Lets introduce a typical crystallographer …

Wile E. Coyote (Genius)

Overconfidentii Vulgaris

(Cristali Coltivatore Optimista)

And the crystal of interest …

Road Runner
(Beep beep)

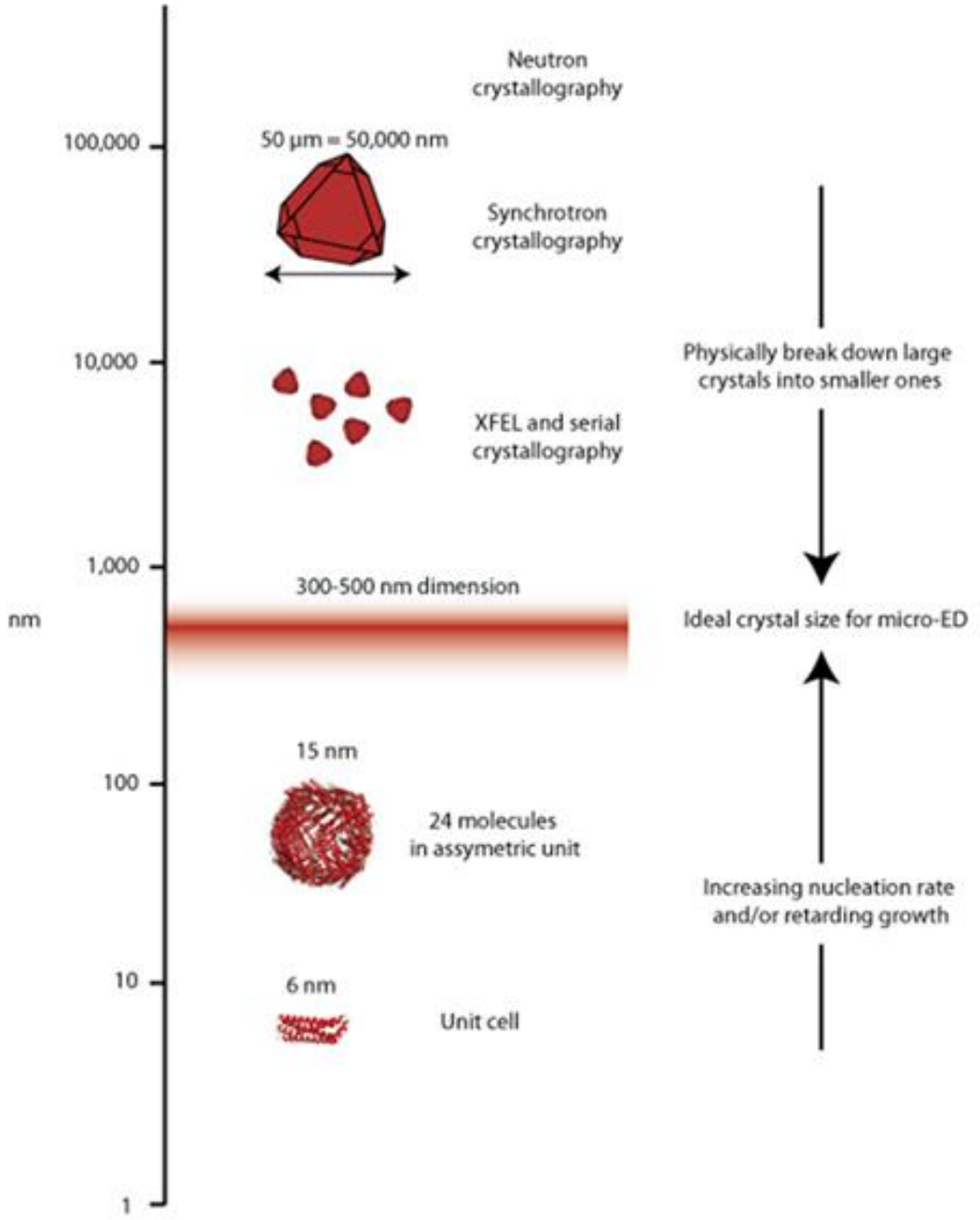
Disappearialis Quickius

(Cristallio Perfetto)

And how the rules of the crystallographer relate to crystallography …

1. **Road Runner cannot harm the Coyote except by going "Beep! Beep!"**
2. **No outside force can harm the Coyote - only his own ineptitude or the failure of Acme products.**
3. **The Coyote could stop anytime - If he was not a fanatic.**
4. **No dialogue ever, except "Beep! Beep!"**
5. **Road Runner must stay on the road - for no other reason than that he's a roadrunner.**
6. **All action must be confined to the natural environment of the two characters -- the southwest American desert.**
7. **All tools, weapons, or mechanical conveniences must be obtained from the Acme Corporation.**
8. **Whenever possible, make gravity the Coyote's greatest enemy.**
9. **The Coyote is always more humiliated than harmed by his failures.**
10. **The audience's sympathy must remain with the Coyote.**

1. **The crystal cannot harm the crystal grower except by not diffracting.**
2. **No outside force can harm the crystal grower - only their own ineptitude or the failure of Hampton research products.**
3. **The crystal grower could stop anytime - If they were not a fanatic.**
4. **No dialogue ever from the crystal.**
5. **The crystal will be on the path between precipitate and clear - for no other reason than it's a crystal.**
6. **All reactions must be confined to the natural environment of the crystal.**
7. **All tools, weapons, or mechanical conveniences must be obtained from Hampton Research.**
8. **Whenever possible, make salt crystals the crystal grower's greatest enemy.**
9. **The crystal grower is always more humiliated than harmed by his failures.**
10. **The audience's sympathy must remain with the crystal grower.**



Getting the ideal crystal

The ideal depends on the need

Typically, synchrotron X-ray data can work with crystals that are a few microns in the smallest dimension.

Tens of microns or larger are useable at any synchrotron source. When size approaches hundreds of microns or more, neutron techniques become available.

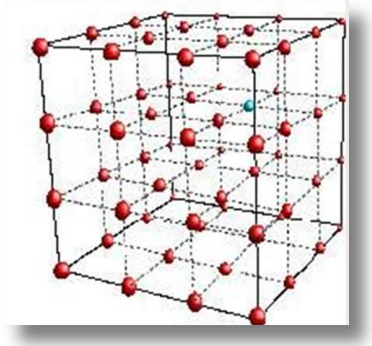
Smaller crystals can be used for serial crystallography at X-ray free electron laser (XFEL) sources, or if the crystal size is below 500 nm, for micro-electron diffraction.

For samples that will not crystallize, above ~60 kDa they can be studied by cryo-electron microscopy (Cryo-EM), and below ~30 kDa, Nuclear Magnetic Resonance (NMR).

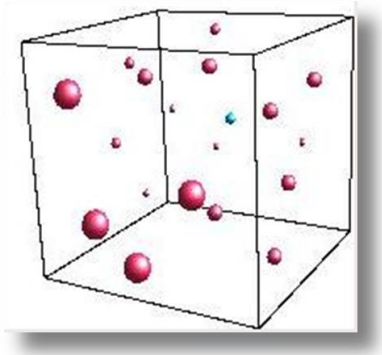
Start with the protein

- A protein that is pure and stable will crystallize more reproducibly and is more likely to provide a structure.
  - Aim for soluble, stable, monodisperse, active, and prepared in minimal buffer.
- Chemicals can stabilize a protein.
  - Ligands, co-factors, and salts all help (can be tested with a melting temperature assay – Nature Protocols 2(9) 2007).
  - Reducing agents (TCEP is the longest lasting) can stabilize the protein – Bollag et. al, Protein Methods, Wiley-Liss Publishing 1996).
  - Protease inhibitors.
  - Chelating agents (EDTA).
  - Buffers help control surface charge distribution.
  - Detergents can attach to hydrophobic patches.
  - Preservatives can stop microbial growth.
  - Glycerol can help storage and aid with later cryoprotection.
- You can significantly increase the maximum concentration, and stability of a protein sample using the optimum buffer.
  - Maximizing solubility can improve crystallization success (Many papers – Acta Cryst D62, 833-842, 2006).
  - Use a pre-crystallization test (Watson and O'Callaghan, Acta Cryst F61, 2005 – sold by Hampton Research).
- Storage is important.
  - Do not lyophilize the sample. If it is stable at room temperature or refrigerated, don't freeze the sample, if you must feeze it, freeze and thaw quickly and prepare small aliquots to minimize freeze-thaw cycles (Deng, Acta Cryst. D60, 203-204, 2004).

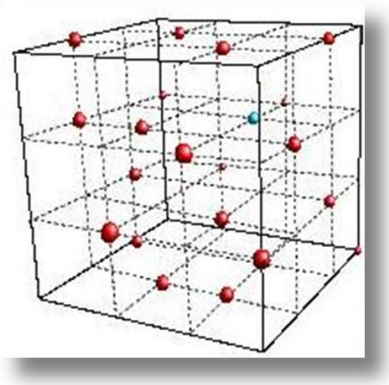
Know how your crystallization screens work



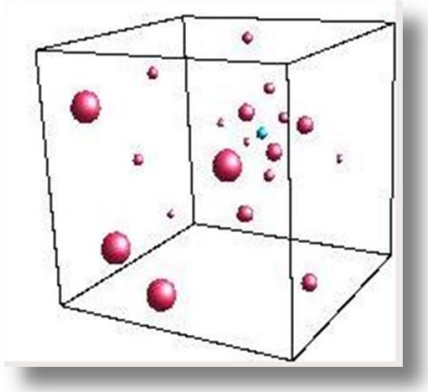
Full matrix – samples everything, inefficient



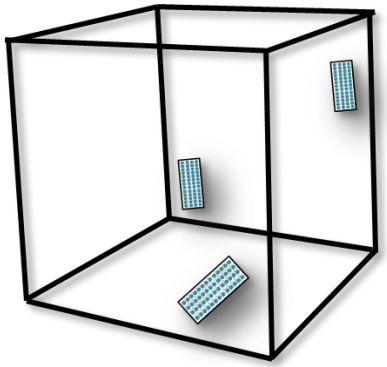
Random – misses regions, does not reduce the number of variables, reduces the number of experiments.



Incomplete factorial – samples everything evenly, does not reduce the number of variables, and reduces the number of experiments by efficient sampling. .



Sparse matrix – look where others had success, reduces the number of variables and experiments but will miss new conditions.



Grid – complete sampling, typically of few variables, samples a small region of space, generally used for optimization.

Each type of screen requires different considerations for the interpretation of results.

Segelke, B. Journal of Crystal Growth, 232, issue 1-4, 553-562, Cox & Weber Journal of Crystal Growth, 90, Issues 1-3, 318-324

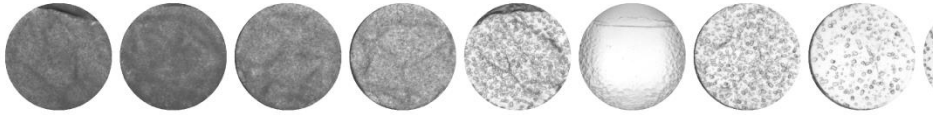
Know your solubility chemistry – Precipitating agents

- Salts – linear impact
  - Anions follow the Hofmeister series for effective precipitation.
  - SO_4^{2-}> HPO_4^{2-} >CH_3CO^{2-}> citrate^{3-} > tartrate^{2-} > HCO_3^- > CrO_3^- > Cl^- > NO_3^- > ClO_3^- >SCN^-
  - The pI of the protein relative to the pH of the solution can reverse order.
  - Chatropic salts unfold proteins and expose interior residues (not commonly used as precipitating agents).
  - Non-chaotropic salts (salting out) preferentially bind water and mask charges between proteins.
- Polymers – logarithmic impact
  - Polyethylene glycol (PEG)
    - Ranges to molecular weight from 200 to over 1 million
    - Traps water and makes it unavailable to the protein
    - At room temperature, PEGs of molecular weight <900 are liquid, a 1000 or greater are solid.
    - Low molecular weight PEG acts as a cryoprotectant.
  - 2-Methyl 2,4-pentane diol (MPD)
    - Properties midway between low molecular weight PEGs and organic solvents.
    - Lowers the chemical activity of water through hydrogen bonding and reduces electrostatic screening of solvent.
    - Particularly effective for nucleic acid crystallization.

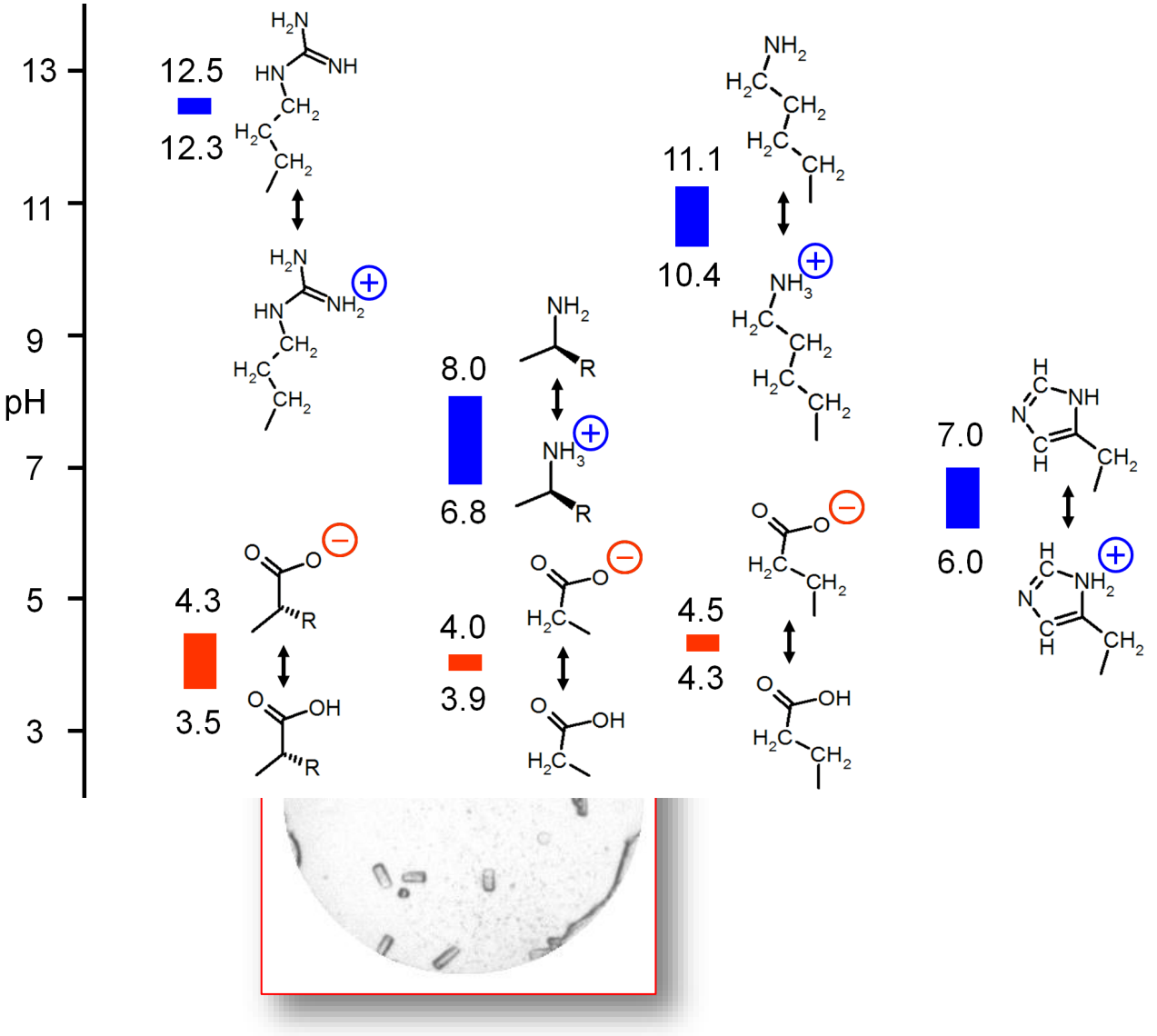
Critical variables – pH, temperature, and ratio

77% protein volume

23% cocktail volume



Luft, et al. (2007). Efficient Optimization of
Drop Volume Ratio and Temperature. Prote



18 %(w/v) PEG 4000

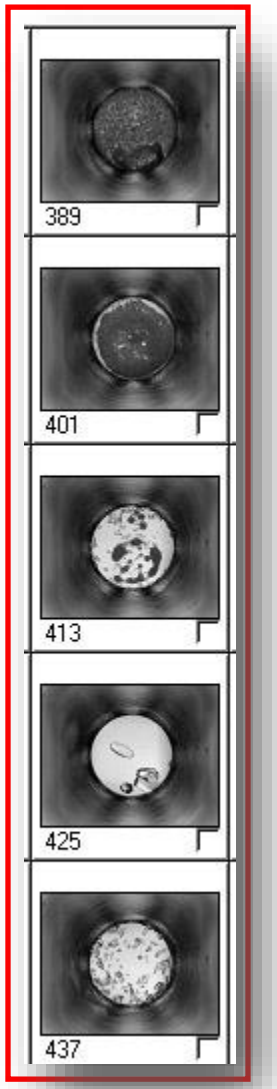
pH 4.50

4.60

4.75

4.90

5.00



pH 4.50

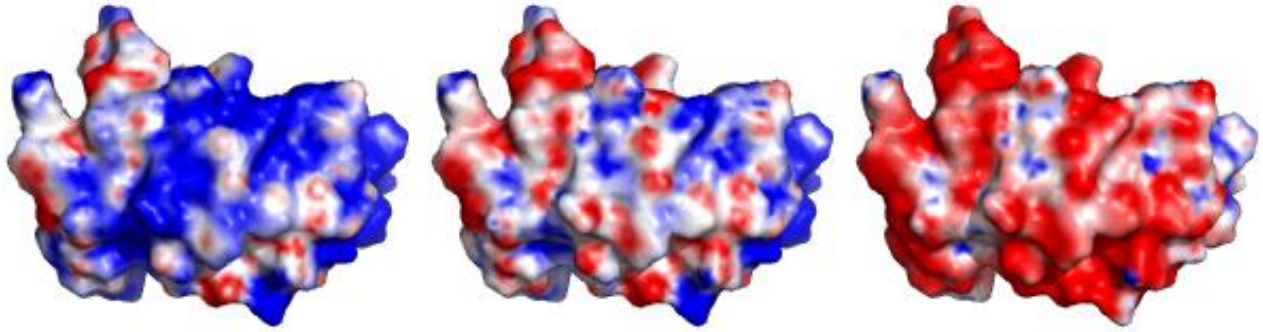
pH 4.60

pH 4.75

pH 4.90

pH 5.00

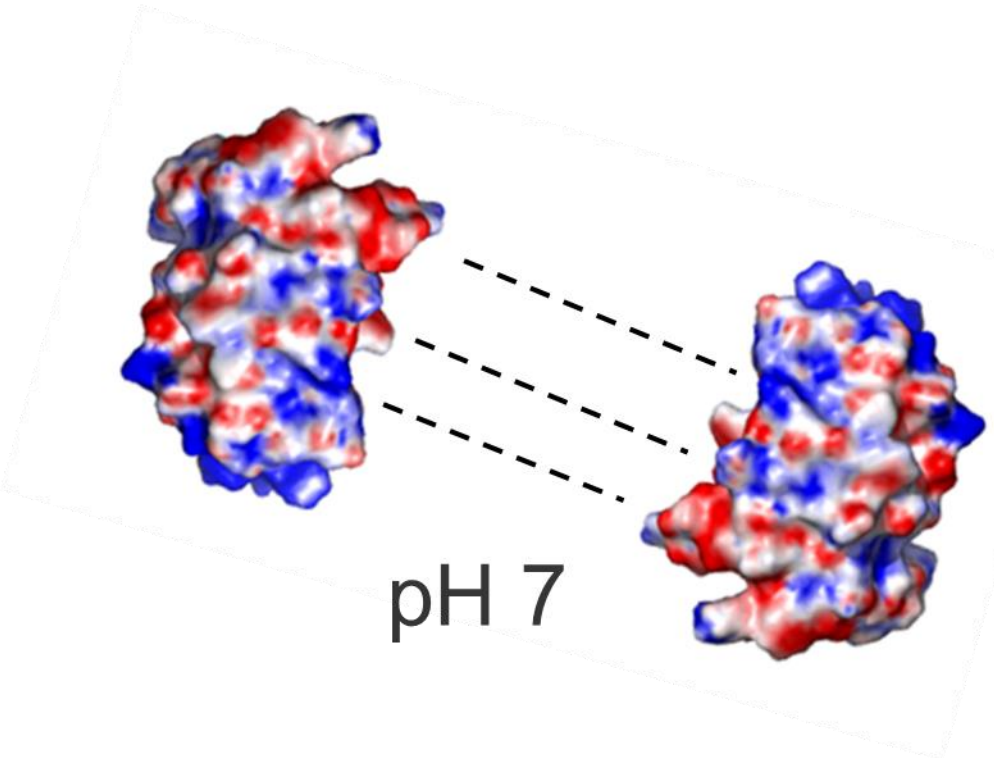
Small pH changes can have big effects



pH 4 pH 7 pH 11

pH < pI
Positive
net charge

pH > pI
Negative
net charge



If the pH is less than the pI, then the protein will have a net positive surface charge

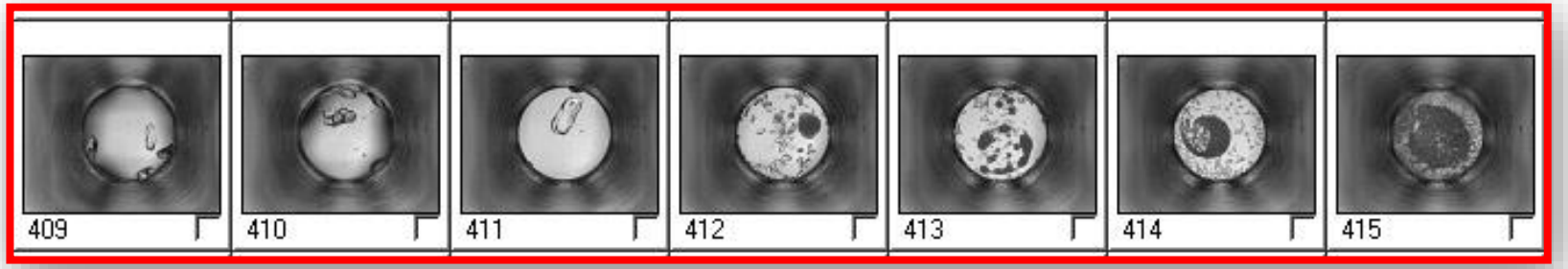
If the pH is greater than the pI, then the protein will have a net negative surface charge

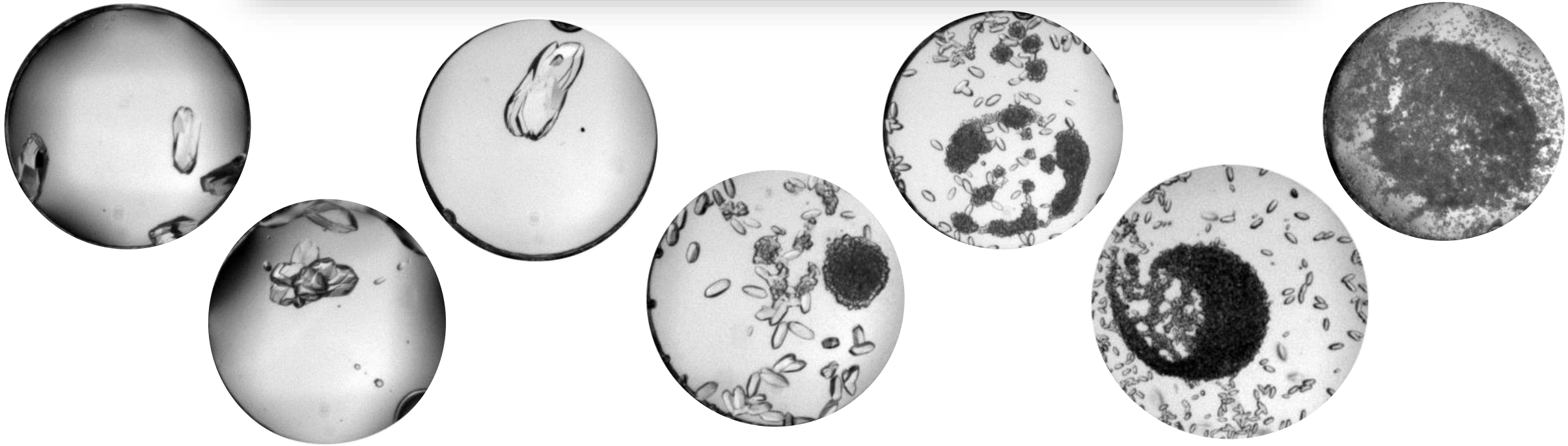
1Z66 domain III of the E protein of tick-borne Langat flavivirus, pI 7.01

%(w/v) PEG 4000

10 12 14 16 18 20 22

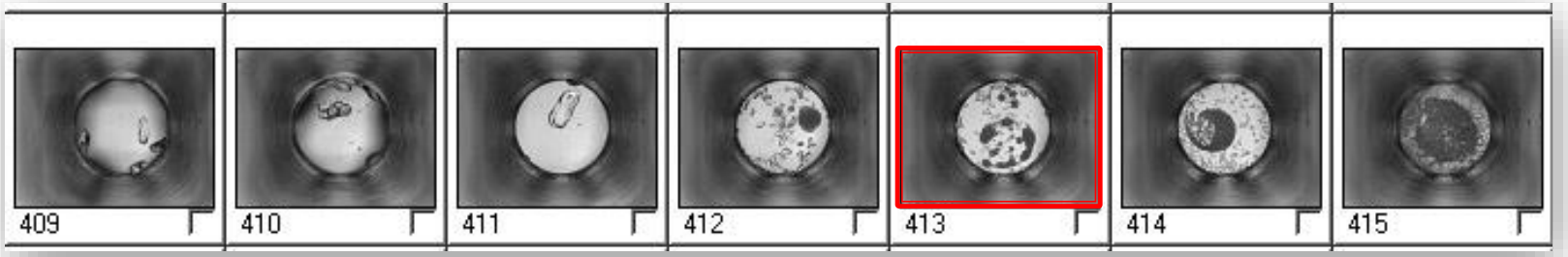
pH 4.75





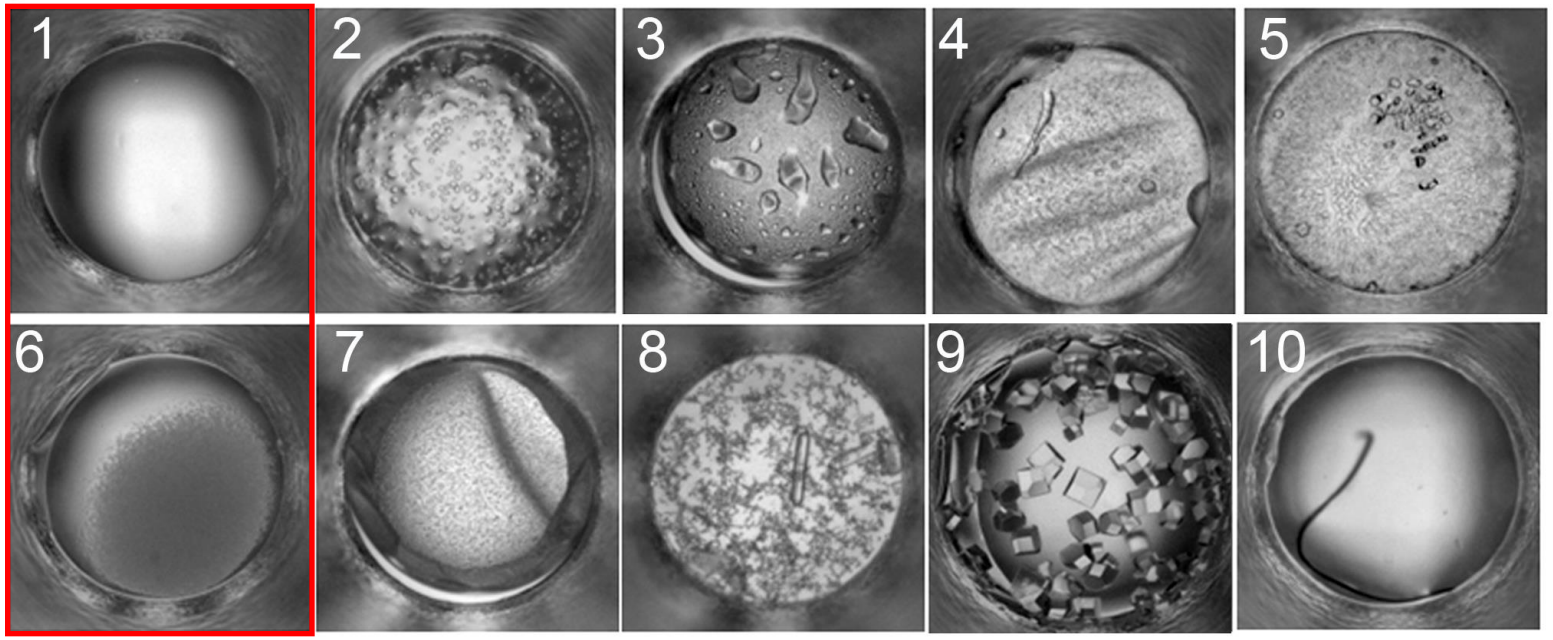
Small changes in PEG concentration can have big effects

%(w/v) PEG 4000

	10	12	14	16	18	20	22
pH 4.75							

18% (w/v) PEG 4000

Typical crystallization experiments' outcomes

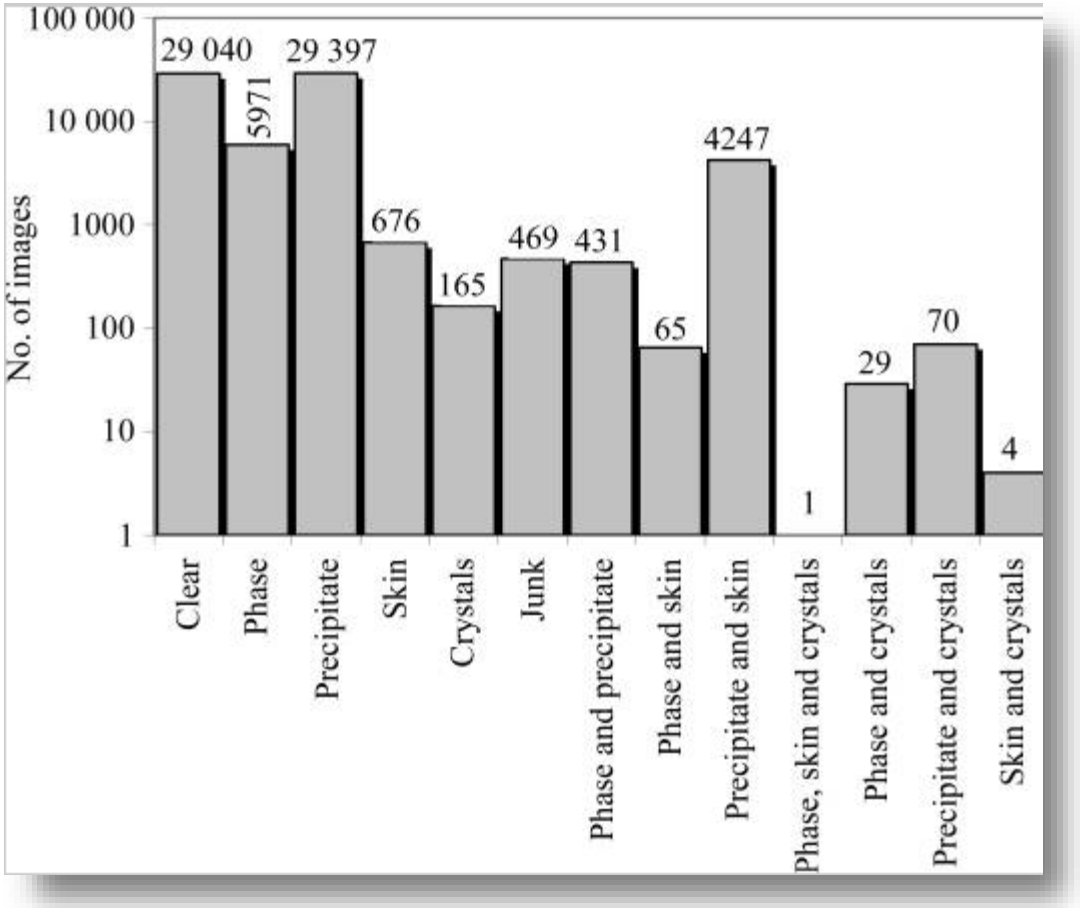


1 clear	~83%	6 precipitate
2 phase separation		7 precipitate + skin
3 phase separation + precipitate		8 precipitate + crystal
4 phase separation + skin		9 crystal
5 phase separation and crystal		10 garbage

Acta Cryst 2008 November 1; D64(Pt 11): 1123–1130.

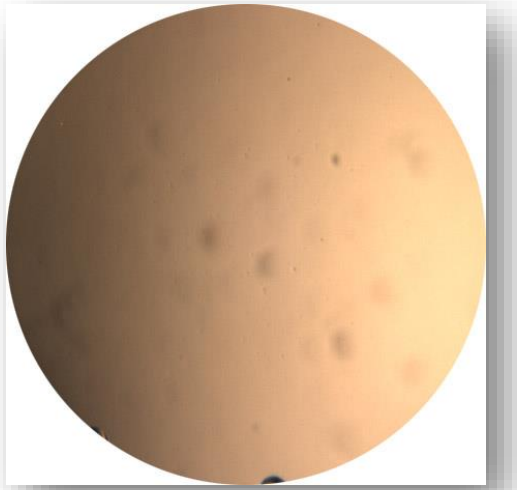
The frequency of outcomes for 70,565 experiments

(96 different proteins x 1536 conditions/screen)



Acta Cryst 2008 November 1; D64(Pt 11): 1123–1130.

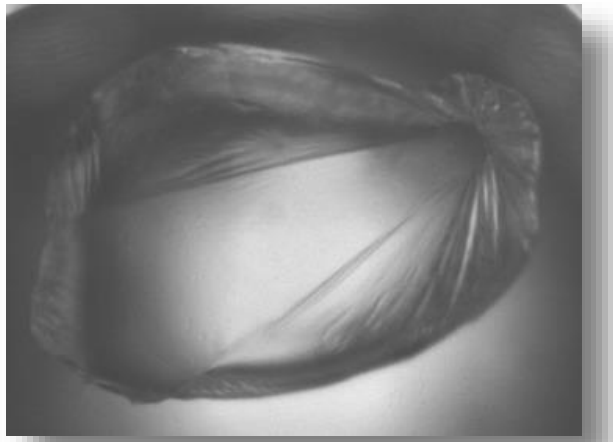
Clear drops are informative



pH range of clear drops						
pH<5	5≤pH<6	6≤pH<7	7≤pH<8	8≤pH<9	9≤pH<10	pH≥10
42	**47**	**94**	**138**	**59**	**71**	**35**
19.2%	**23.5%**	**30.7%**	**36.0%**	**48.8%**	**33.0%**	**38.0%**

Skin formation

- A tough, plastic-like layer of denatured protein
  - Can be sticky like a spider's web
- Does not preclude crystal formation
- Easily mistaken for crystals
- Forms in batch and vapor diffusion experiments

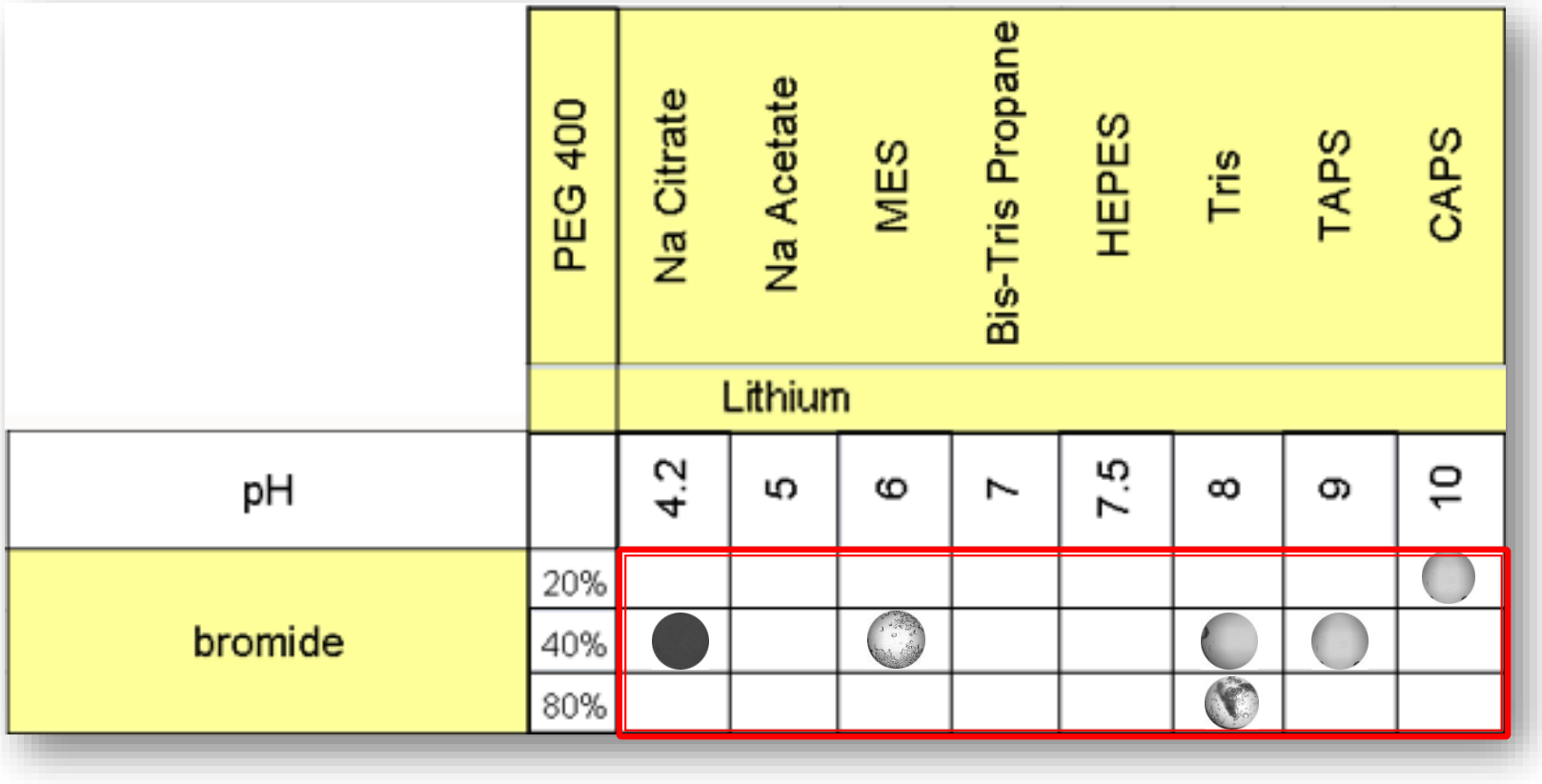




Phase separation: the protein is concentrated into hydrophobic droplets

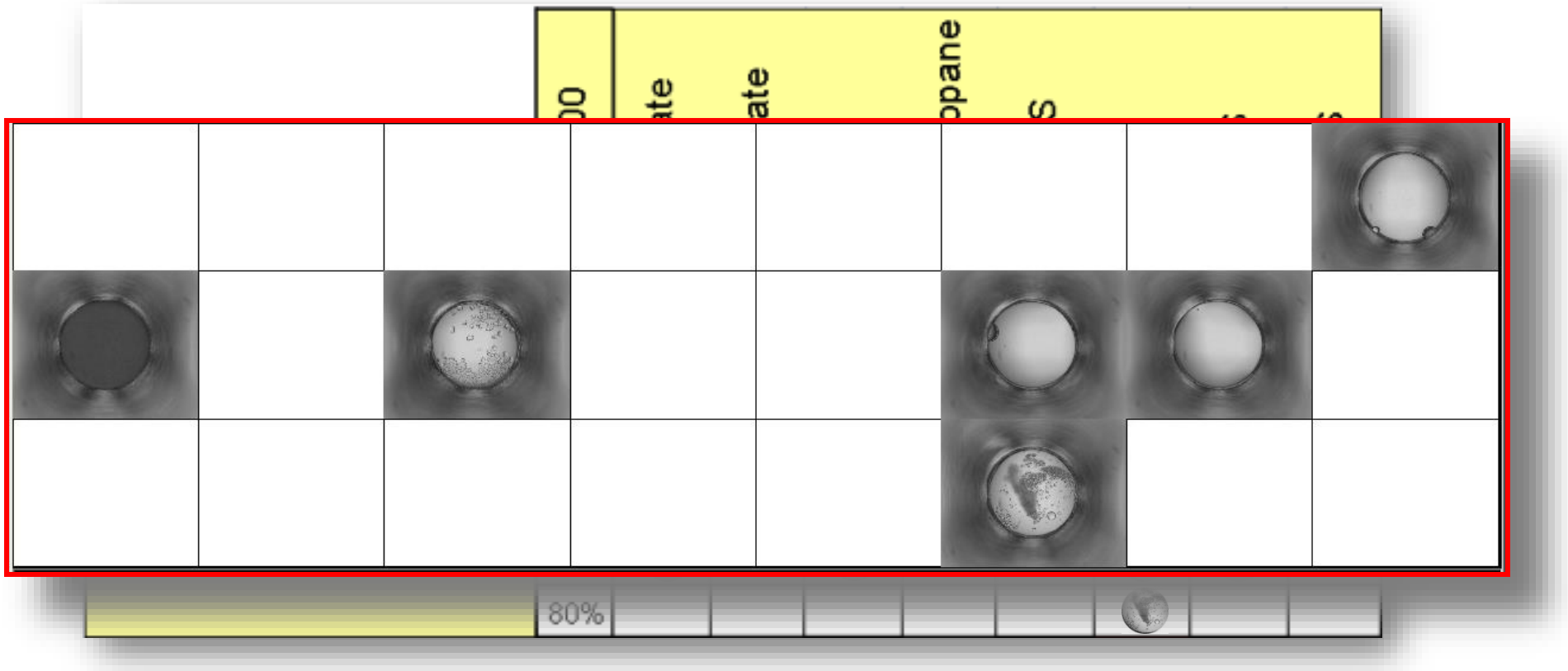
Consider the chemical design of the crystallization experiments and their respective outcomes as two different sets of data.

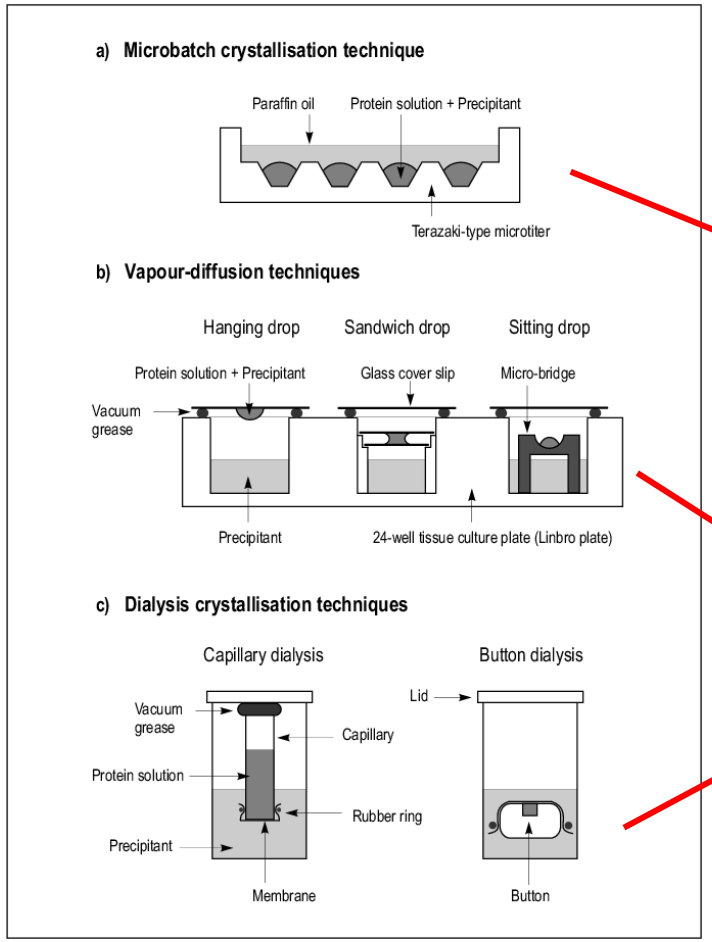
The data is related but distinct.

Place the *protein's reaction to the cocktails* on this coordinate system



There is a reaction between the protein and the different cocktails





Different techniques traverse the crystallization space differently.