Negative Staining Beyond Uranyl Acetate

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and
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Phage capsids collect by CDW on JEOL 1230 TEM

TODAY'S DISCUSSION

- Negative Staining Beyond UA
  - What is Negative Staining
  - How to Negatively Stain Samples
  - Which Stain to Choose
  - Troubleshooting
- Return to Lab Q&A
  - COVID Research at BioCryo
  - Making Sense of Reopening

Come Talk to Us

Eric "W." Roth

they/them/their's

Office: Silverman B545

"Mastery through endless repetition"

"Happy Microscopy!"

Charlene "Bene" Wilke

they/them/she/her

Office: Hogan 5-125

"If you ever have a chance to work on Bacteria-Phage, jump on it! You'll be amazed what you see."

What is Negative Staining?

Qualitative contrasting of purified biological or soft material samples

200 kDa up to several MDa for EM observation

Depositing stain on the grid results in an EM image resembling that of a photographic negative.

"We are mostly staining the background."

Enzymes, annelid hemoglobins, molluscan hemocyanins, ribosomes, isolated cellular organelles, membrane fractions, bacterial cell walls and membranes, filamentous protein structures, liposomal structures any other soluble protein molecules and components, polymer spheres, nano-constructs…

What is Negative Staining?

- *Easy to learn and perform*
- *Rapid (<30 minutes on bench)*
- *Does not require specialized equipment*

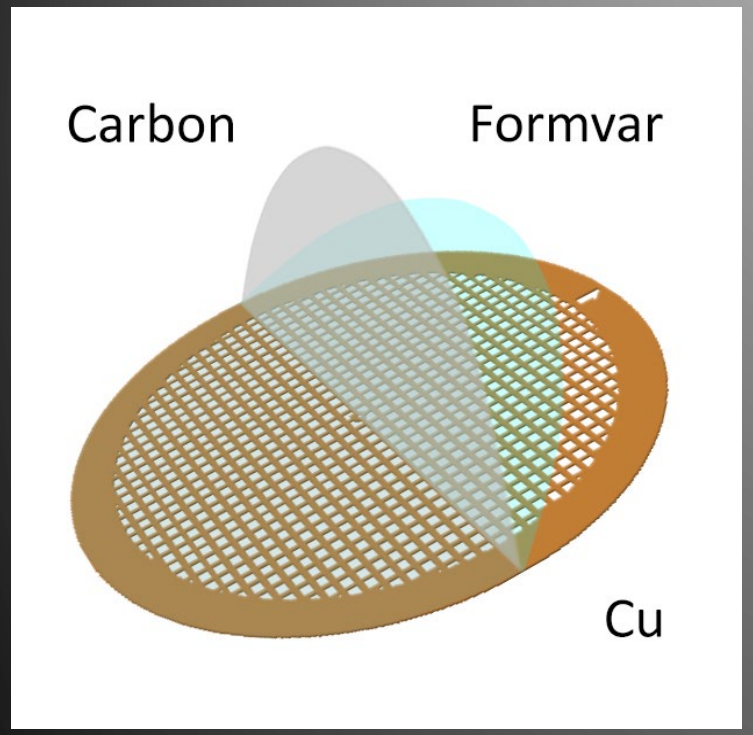
- ✓ *Tweezers*
- ✓ *Grids (carbon membrane)*
- ✓ *Stain*
- ✓ *Plasma Cleaner or Glow Discharger*
- ✓ *Filter Paper*

What is Negative Staining?

Protocol? How to?

"Everyone has different recipes to make cookies, but most cookies are delicious."

How to Negatively Stain Samples

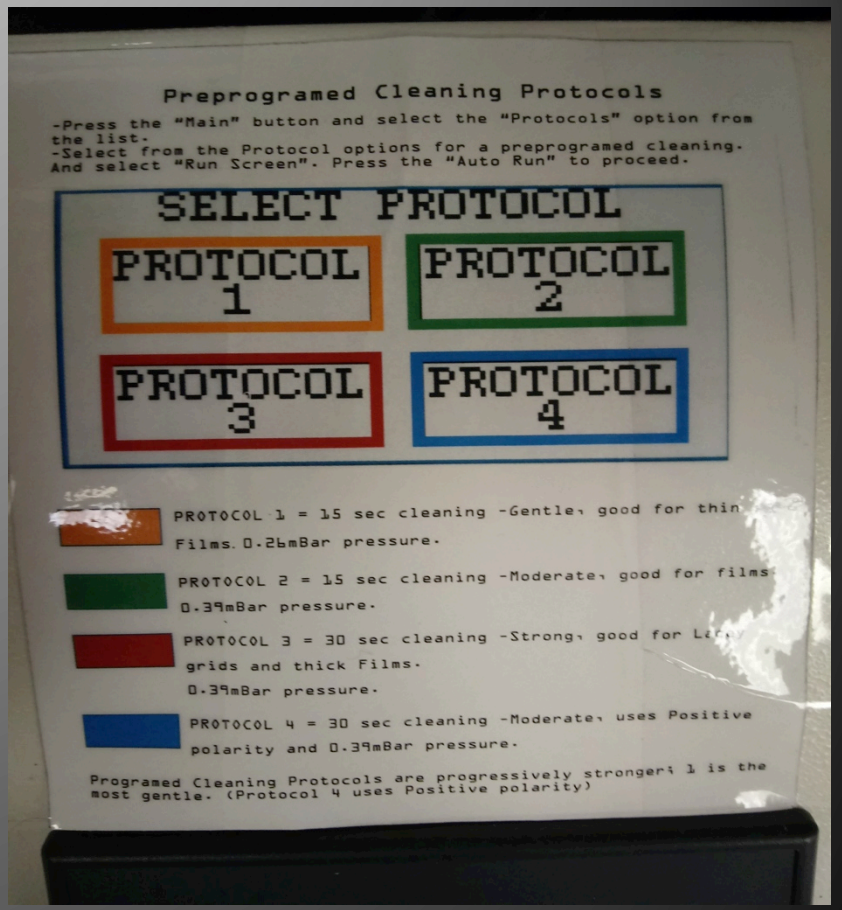


GRID PREP

Grid with a support film:
Carbon Film, Graphene Oxide, etc.

Grid material:
Cu, Ni, Au?

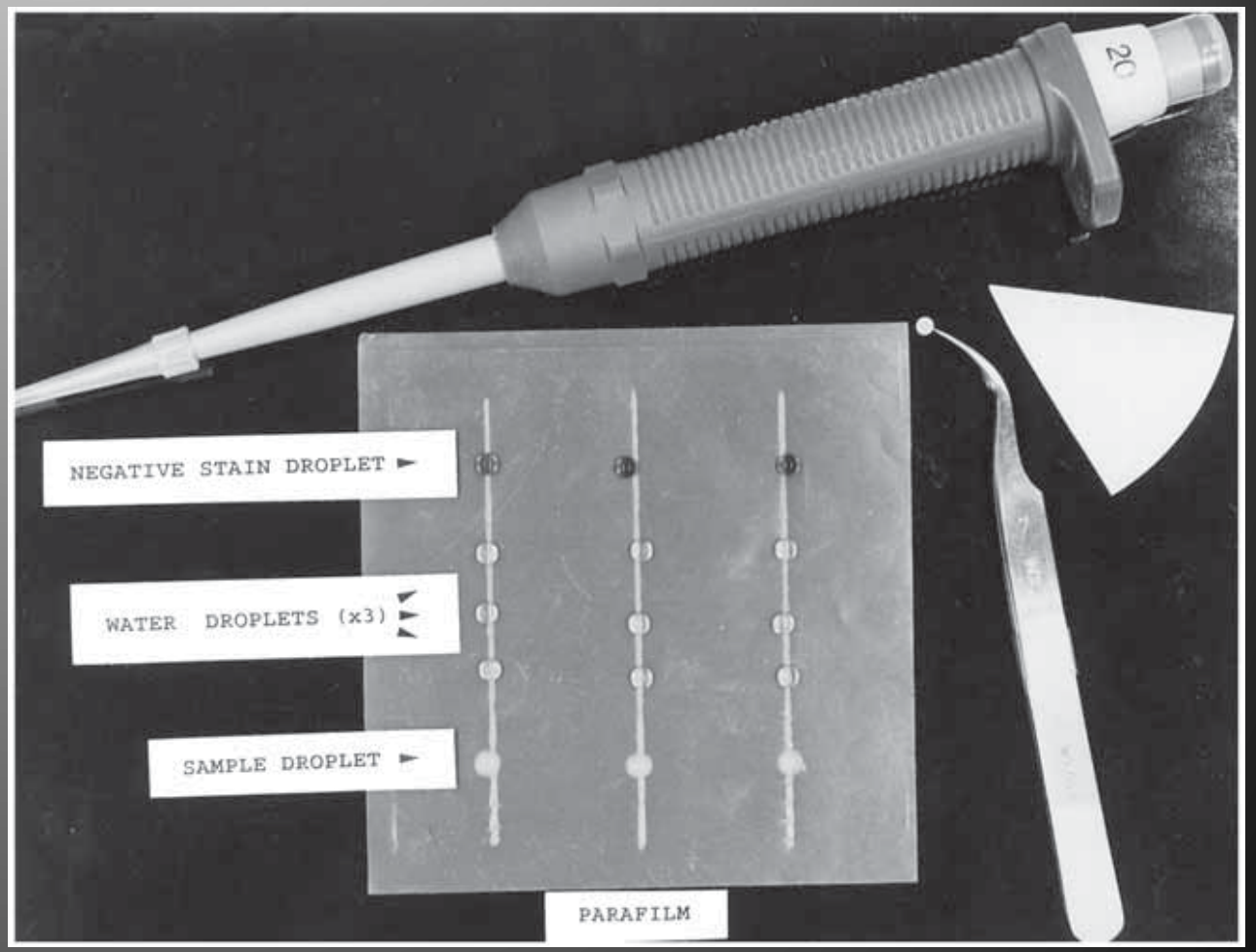
Glow Discharge / Plasma Cleaning
Why, When and How long



How to Negatively Stain Samples

BENCH SET UP

- Sample
- Wash solution, di water
- Stain
- Grids
- Parafilm or petri dish
- Pipetter P10, P200
- Forceps, preferably negative action and anti- capilary
- Filter paper
- Grid box for storage




Electron
Microscopy
Sciences
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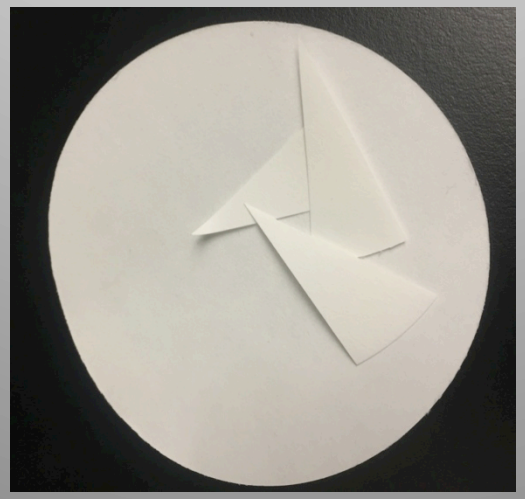
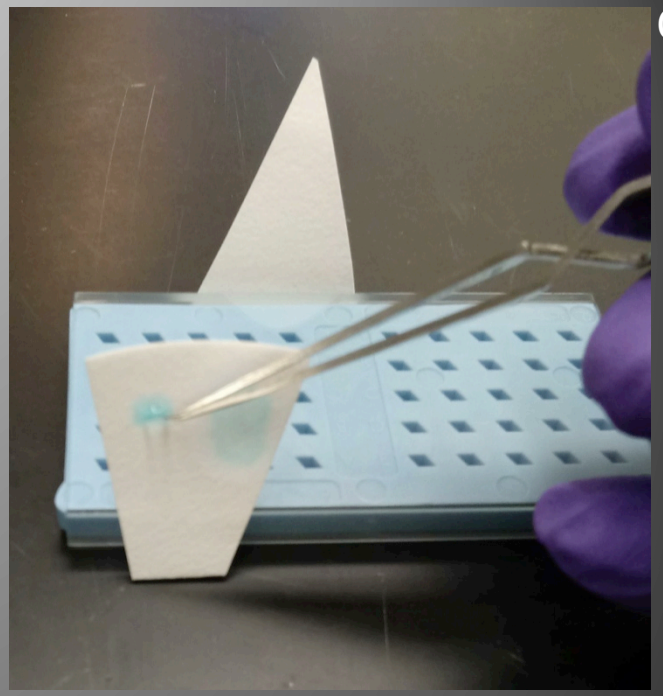
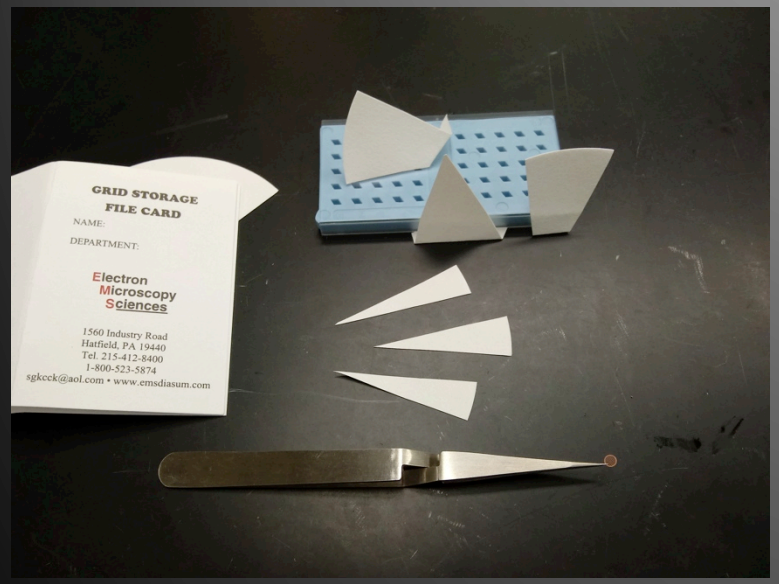
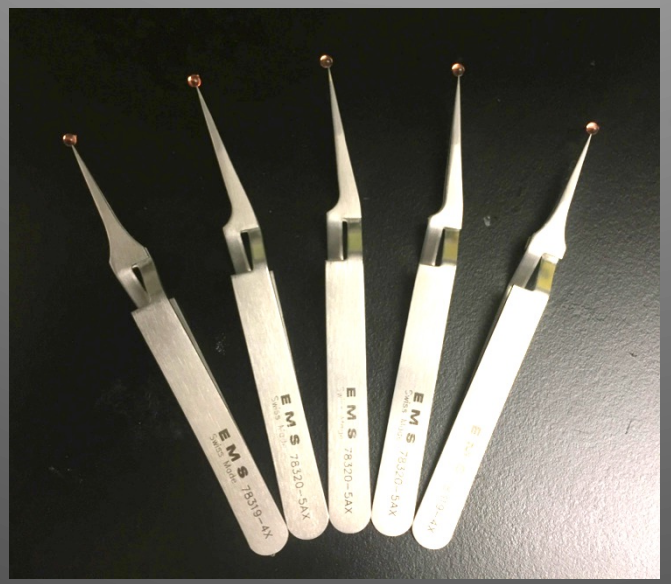
CF300-CU
50/pk
CARBON FILM 300 MESH,
COPPER






GRID STORAGE
FILE CARD
NAME:
DEPARTMENT:
Electron
Microscopy
Sciences
1560 Industry Road
Hatfield, PA 19440
Tel. 215-412-8400
1-800-523-5874
sgkcck@aol.com • www.emsdiasum.com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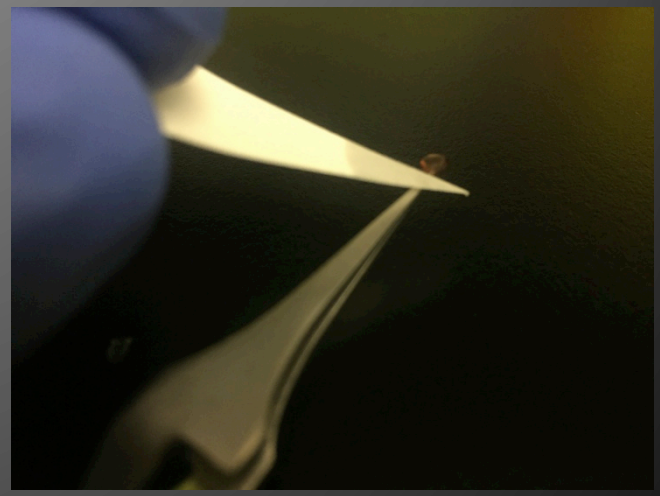
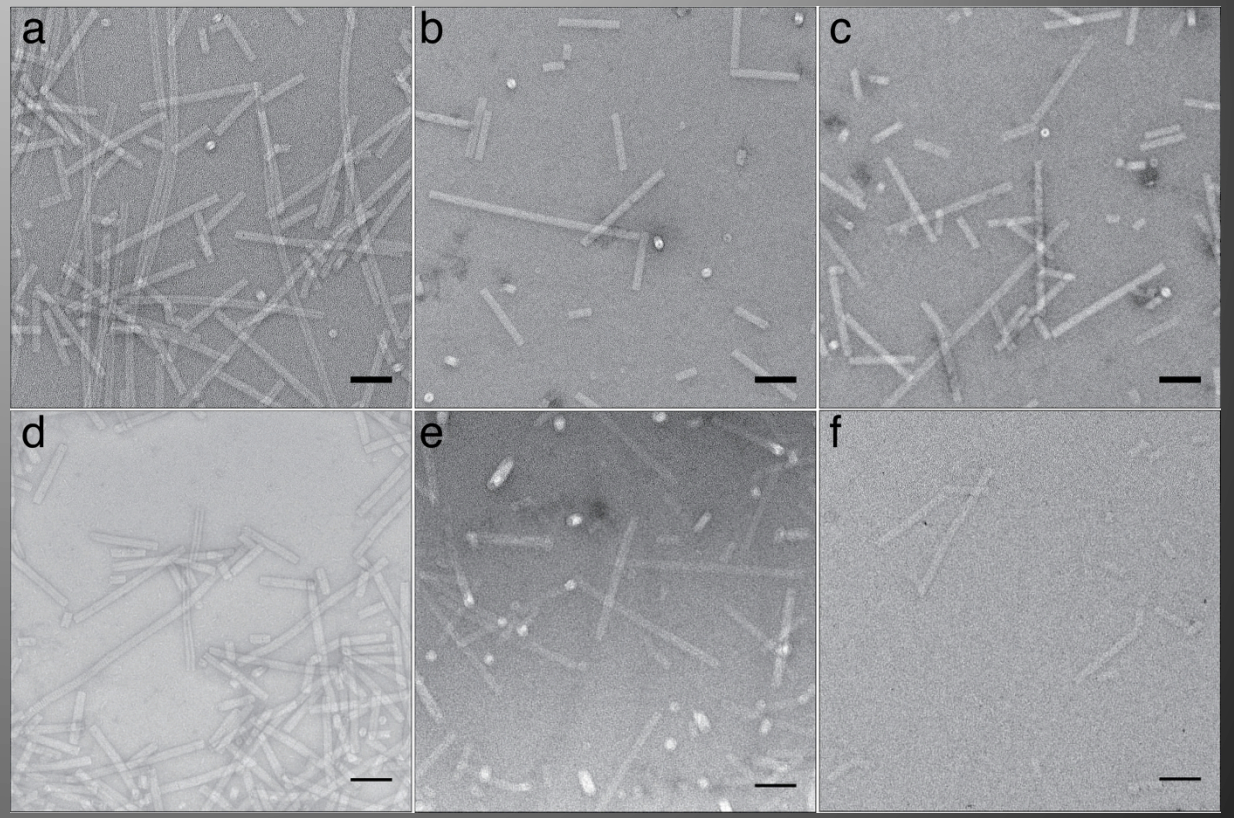


Which Stain to Choose?

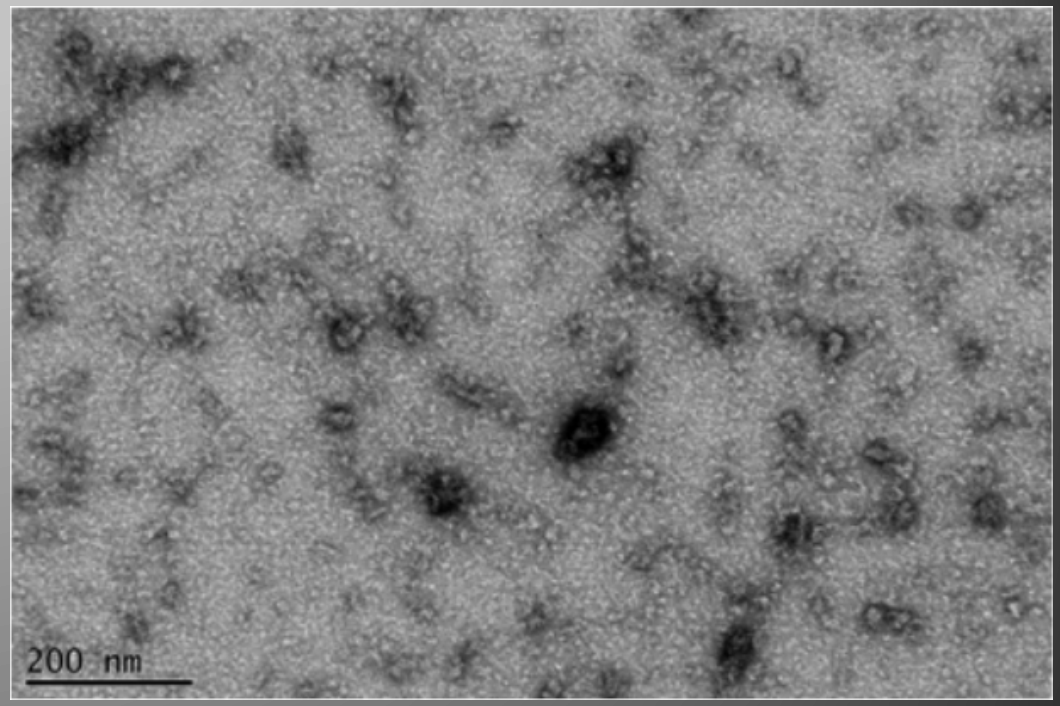
Example micrographs of Tobacco Mosaic Virus stained with
(A) 1% UF (B) 2.5% TmAc
(C) 2.5% ErAc. (D) 1% UA
(E) 2.5% GdAc and (F) 2.5% SmAc.
Scale bars are 100 nm



Scarff, Charlotte A., et al. "Variations on negative stain electron microscopy methods: tools for tackling challenging systems." *JoVE (Journal of Visualized Experiments)* 132 (2018): e57199

Uranyl Acetate

- Easy to make (1-3% (saturates at >3%))
- Easy to use, long term storage @4C in dark
- High Electron Density / Contrast
- Low pH! Not recommended some samples
- Fixes some viruses and biological material
- Can precipitate at neutral pH and with many salts (sodium phosphate, etc.)



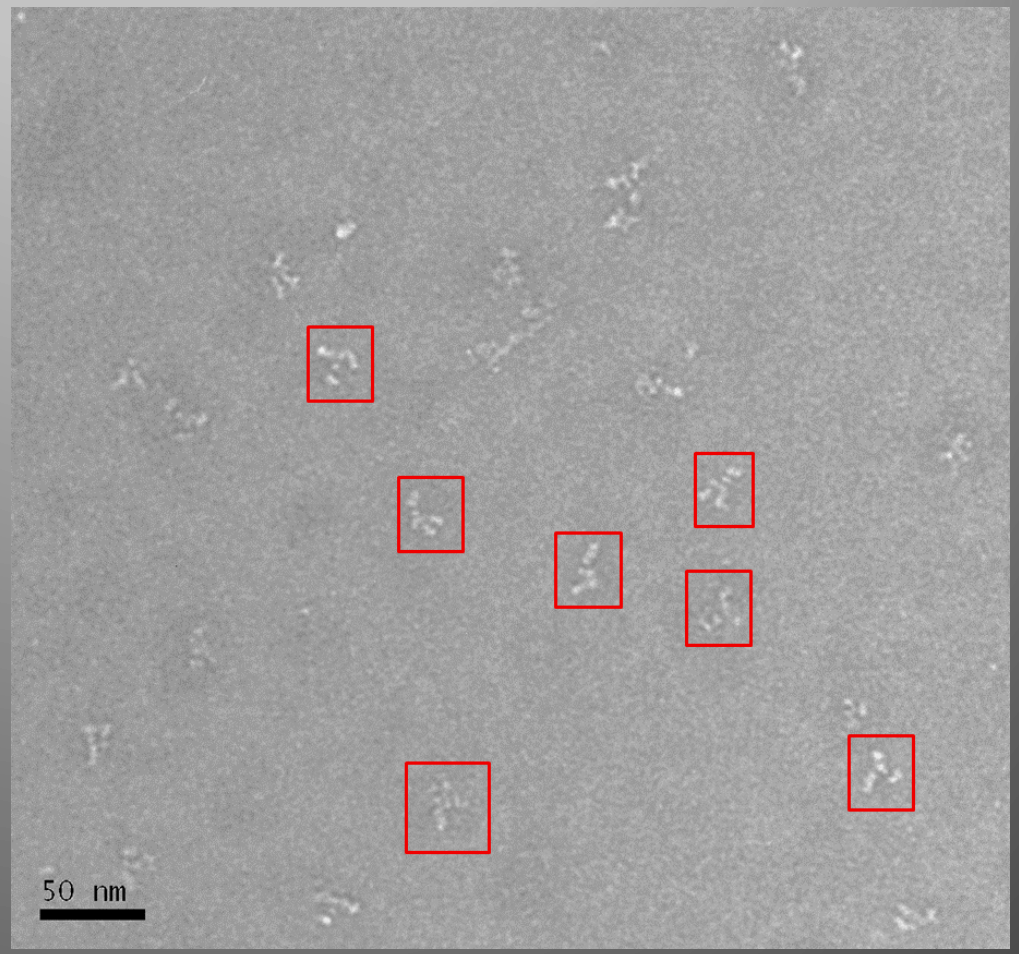
Supramolecular particle formation in E1C phase

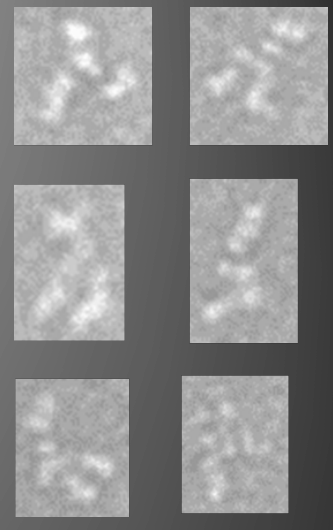
Dai, Min, Jennifer Haghpanah, Navjot Singh, Eric W. Roth, Alice Liang, Raymond S. Tu, and Jin Kim Montclare. "Artificial protein block polymer libraries bearing two SADs: effects of elastin domain repeats." *Biomacromolecules* 12, no. 12 (2011): 4240-4246.

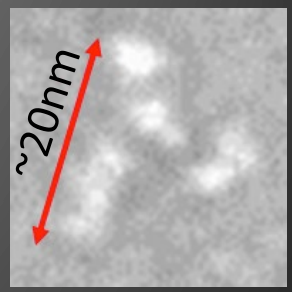
Uranyl Formate

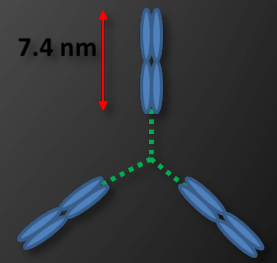
- *Challenging to make – must use degassed DI water, can "break" easily what adding KOH*
- *Use same day or store in aliquots at -20C in dark*
- *Smaller grain size/superior morphology Vs. UA*

Macro Molecules imaged with UF prior to cryoTEM



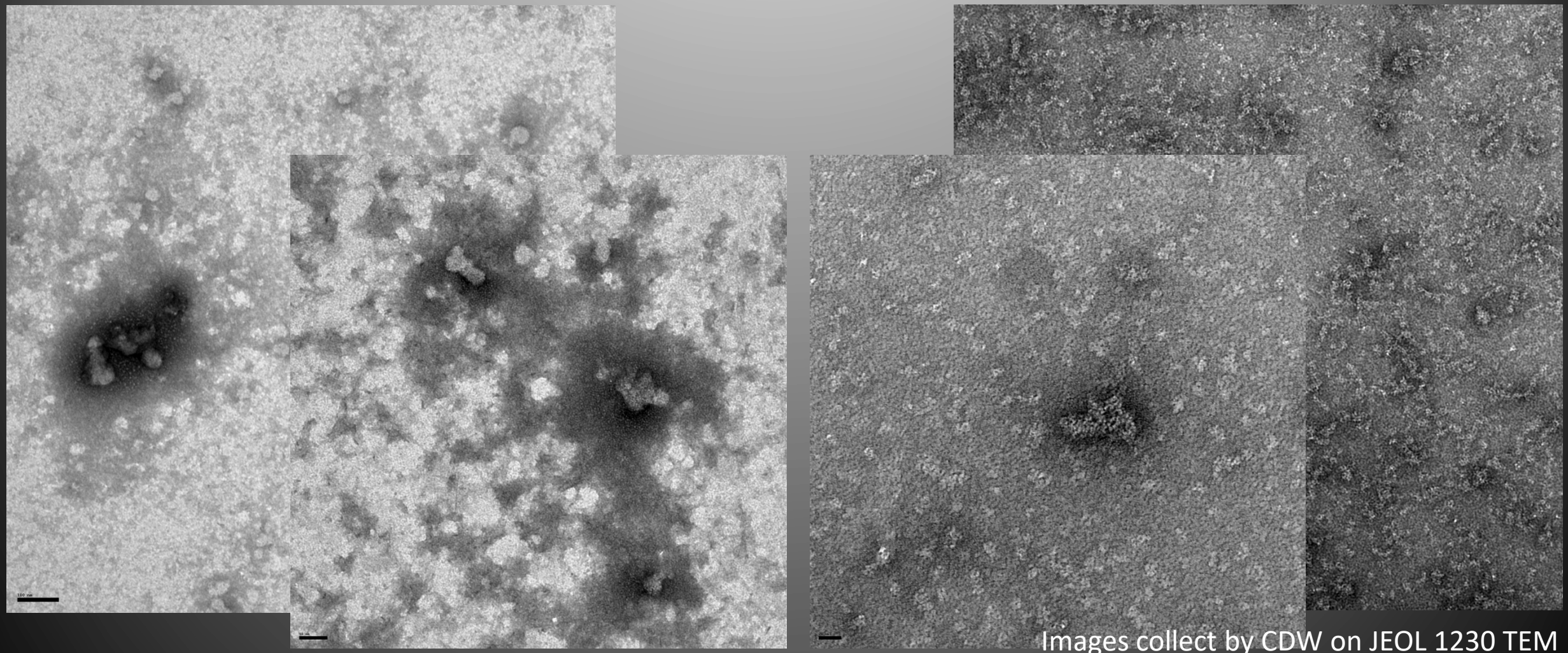






Sample: Justin Modica (Mrksich Group, Northwestern)
TEM: Sonali Dhindval (VPD Group, Northwestern)
Captured on JEOL ARM300 TEM

Uranyl Acetate Vs. Uranyl Formate

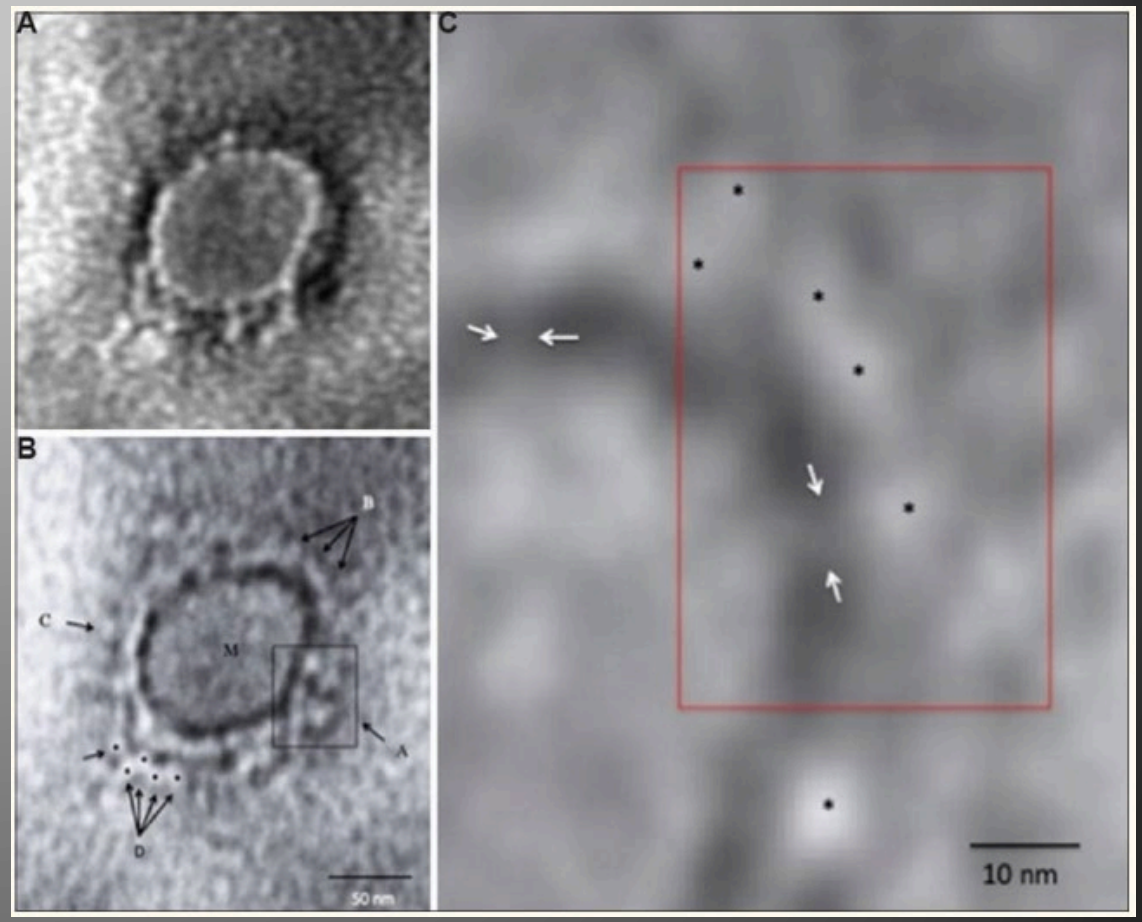


Images collect by CDW on JEOL 1230 TEM

Phosphotungstic Acid (PTA)

- Easy to make (1-3%)
- Stable at various pH 0.5 to pH 8
- Considered a positive stain
- Can disrupt membranes
- Less likely to precipitate with salts
- Less contrast than UA/UF

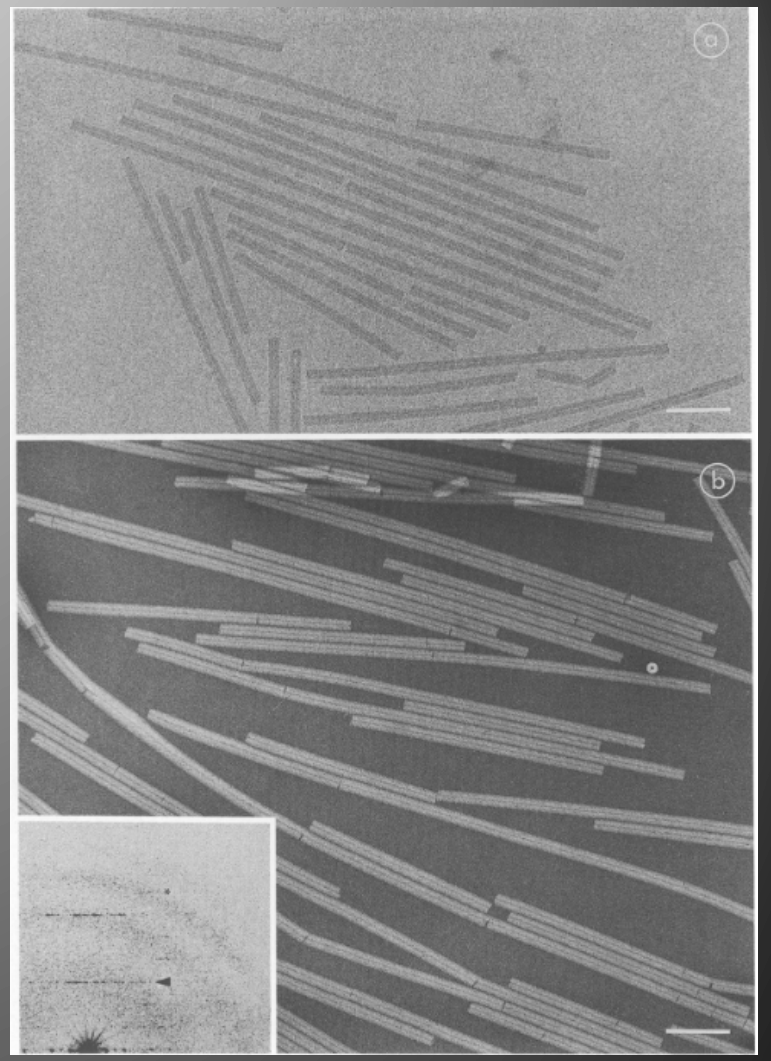
Negative stain of SARS-CoV-2 VP in focus (a), and defocus (b) to resolve virus envelope in finer detail, (c) high mag and processed image illustrating 'stalk' connecting peplomer to virion surface



Prasad, Sharda et al. "Transmission electron microscopy imaging of SARS-CoV-2." The Indian journal of medical research vol. 151,2 & 3 (2020): 241-243. doi:10.4103/ijmr.IJMR_577_20

Ammonium Molybdate

- *Easy to make (1-2%- or higher)*
- *Unstable > pH 7.0 (forming crystals when drying)*
- *Good for osmotically-sensitive organelles*
- *Lower contrast than other stains*



Unstained cryoTEM (a) Vs. Negative Stain cryoTEM (b) of tobacco mosaic virus. Staining allowed for higher resolution imaging without destroying morphology.

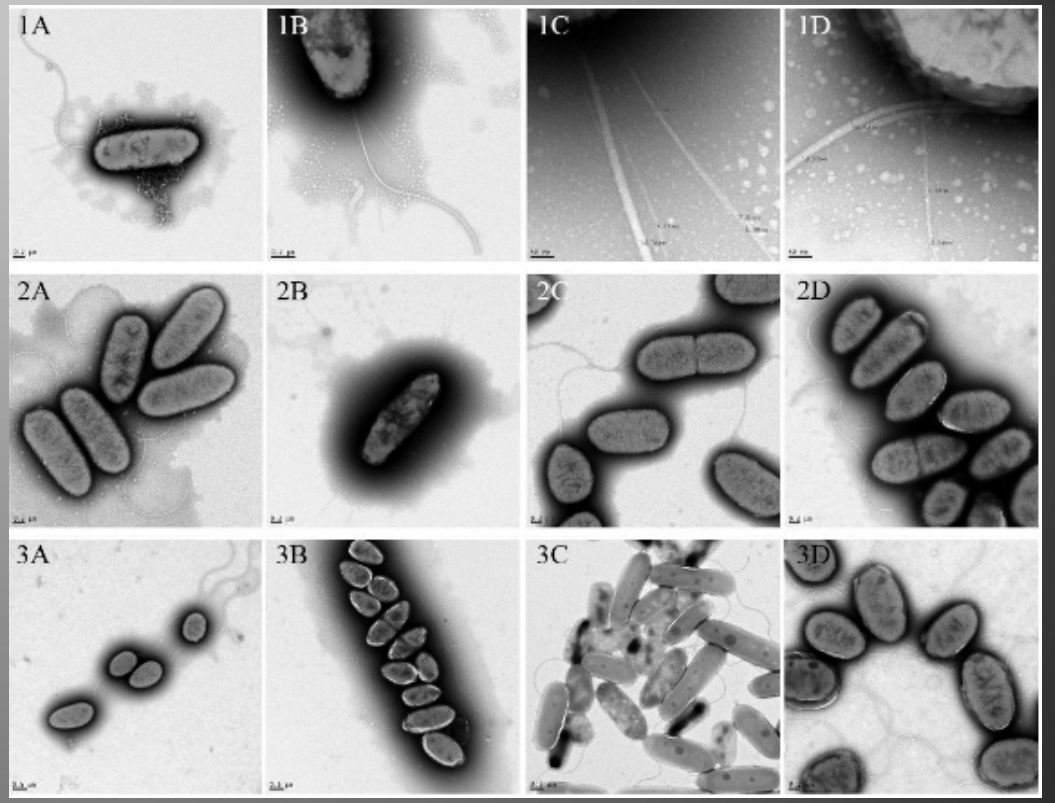
Adrian, Marc, et al. "Cryo-negative staining." *Micron* 29.2-3 (1998): 145-160.

Methylamine Tungstate

- *Must be made fresh (2%) (does not store well)*
- *Does not damage delicate structures as much as PTA*
- *Lower contrast then UA, but good resolution*
- *Commercially available ("NANO-W" nanoprobes.com)*

Negative staining of Pseudomonas aeruginosa, a key player in Cystic Fibrosis infections.

"This strategy yielded high contrast and fine detail with very little non-specimen background."

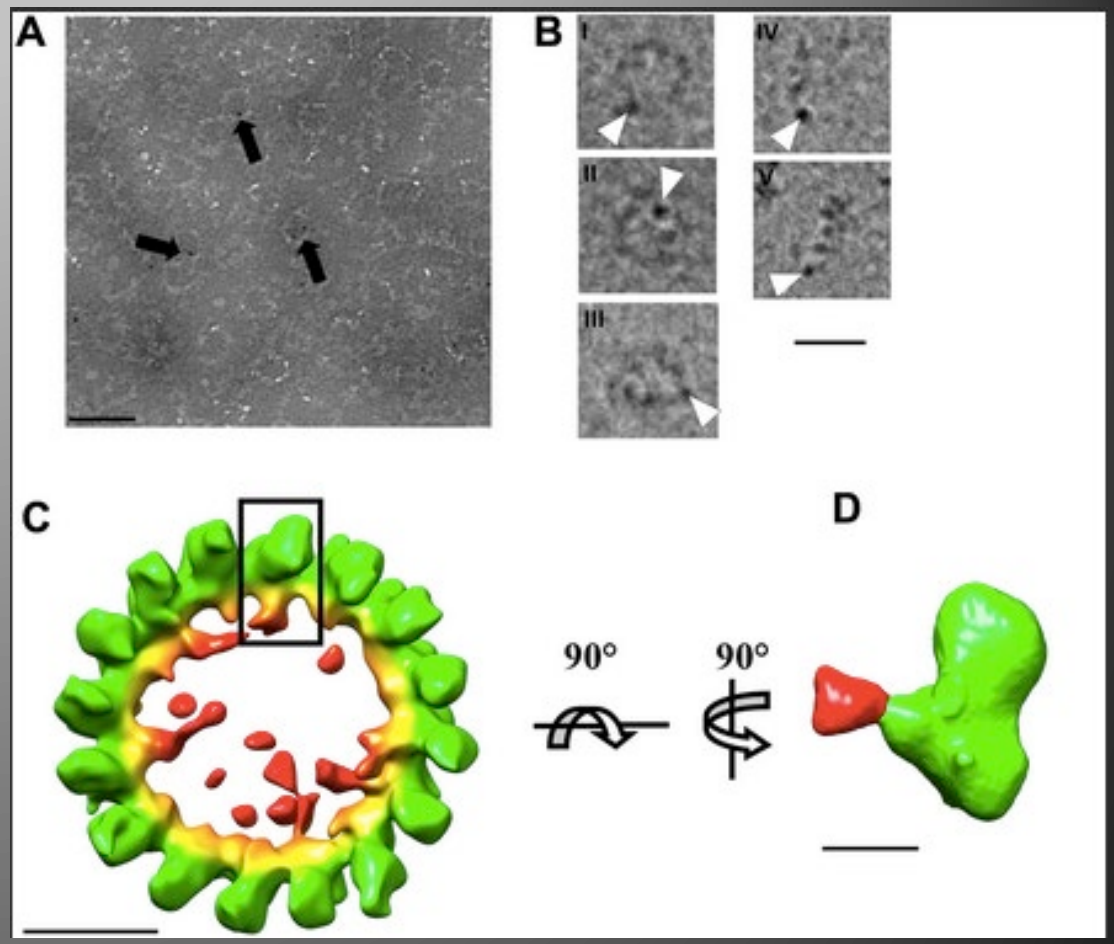


Chance, Deborah L., and Thomas P. Mawhinney. "Using Negative Staining TEM to Study Structure/Function Relationships of Cystic Fibrosis Host-Adapted Opportunistic Pathogen Pseudomonas aeruginosa." *Microscopy and Microanalysis* 23.S1 (2017): 1354-1355.

Methylamine Vanadate

- *Physiological pH (7-8)*
- *Smooth background*
- *Lower contrast than UA, but high resolution*
- *Less unwanted, "positive" staining*
- *Commercially available ("NanoVan" nanoprobes.com)*

NanoVan used as a negative stain in combination with immuno gold labeling to identify DRP1 (a protein resposible for mitochondrial membrane fission) molecules (A) prior to cryoTEM analysis and 3D reconstruction (B, C, D)



Basu, Kaustuv, et al. "Molecular mechanism of DRP1 assembly studied in vitro by cryo-electron microscopy." *PloS one* 12.6 (2017): e0179397.

Other Stains

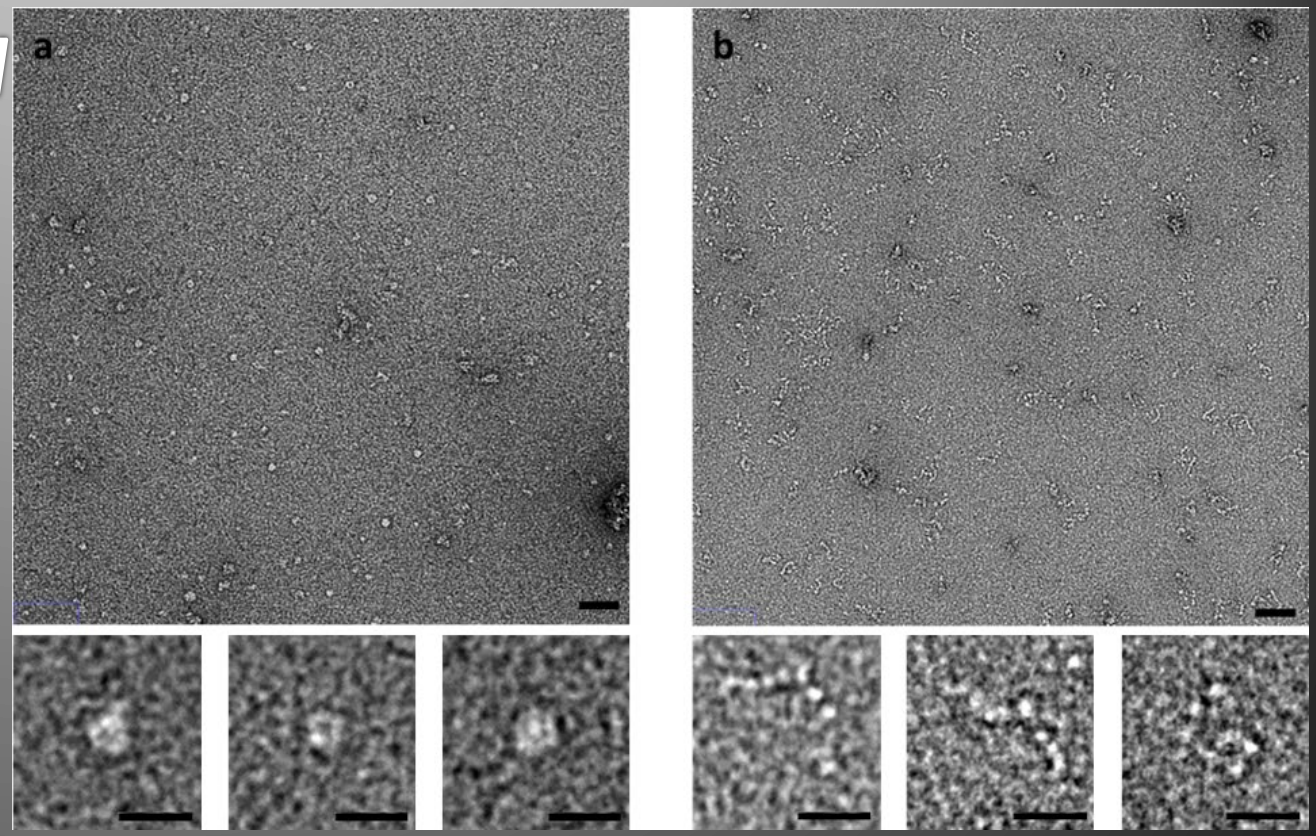
Less Common Stains

- Gold Thioglucose
- Lanthanum Acetate
- Platinum Blue
- Lithium Tungstate
- Sodium Zirconium Glycollate
- Tunstoborate
- Alluminum Formate
- Uranyl Oxalate
- Uranyl Sulphate

Troubleshooting

"Your challenge could be resolved by changing any one step. It takes trial and evaluation."

- *Staining Method: Floating, Blotting, Flicking*
- *Sample Charge*
- *Timing*
- *Sample pH V.s Stain pH*

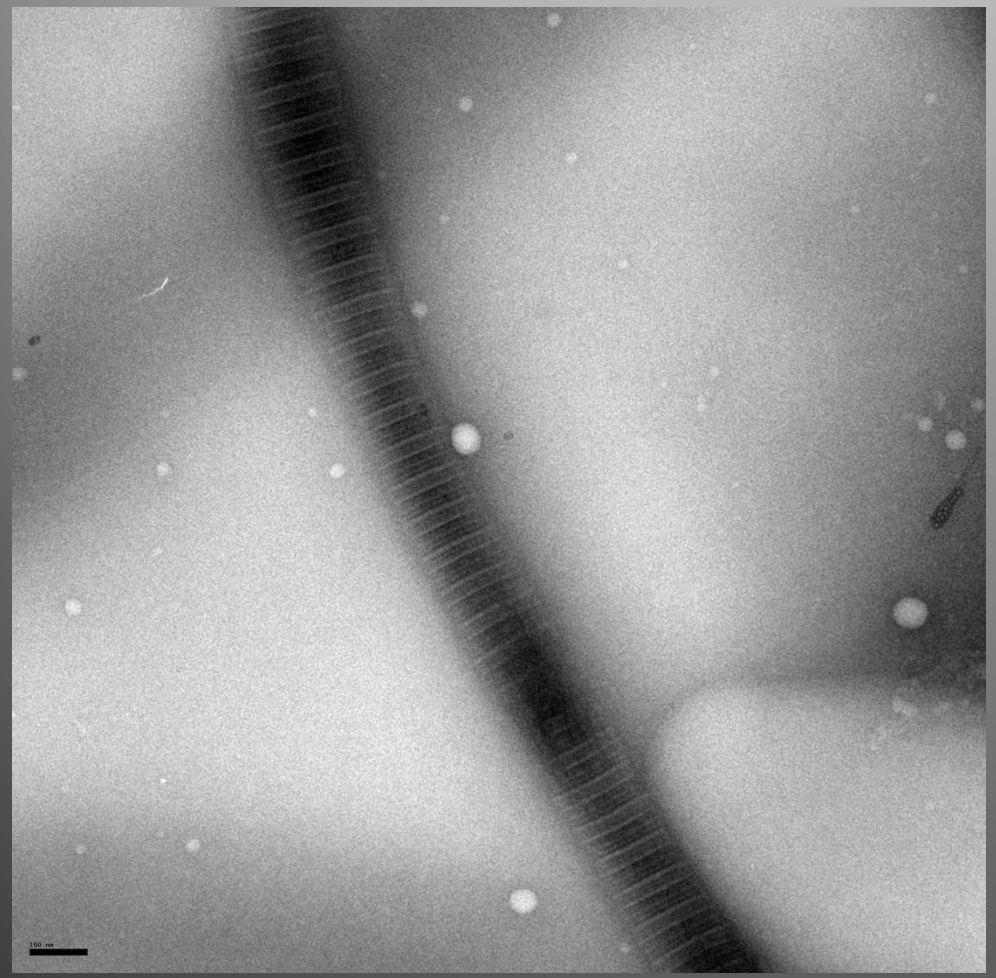


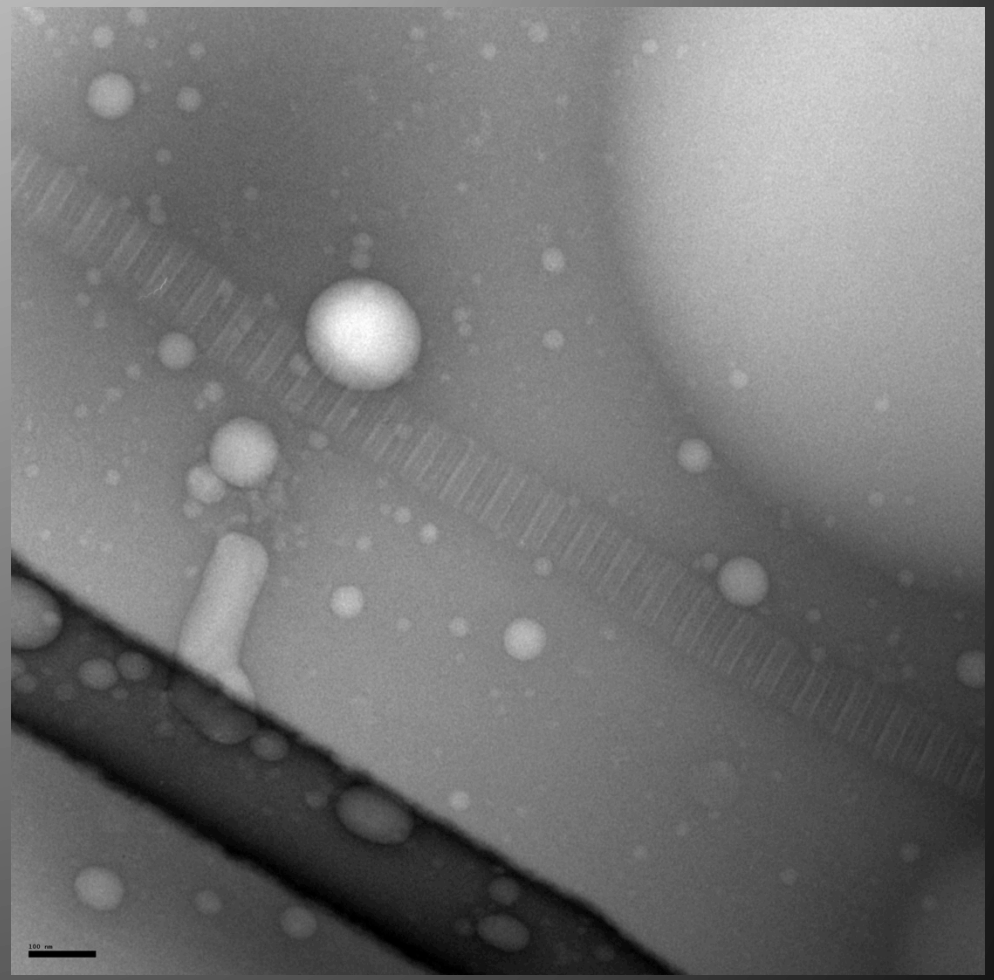
Scarff, Charlotte A., et al. "Variations on negative stain electron microscopy methods: tools for tackling challenging systems." *JoVE (Journal of Visualized Experiments)* 132 (2018): e57199

Troubleshooting

Inconsistent Staining

Positive Vs. Negative Staining Effects with (UA) on same grid



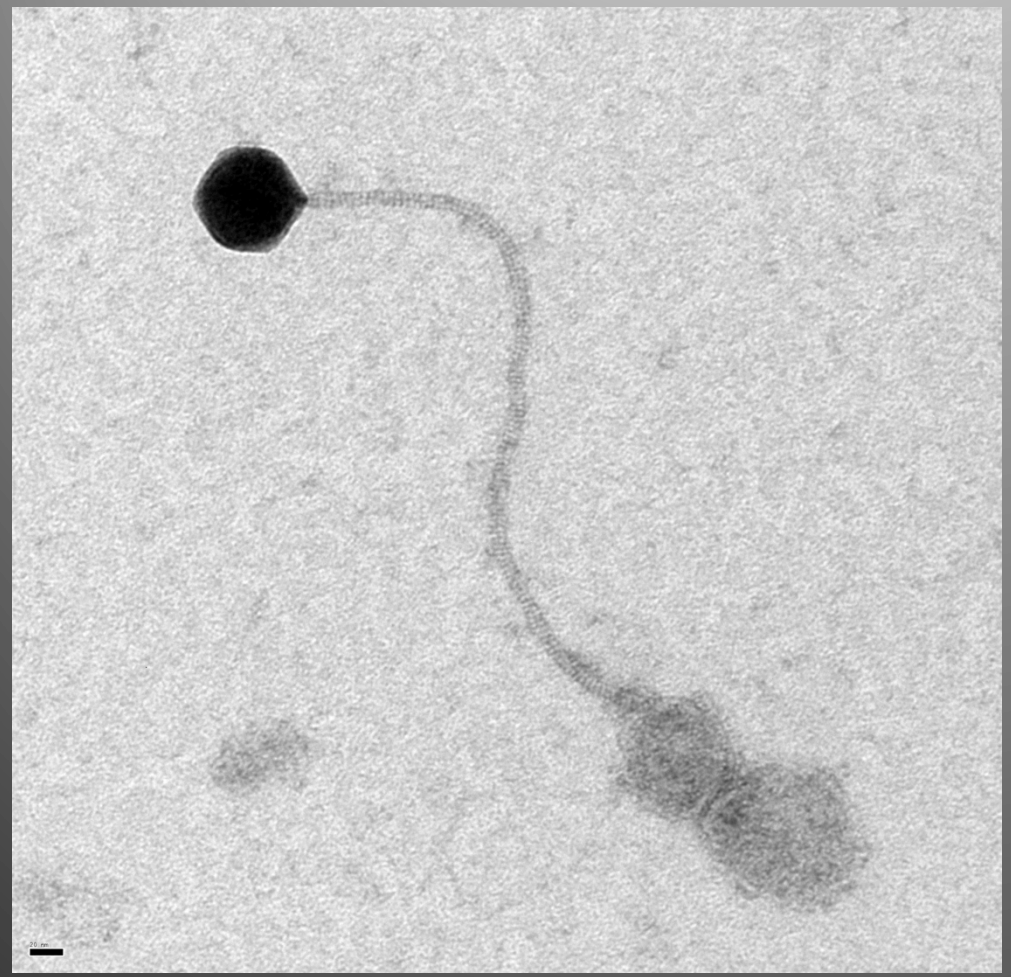


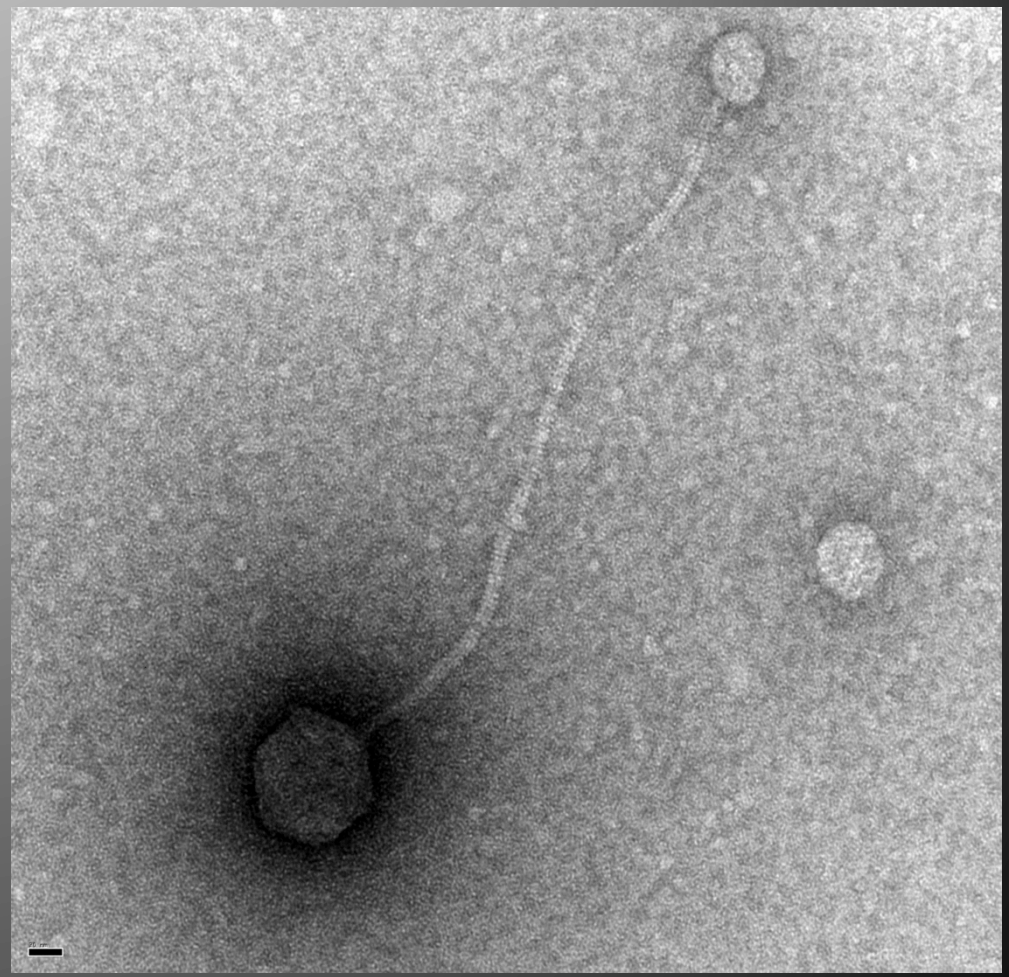
Collagen Fibers Collect by CDW on JEOL 1230 TEM

Troubleshooting

Inconsistent Staining

Positive Vs. Negative Staining Effects with (UA) on same grid



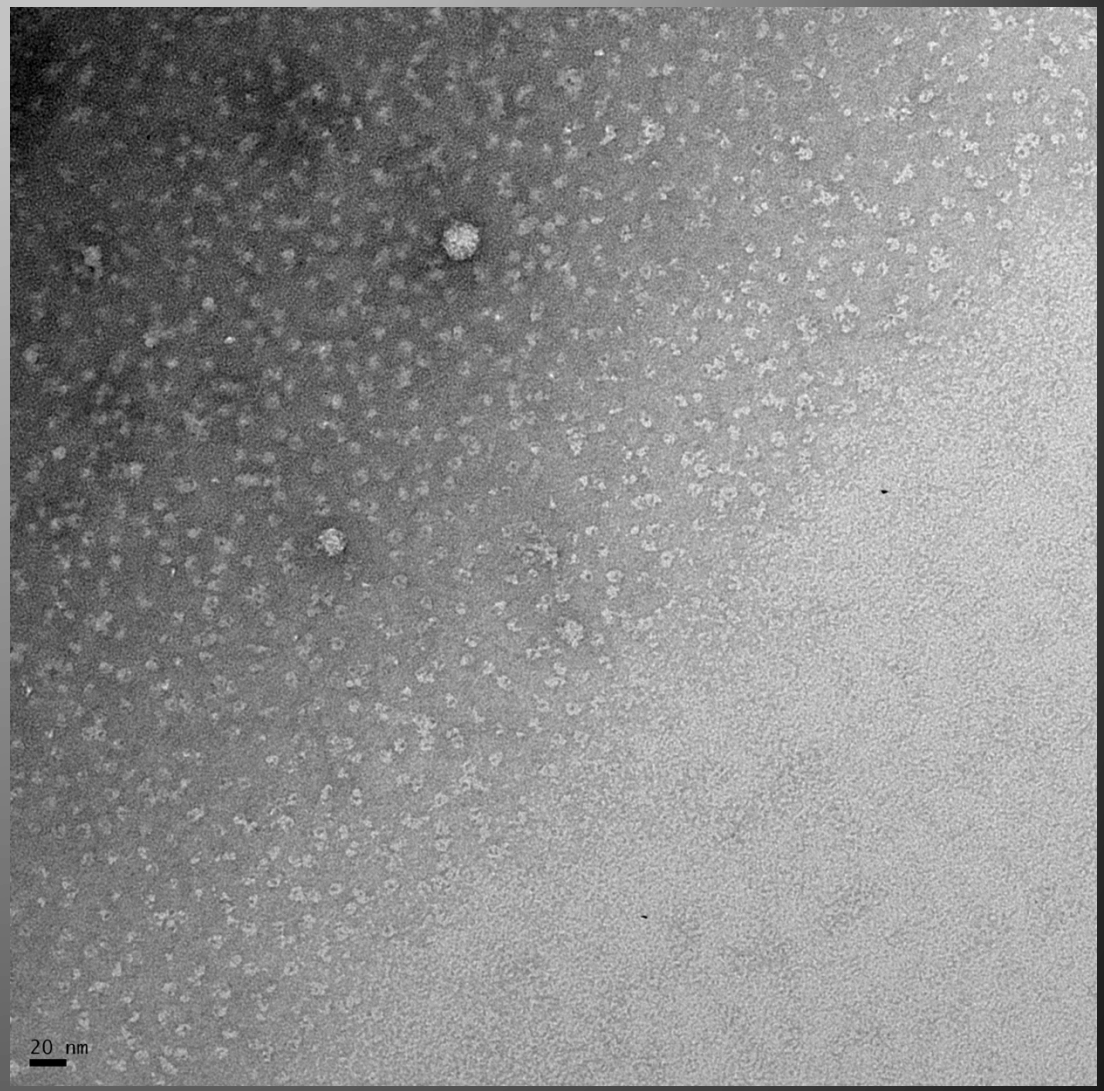


Phages
Collect by CDW on JEOL 1230 TEM

Troubleshooting

Inconsistent Staining

Gradient effect as stain settles and pools unevenly. Staining spans from slightly positive with a dark background (upper left), to stongly stained background (middle band), to pure negative (lower right)



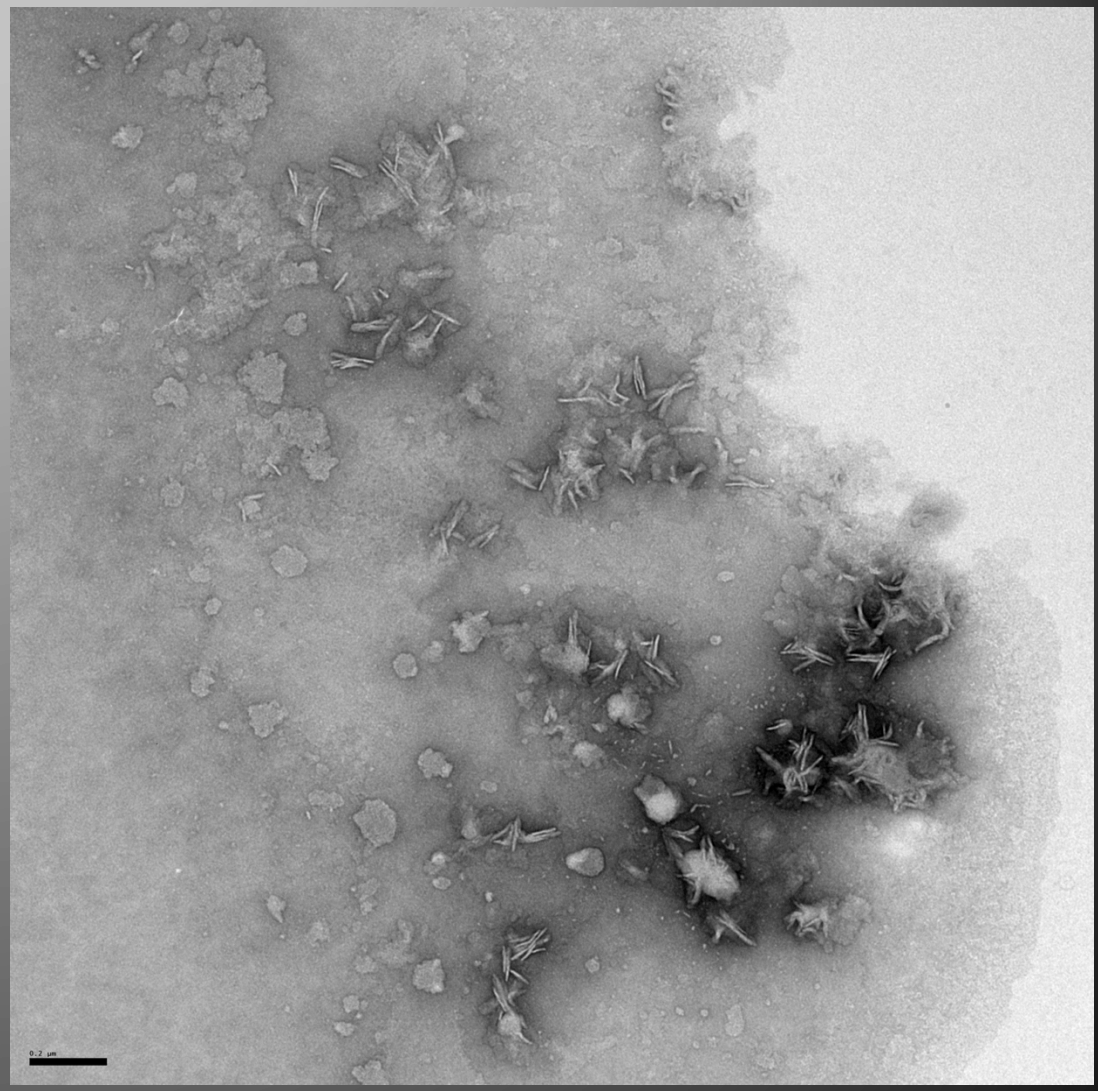
Macro Molecules Collect by CDW on JEOL 1230 TEM

Troubleshooting

Mistaking Buffer Salt for Sample

Work with a high salt concentration sample or insufficiently rinsing sample prior to negative stain can result in the formation of salt crystals on the grid.

No, this is not your sample.



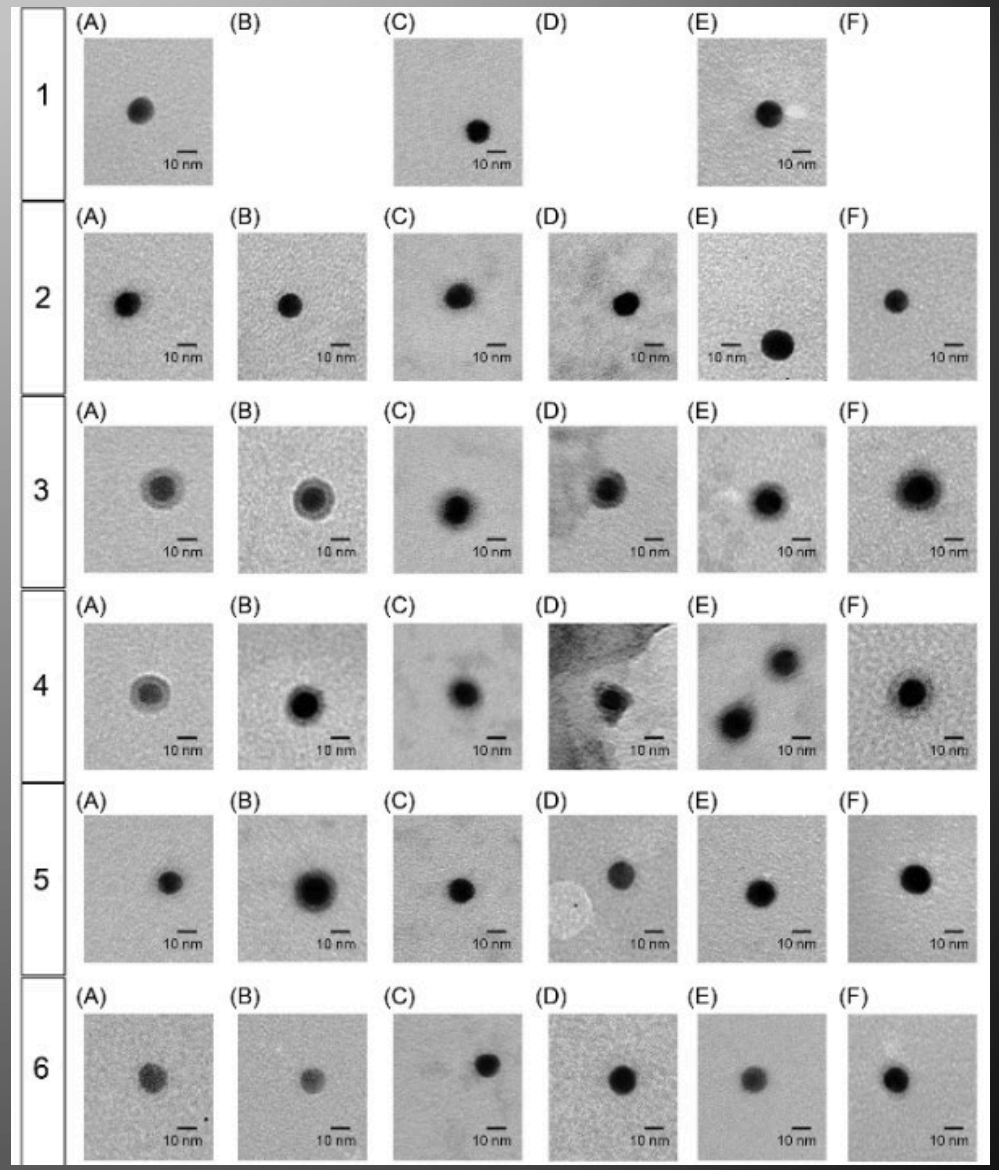
Buffer Salts Collect by CDW on JEOL 1230 TEM

pH influencing data

Gold Nano Particles with Polymer Coating

1) No Staining 2) Uranyl Acetate 3) PTA pH 0.5
4) PTA pH 3.0 5) PTA pH 5.0 6) PTA pH 7.0

Polymer shell invisible
with UA and higher pH PTA

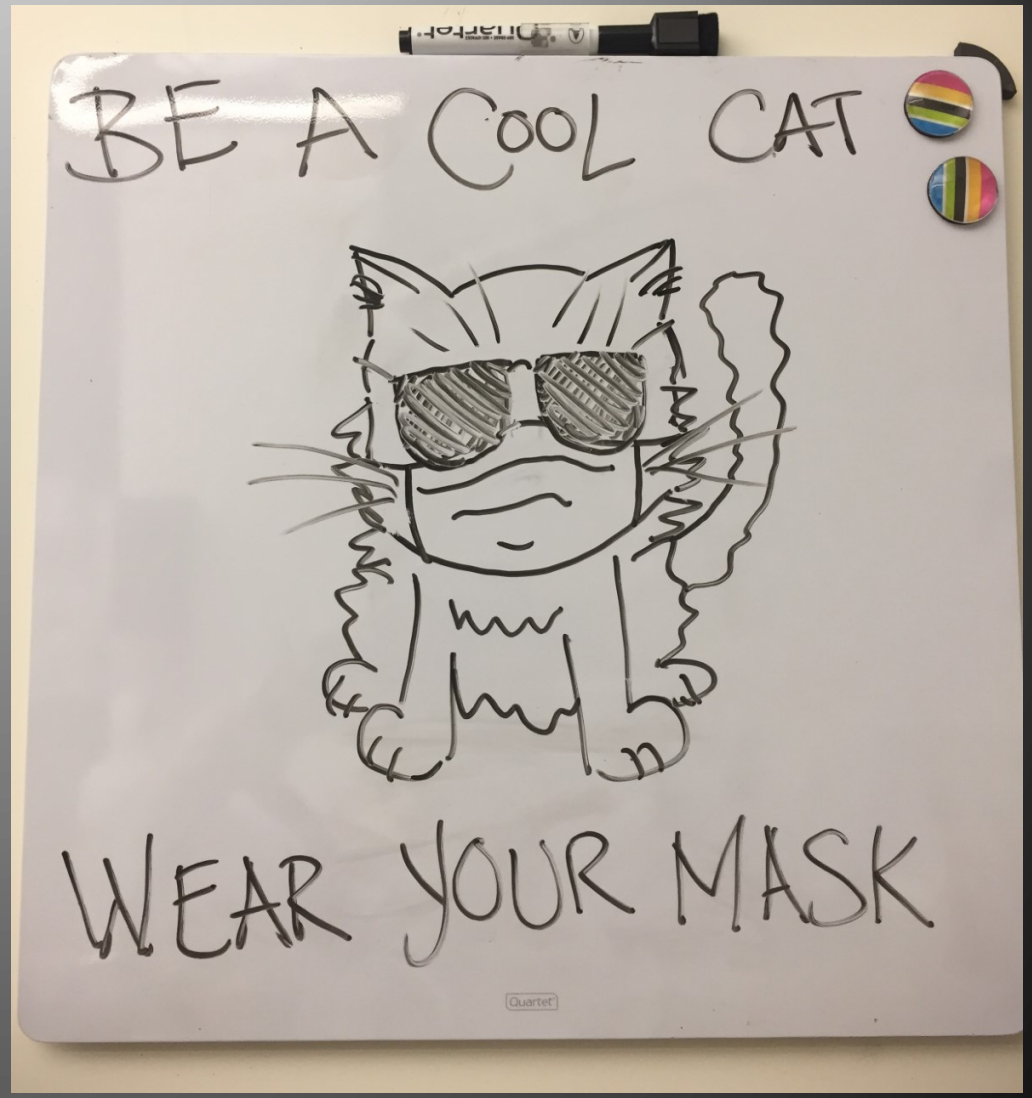


Pyshnaya et al. **"Surprises of electron microscopic imaging of proteins and polymers covering gold nanoparticles layer by layer."** ***Colloids and Surfaces B: Biointerfaces vol. 150, (2017): 23-31. doi: 10.1016/j.colsurfb.2016.11.007***

pH Influencing Morphology

Uranyl Acetate	*pH 4.2-4.5*
Uranyl Formate	*pH 4.0-5.0*
Phosphotungstic Acid (PTA) *(considered positive stain)*	*pH 0.5-8.0*
Ammonium Molybdate	*pH 5.0-7.0*
Methylamine Vanadate (NanoVan)	*pH 8.0*
Methylamine Tungstate	*pH 6.0-7.0*
Sodium Silicotungstate	*pH 5.0-8.0*

Return to the Lab Safety Q&A



COVID Research at BioCryo

- Yes, we are accepting COVID samples (infected tissue and virus particles)
- Virus Particles must be *deactivated* (tissue or VP's 24 hours in Glutaraldyhyde)
- We are not a BSL 2 or BSL 3 lab, -NO LIVE SAMPLES-
- Clear communication is required
- Must disinfect outside of tube/container prior to delivery

Making sense of Reopening

A/B- only access through elevator, Limited capacity in Hogan 5-150 (4 people maximum) and Silverman B555 (2 people in room, 1 at bench)

PPE & Hygiene

- Must wear gloves and mask on equipment
- Wipe down high-touch areas with 70%ETOH and paper towel

Microscope Usage

- Only usable by researchers with 24/7 access
- Reservations made by staff 2 days in advance, 9AM-6PM
- Please, start sessions with your phone
- No Trainings
- 1 Person/Room, Follow Traffic signs