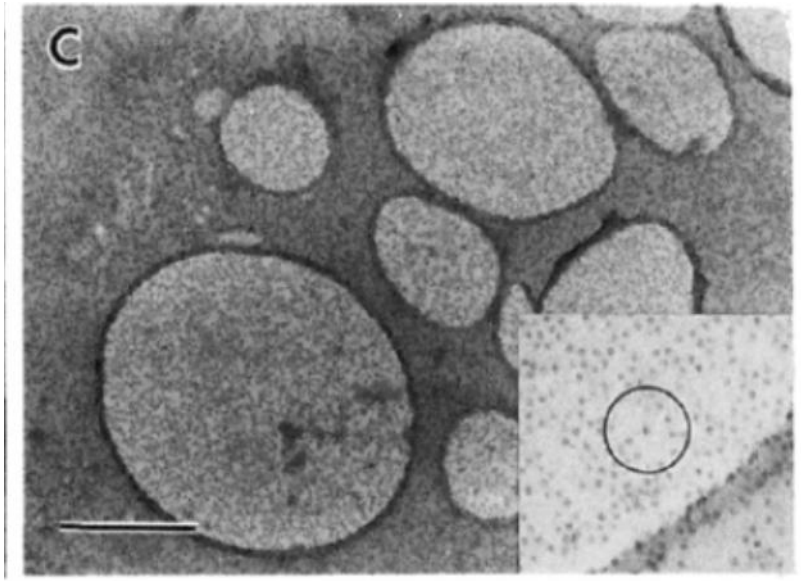
Raffaini & Ganazzoli (2010)
Langmuir 26: 5679-5689

Glaeser (Submitted) Current Opinion in Colloid and Interface Science

SURPRISINGLY, MONOLAYER-FILMS OF DENATURED PROTEINS CAN ALSO SERVE AS A STRUCTURE-FRIENDLY SUPPORT FILM

- In many other contexts it is accepted that additional particles adsorb to a denatured monolayer at the air-water interface
- A sacrificial layer of denatured protein can actually be a good thing!
- Evidently this does not work for every protein

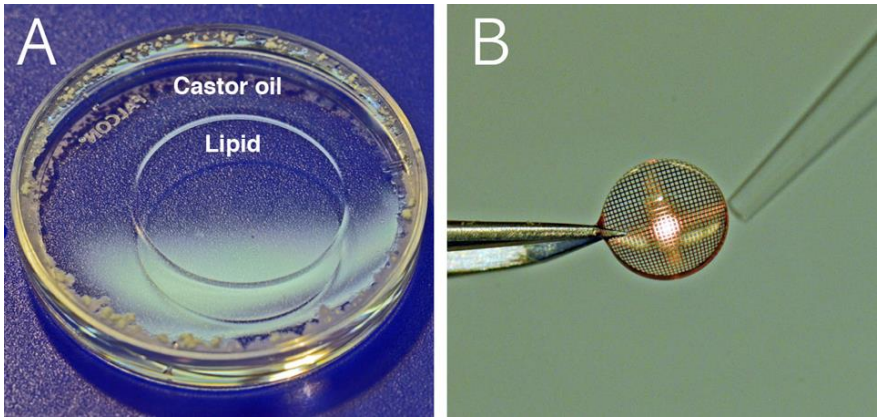


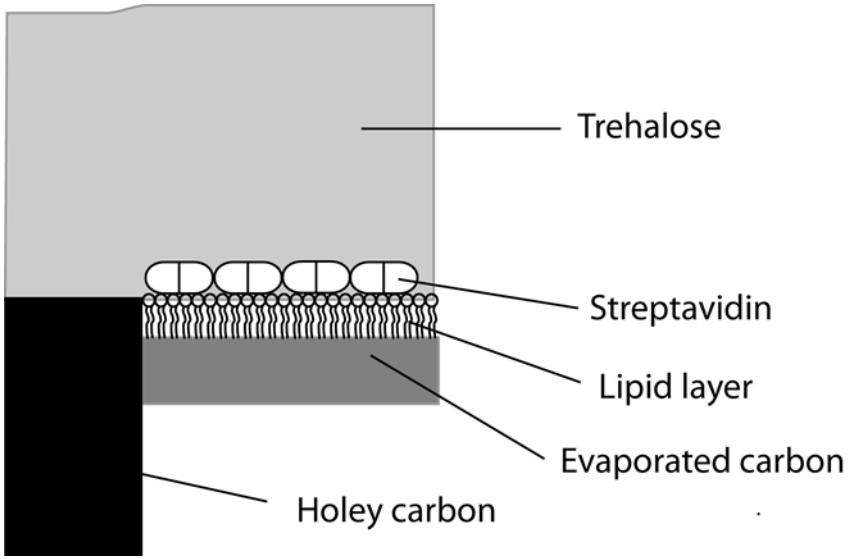


Yoshimura, Schebanyi, & Baumeister (1994) Langmuir 10:3290-3295

THE BERKELEY PROGRAM TO DEVELOP STREPTAVIDIN (SA) AFFINITY GRIDS

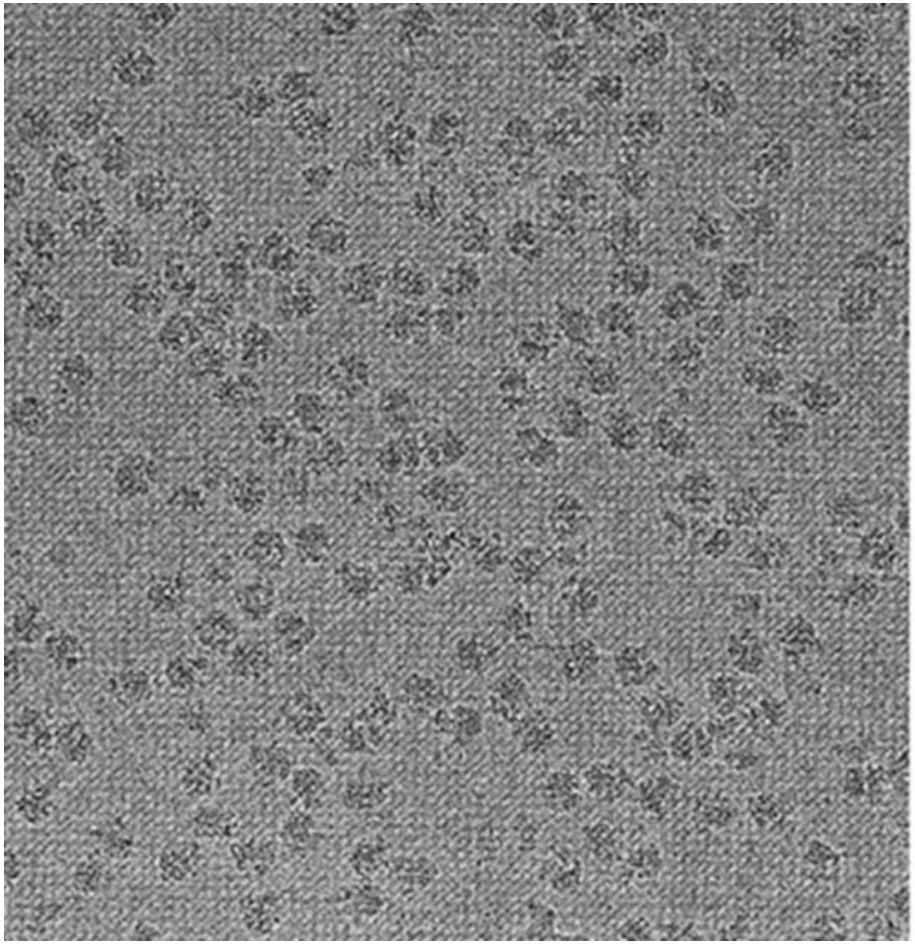
- SA crystals are grown on-grid - this enhances reproducibility
- Embedding in trehalose confers long shelf-life
- Carbon-backed for mechanical stability
- Biotinylated particles overcome preferential orientation

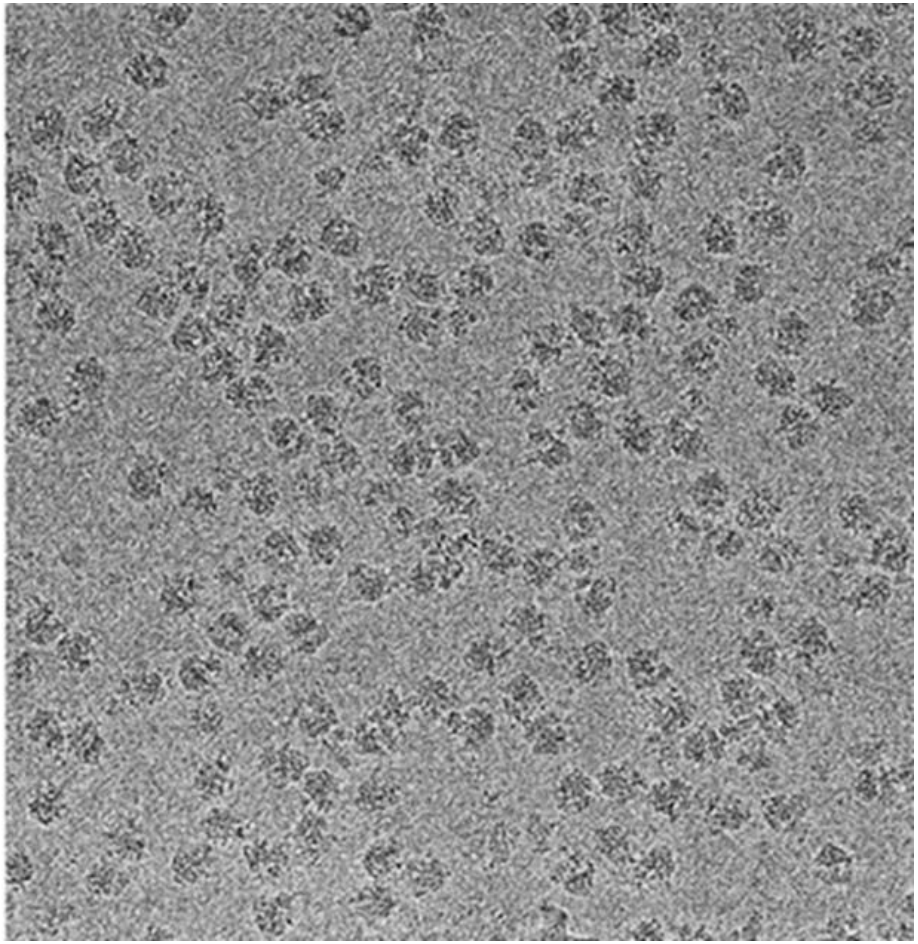




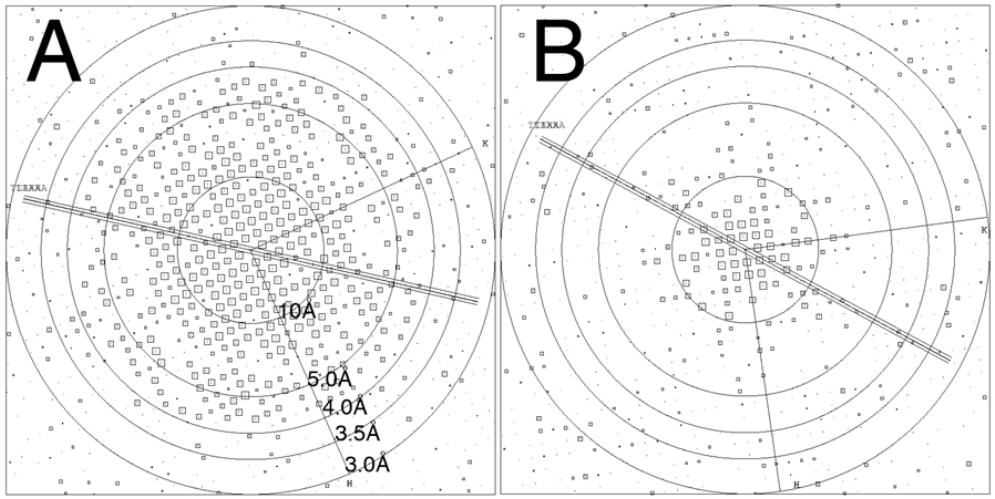
Han et al. (2016) J Struc Biol195:238-44

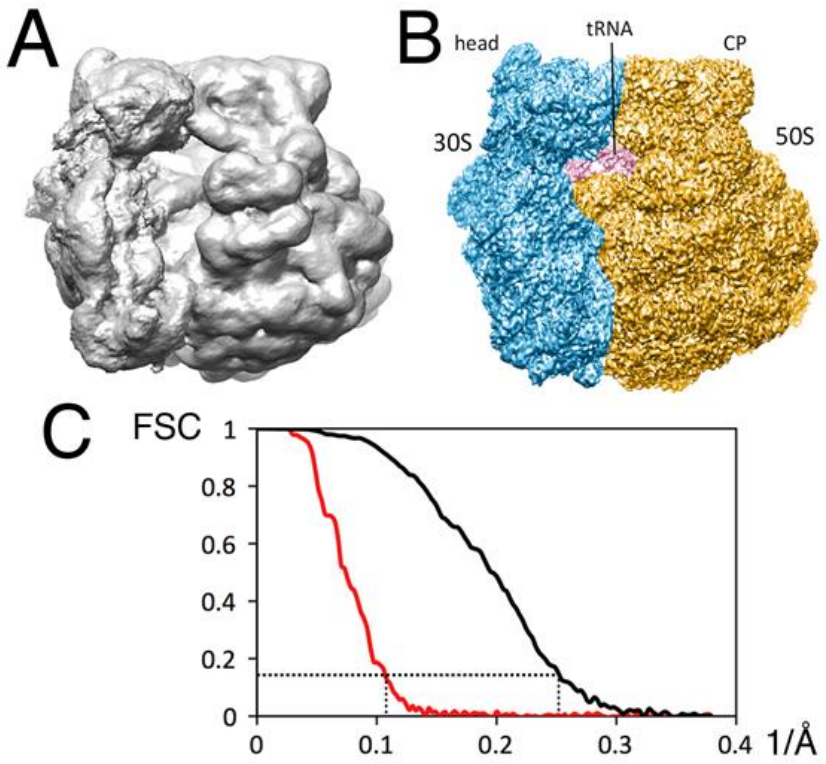
THE SA-CRYSTAL MOTIF IS EASILY REMOVED BY FOURIER FILTERING

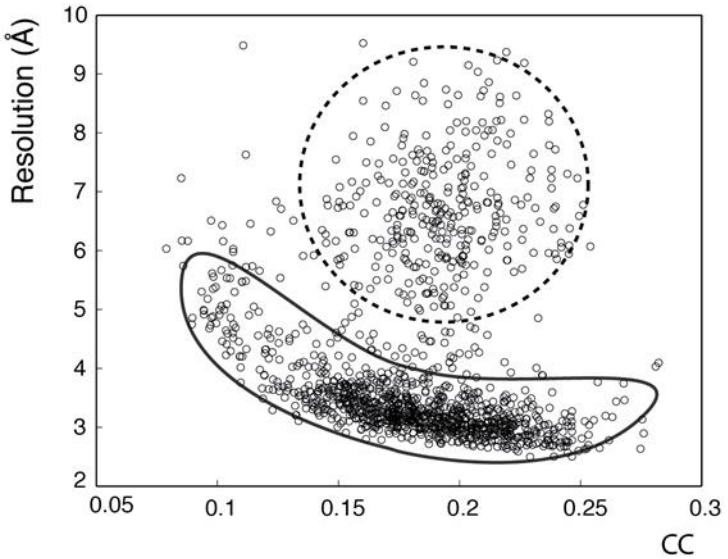




SA CRYSTALS PROVDE AN INTERNAL STANDARD FOR THE IMAGE QUALITY





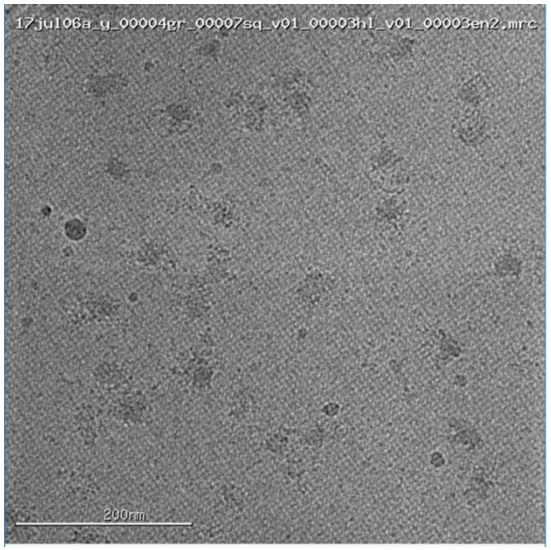


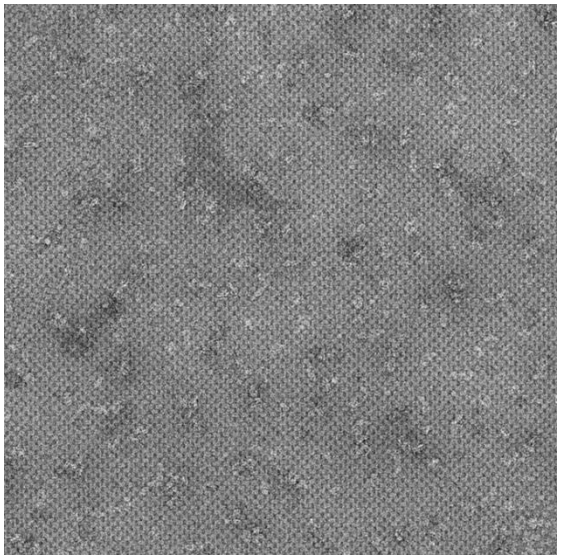
Han et al. (2017) J Struct Biol *In Press*

FSC curves for volumes reconstructed from only 22,697 particles: good SA *vs* poor SA

PRELIMINARY RESULTS FROM USE OF SA-AFFINITY GRIDS IN OTHER LABS

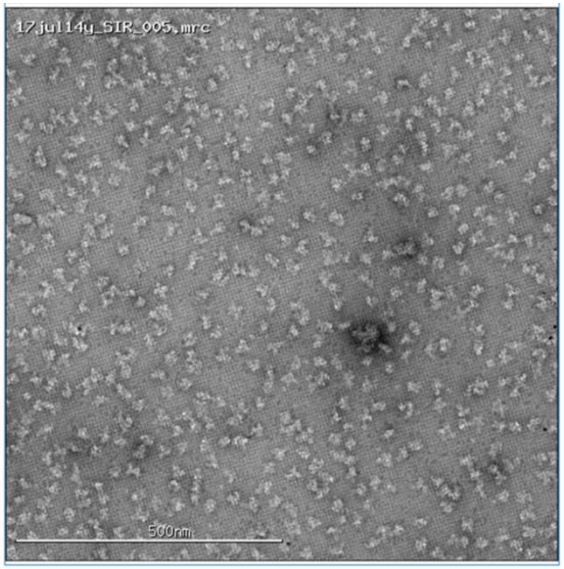
Nicole Haloupek
~1 MDA particle
Nogales lab

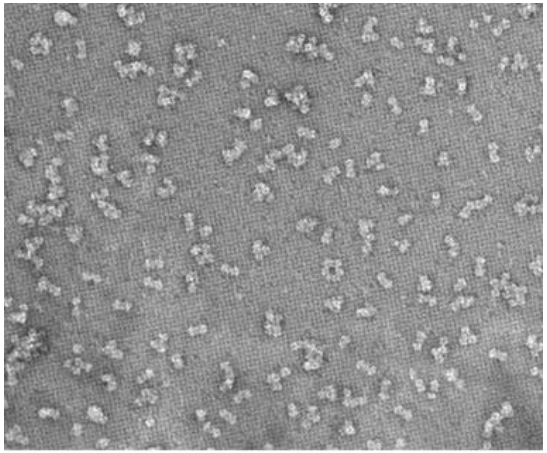




Simon Poepsel
~250 kDa
particle
Nogales lab

Beth Stroup
FSU
800 kDa
particle





Marlovits lab
~500 kDa
particle

CHANGE OF SUBJECT

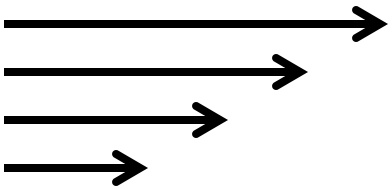
REGARDING HAZARDS DUE TO SHEAR: WHAT I HAVE FOUND OUT SO FAR

SHEAR CAN BE SIGNIFICANT FOR FILAMENTS

- TMV, microtubules, F-actin fibers etc. are often oriented by flow
- F-actin can "change" conformation & fibers can break (Egelman)

THE RELEVANT PARAMETER IS CALLED "FLOW SHEAR RATE"

- Definition: Gradient of fluid velocity perpendicular to the direction of flow



- $\frac{\Delta V}{\Delta Z}$; the units are s^{-1}
- Small, globular subunits are NOT at risk Jaspe & Hagen (2006) Biophysical J 91:3415-24
  - A shear rate of 10^7 s^{-1} is needed to unravel a compact protein
  - Shear rates greater than 10^5 s^{-1} are difficult to produce experimentally

IT IS STILL UNKNOWN WHETHER SHEAR CAN STRIP SUBUNITS, OR DEFORM COMPLEXES WITH SOFT CONTACTS

- Flexible or weakly-bound complexes are clearly at greater risk

Mini-talk-within-a- talk

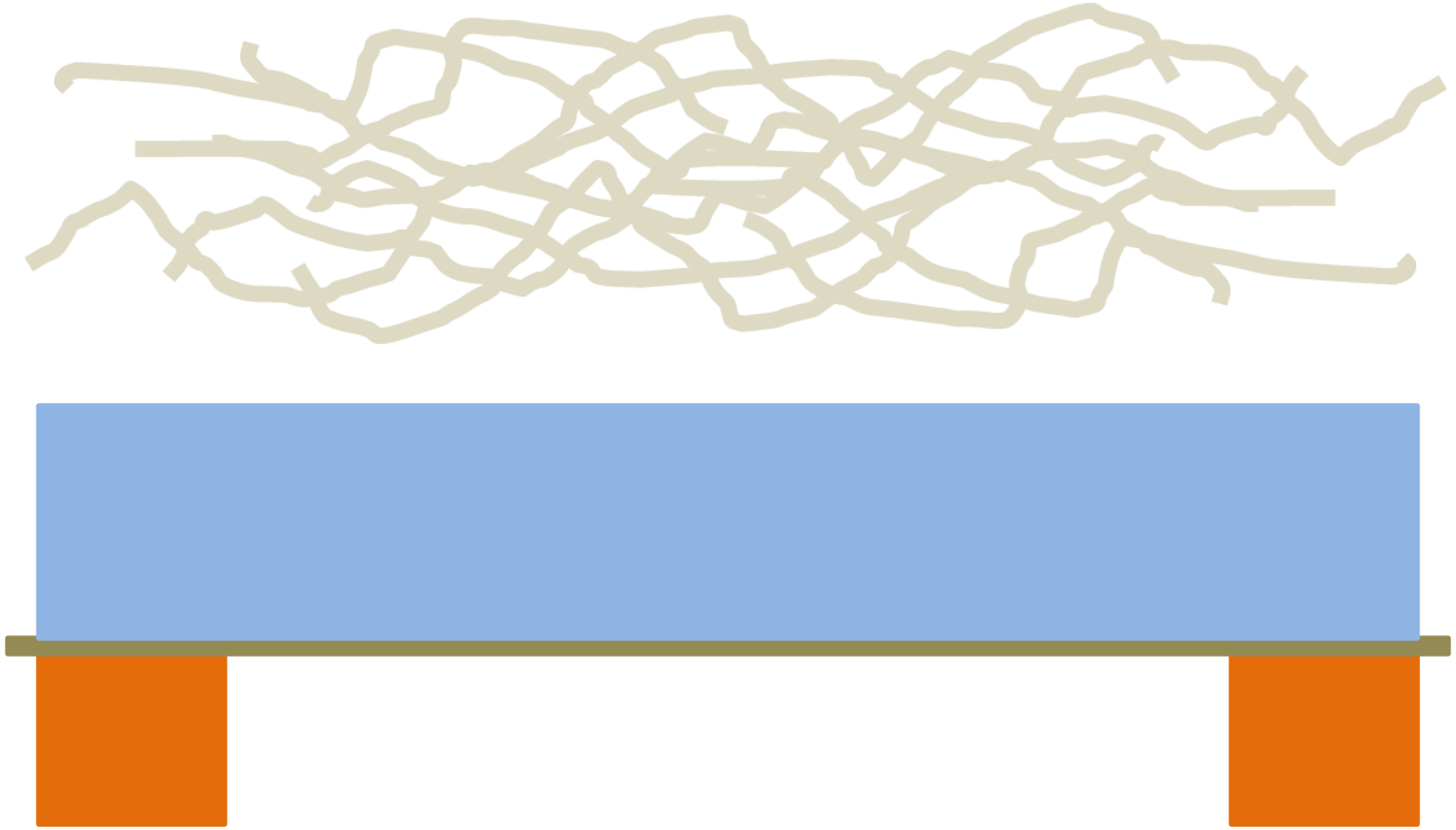
HYPOTHESIS

WHAT MAY HAPPEN DURING BLOTTING OF EM GRIDS

R. M. Glaeser

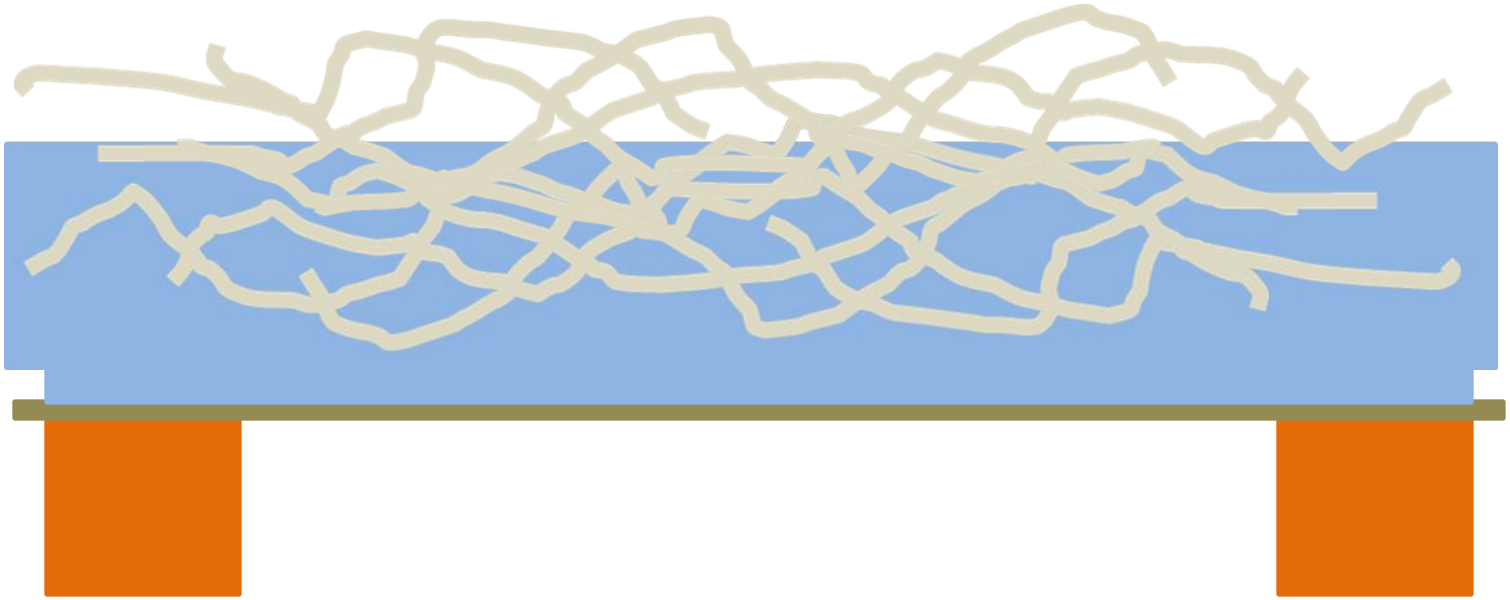
2017/09/22

Filter paper is poised above a puddle of water that was placed on the hydrophilic surface of a support film, on an EM grid



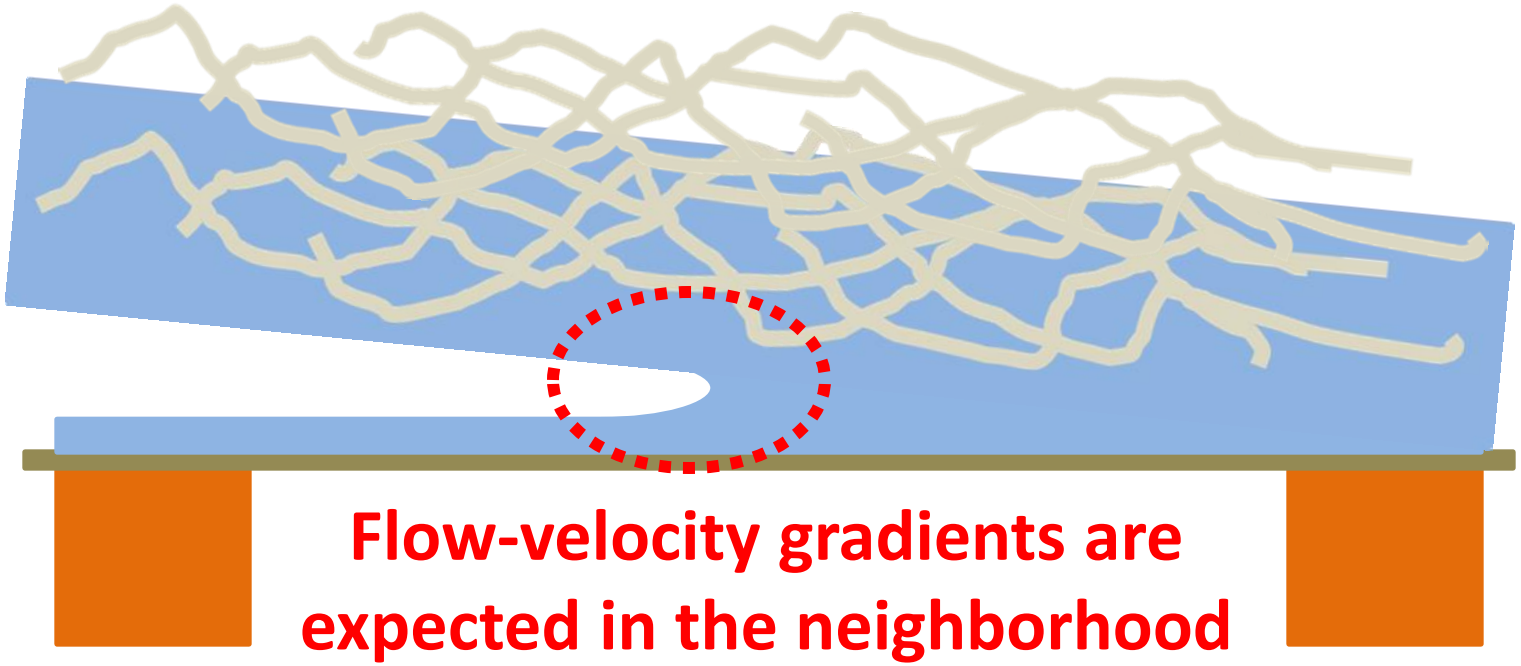
When the filter paper is pressed onto the puddle

bulk water does not rupture between the wet filter paper and the grid



Instead, the interface between the filter paper & grid remains well-lubricated

Rupture only occurs when the filter paper is "peeled" away from the grid. The meniscus then sweeps to one side, leaving thin films of water on the two hydrophilic surfaces

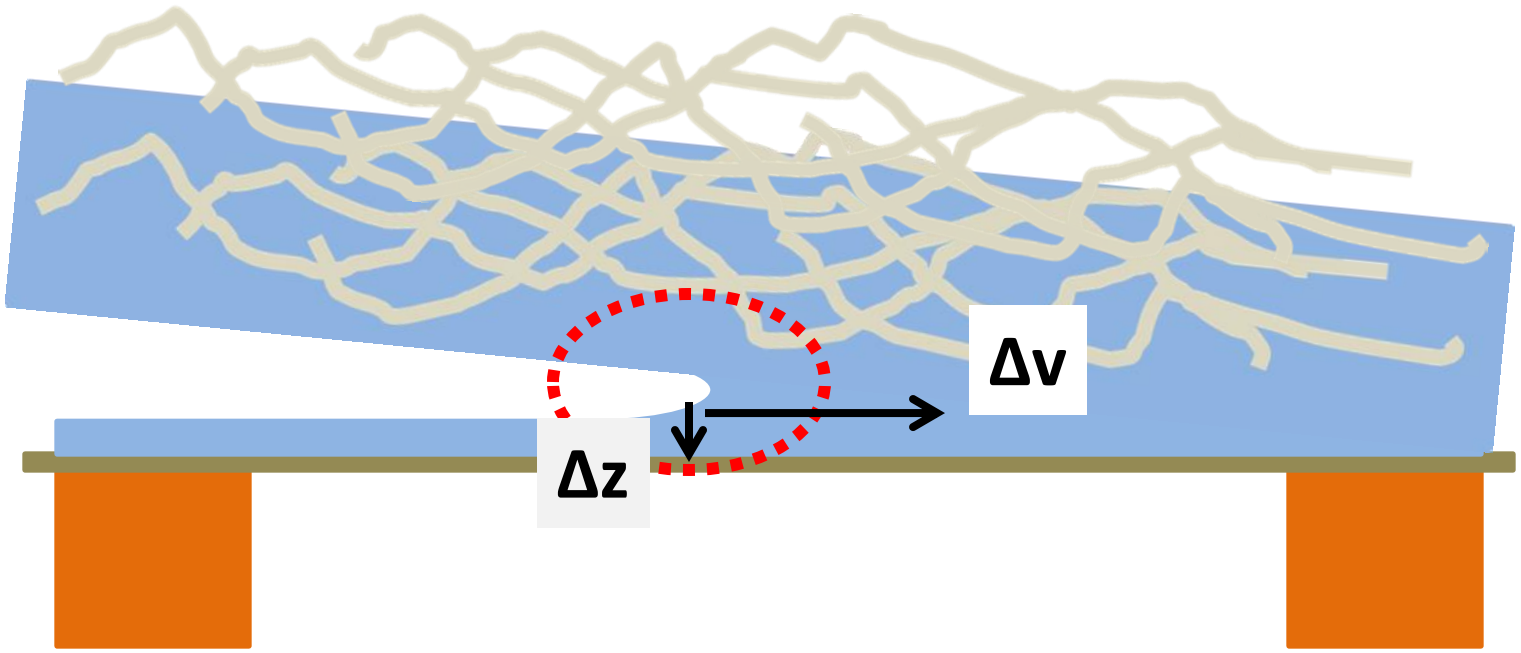


Flow-velocity gradients are expected in the neighborhood of the meniscus

ESTIMATING A WORST- CASE POSSIBLE-VAULE FOR THE SHEAR RATE:

$$\frac{\Delta v}{\Delta z} = \frac{10\ m/s}{1\mu m} = 10^7 s^{-1}$$

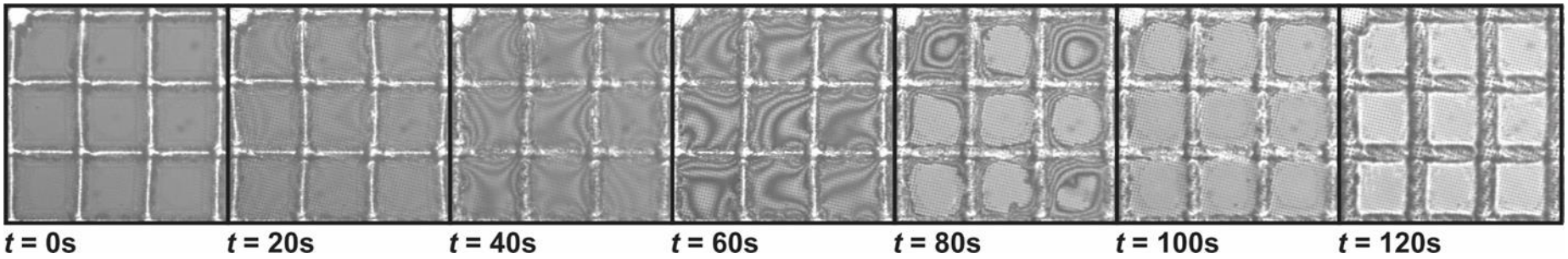
which is thought to be enough to unfold even small, compact proteins

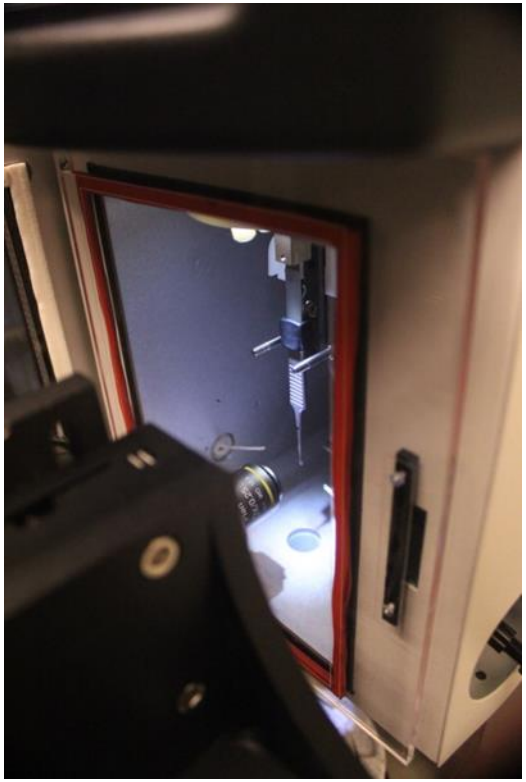


A MUCH SLOWER RATE OF REMOVAL OF WATER MAY (?) BE NEEDED FOR SHEAR-SENSITIVE PARTICLES

- **As the applied sample is wicked or removed from the support film, one cannot avoid that gradients of flow velocity will be present**
- **These gradients – i.e. the shear rate – will be larger, the faster one arranges to remove excess sample**
- **Blotting with filter paper offers little opportunity to control (slow down) the fluid velocity during thinning**
- **This problem motivates looking at ways other than blotting to thin cryo-EM samples**

WORK IN PROGRESS : THINNING AT HIGH HUMIDITY WITHOUT BLOTTING



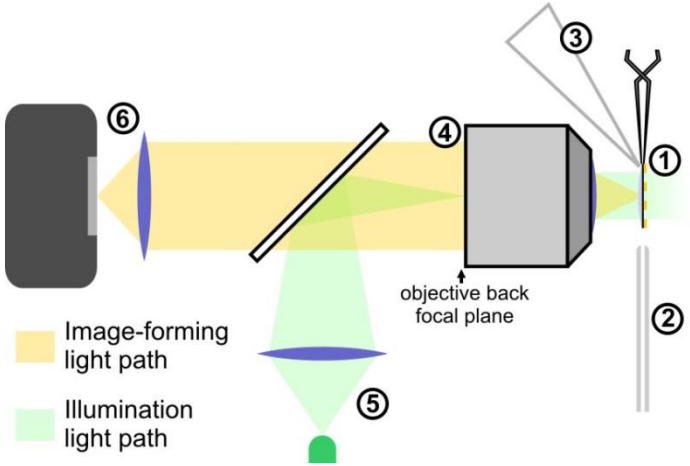


Time series illustrates removal of excess buffer from an EM grid

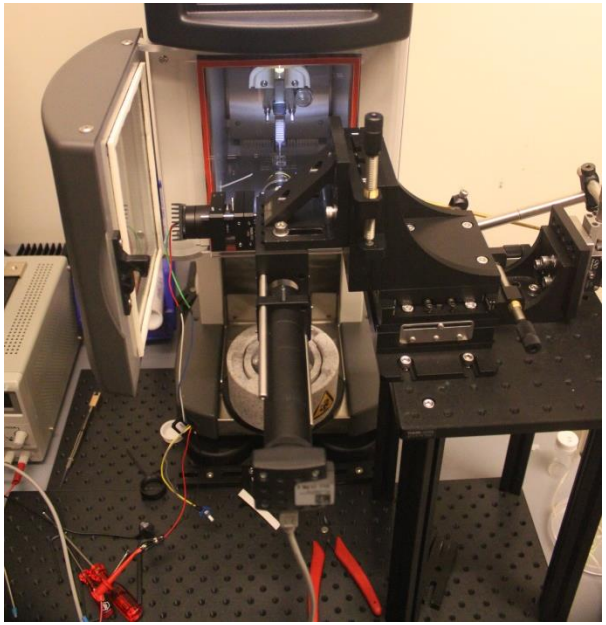
Frames from a video showing liquid thinning on a streptavidin affinity grid

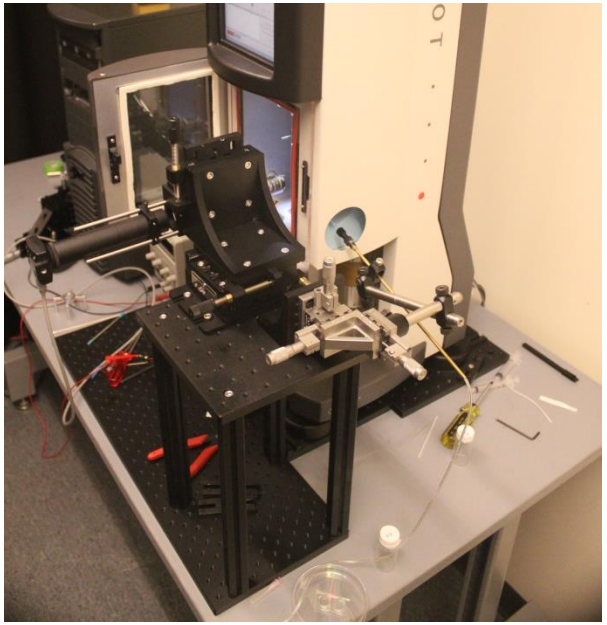
- **Thinning occurs because there is a gradient of nonafluorobutyl methyl ether vapor across the face of the grid**
- **This generates a gradient in surface tension, which in turn thins the area with lowest surface tension (Marangoni effect)**

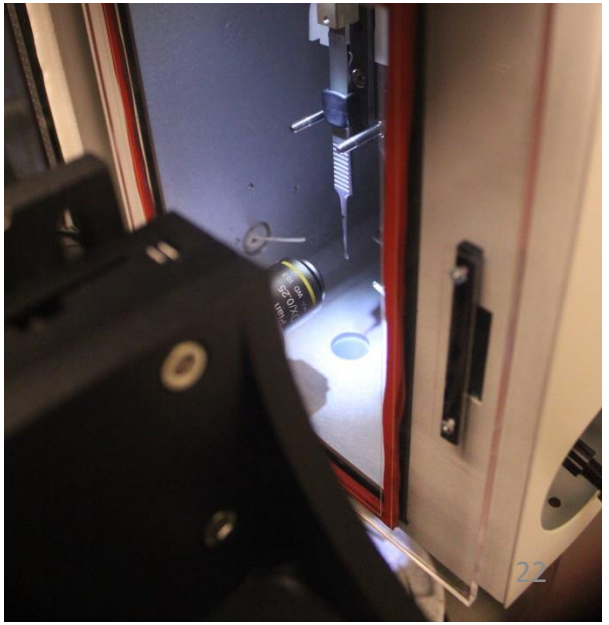
EXPERIMENTAL SET-UP FOR MARANGONI THINNING



1) EM grid (held in forceps)
2) Capillary containing nonafluorobutyl methyl ether
3) Filter paper to absorb displaced sample
4) Objective for imaging film thickness (reflected light)
5) Monochromatic source
6) Camera







ACKNOWLEDGMENTS

All aspects

- **Bong-Gyoon (“BG”) Han (LBNL)**

SA affinity grids & ribosomes

- **Jamie Cate (MCB & Chem, UCB)**
  - **Arto Pulk; Zoe Watson**

Marangoni effect experiments

- **Dan Fletcher (Bioengineering, UCB)**
  - **Michael Vahey**