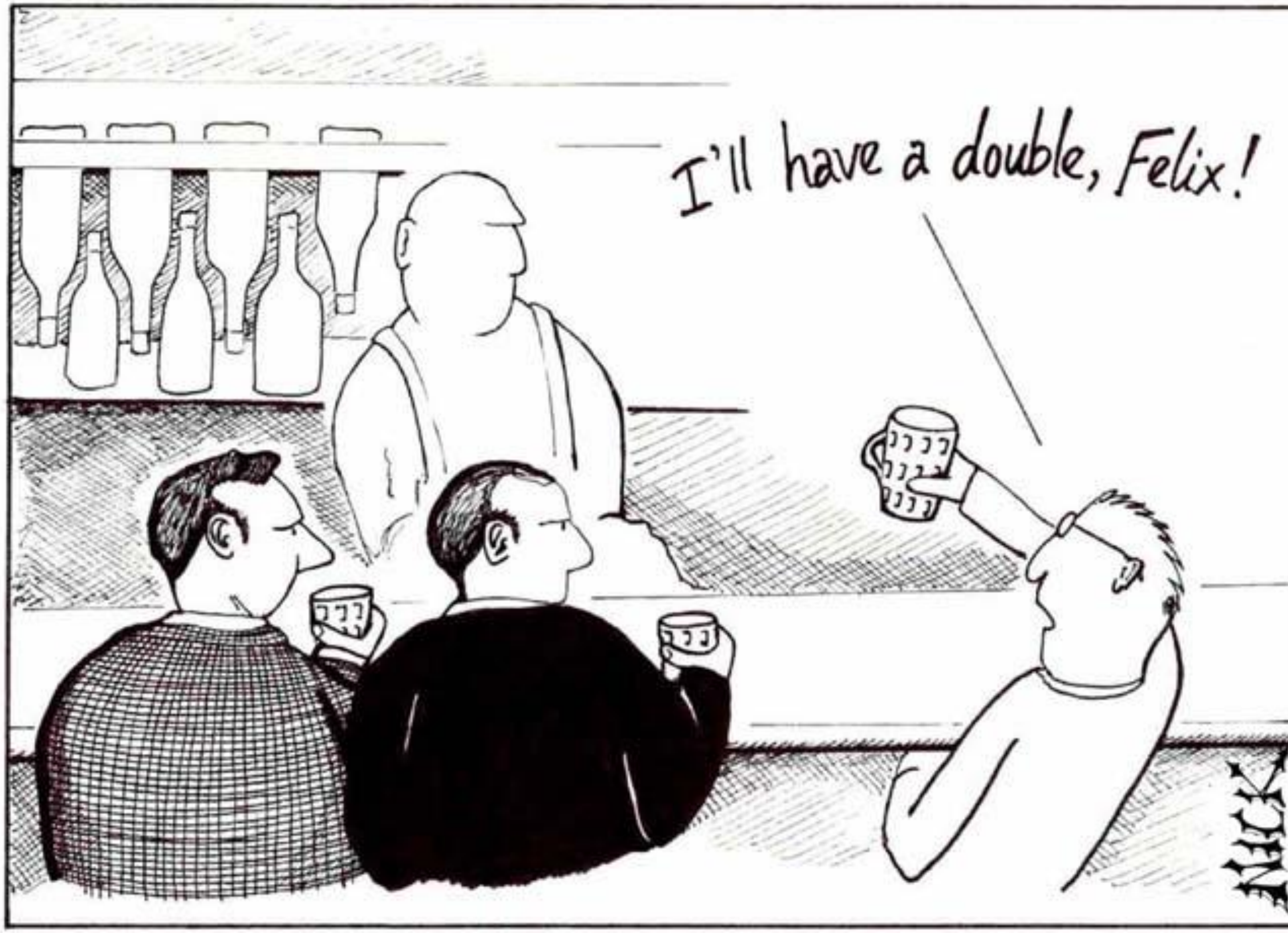
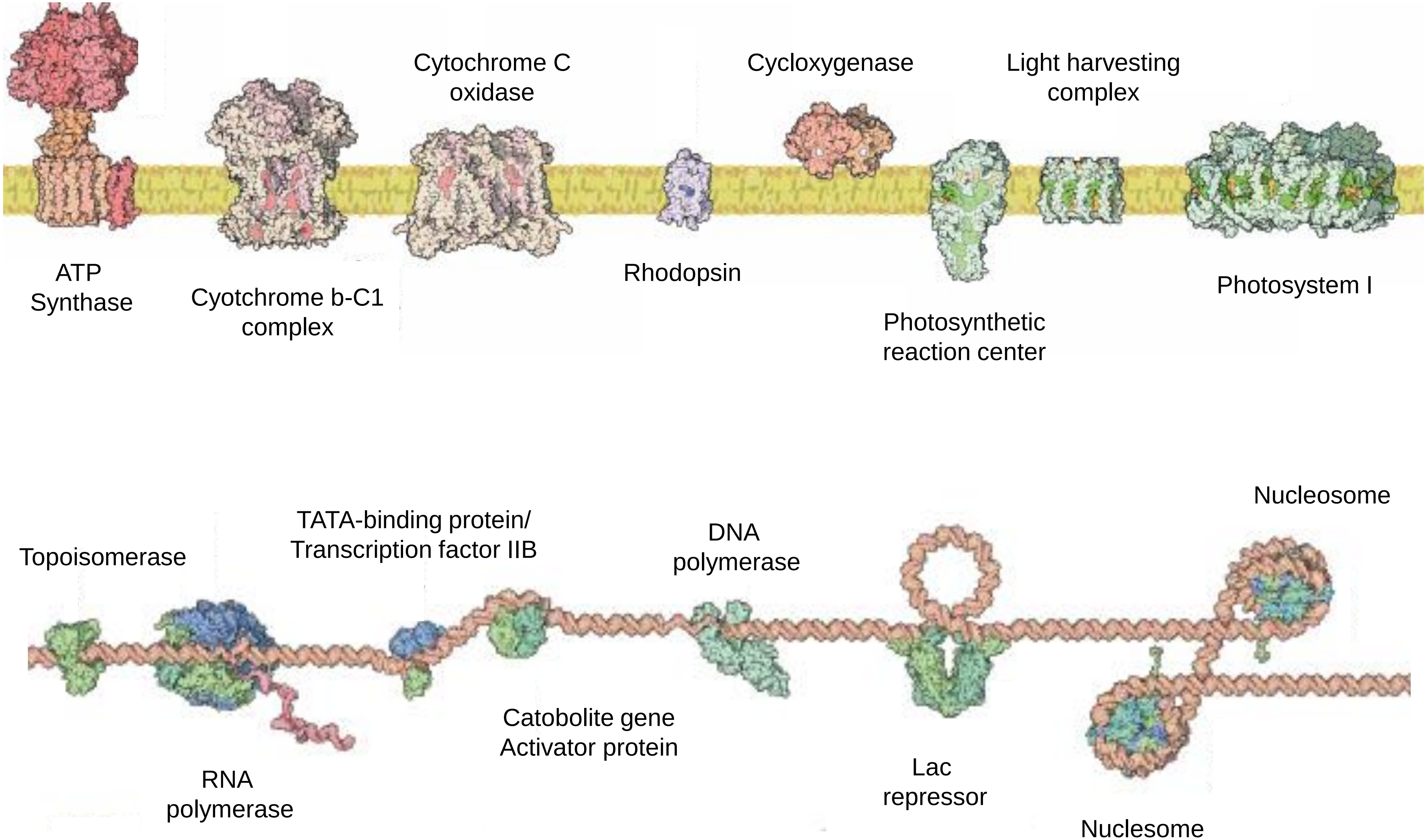
‘Where to go next and not loose ‘all’ your hair’

Edward Snell



Cambridge, 1953. Shortly before discovering the structure of DNA, Watson and Crick, depressed by their lack of progress, visit the local pub.



Form (or structure) gives a **clue** to the function.

Adapted from Molecular Machinery: A tour of the Protein Data Bank, http://www/rcsb/org


SR-71

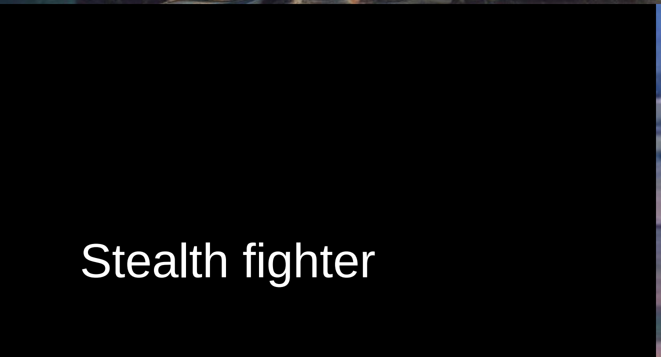

Space shuttle


B52

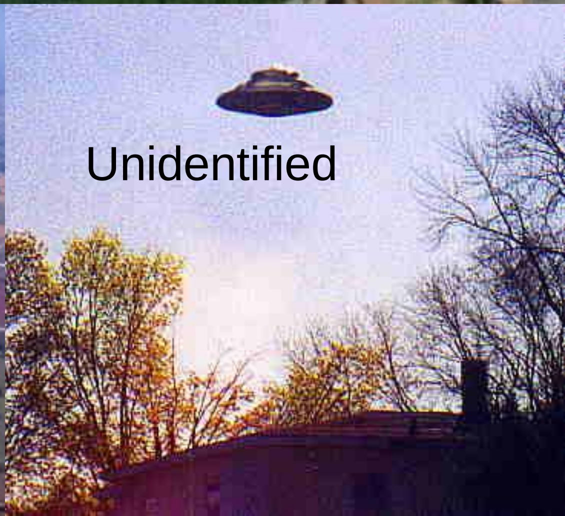

Hot air balloon


Hang-glider


Biplane


Stealth fighter


Spitfire


Unidentified

Sleek,
very mobile?
Only flies
vertically?
Excretes numerous droppings
Needs others for
reproduction
Symbiotic relationship?
Two wings, must fly
really high?
Nothing there,
false observation?
False eyes to scare predators
Still unidentified

Crystallographic information provides an average picture of the macromolecule

Biological information is also needed to explain that picture.

The research proposal

Aim 1.

Aim 2.

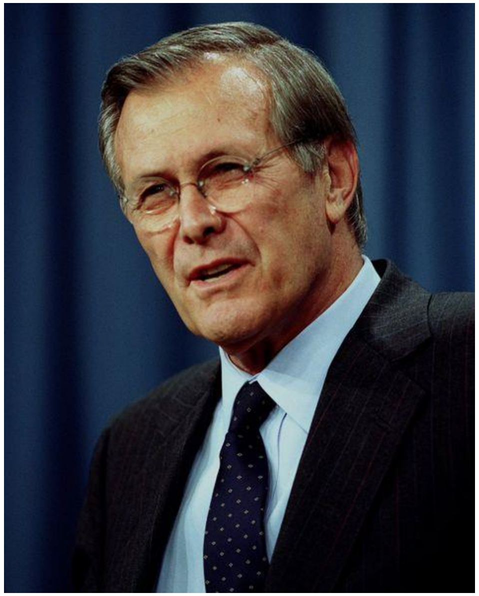
80% of crystallization is failure

Failure …

- I have not failed. I've just found 10,000 ways that won't work – Thomas Edison
- Failure is instructive. The person who really thinks learns quite as much from his failures as from his successes – John Dewey
- A life spent making mistakes is not only more honorable but more useful than a life spent in doing nothing – George Bernhard Shaw.
- Genius is one percent inspiration and ninety-nine percent perspiration – Thomas Edison.
- Crystallization is one percent inspiration and ninety-nine percent optimization – Unknown crystal grower.
- **Rule 2 – Be an optimist.**
- Garbage In, Garbage Out – From a syndicated article about the first stages of computerisation of the US Internal Revenue Service that appeared in several US newspapers on 1 April 1963.

The Essence of Crystallization

- There are known knowns. These are things we know that we know. There are known unknowns. That is to say, there are things that we know we don't know. But there are also unknown unknowns. There are things we don't know we don't know.

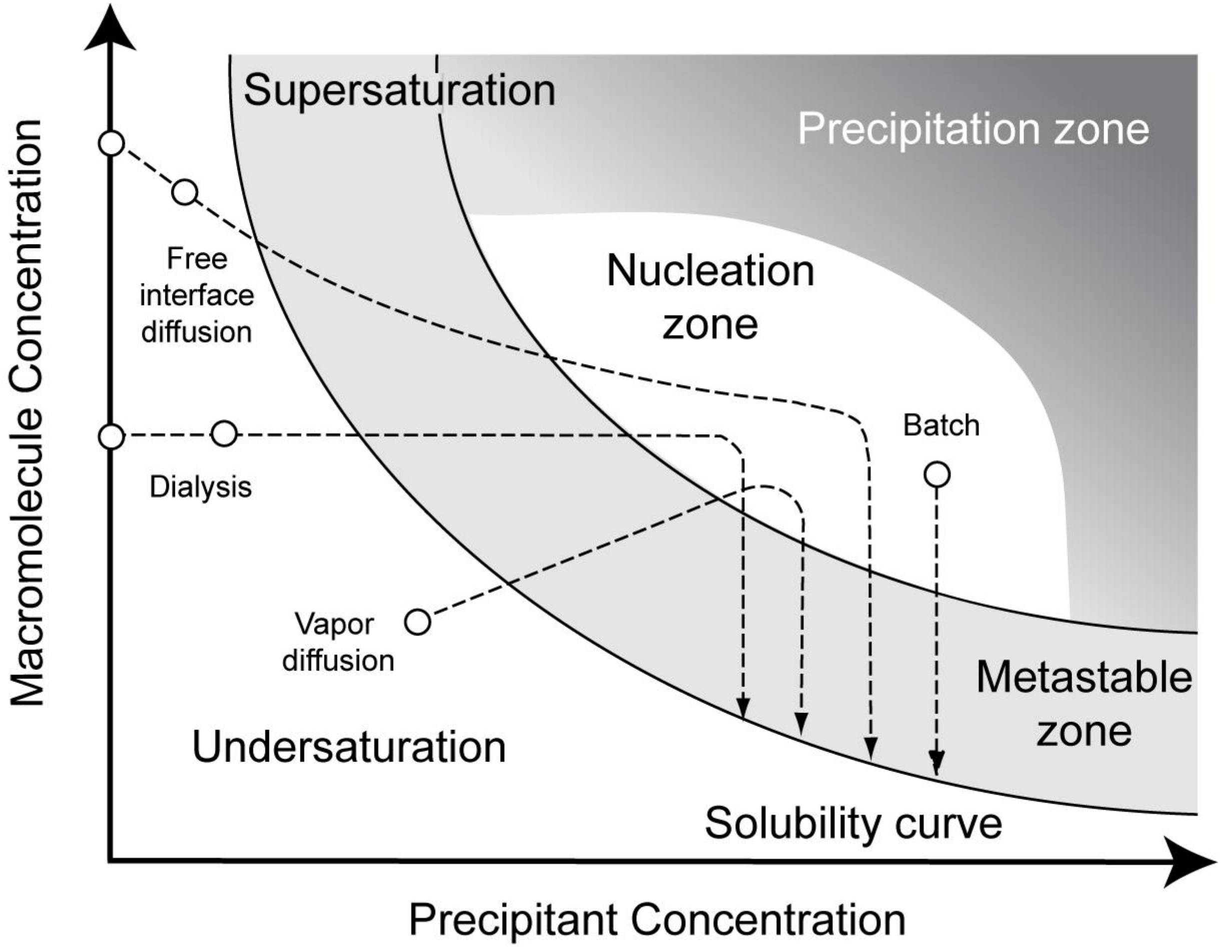


- Donald Rumsfeld, Feb 12th 2002.

- **Rule 3 – Write everything down.**

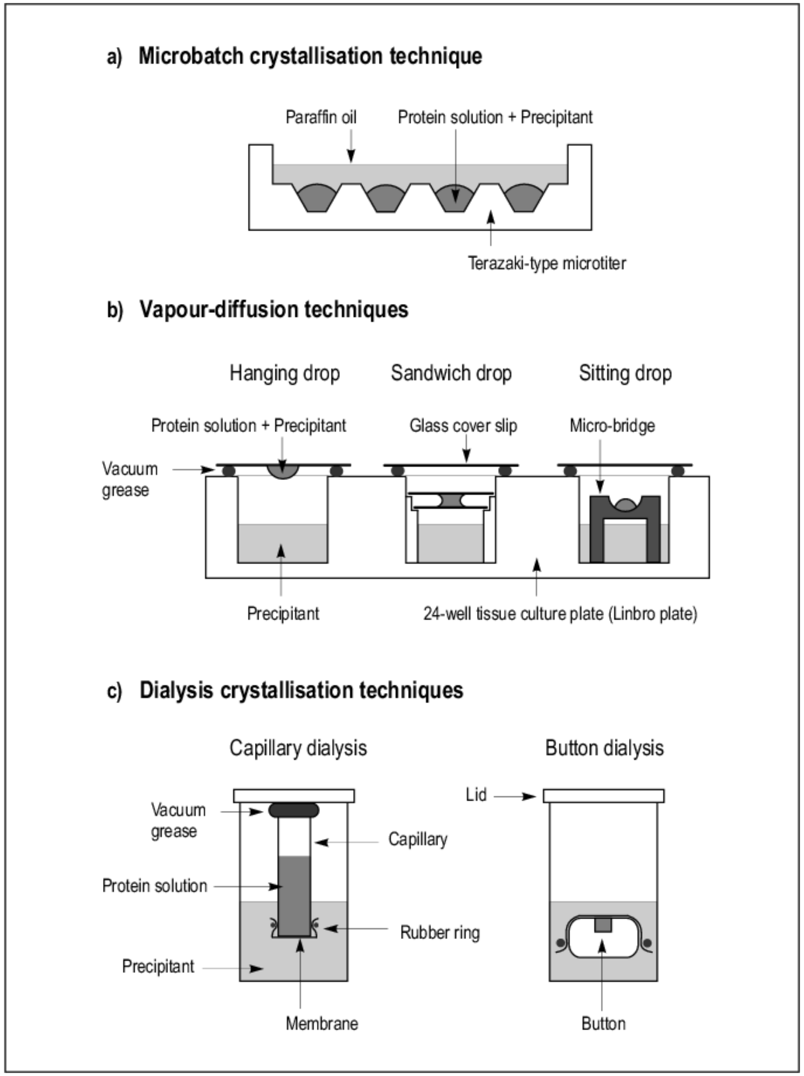
Shamelessly copied from a slide by Ted Baker

Simplified phase diagram for crystallization



- **Rule 4 – Know this diagram by heart.**

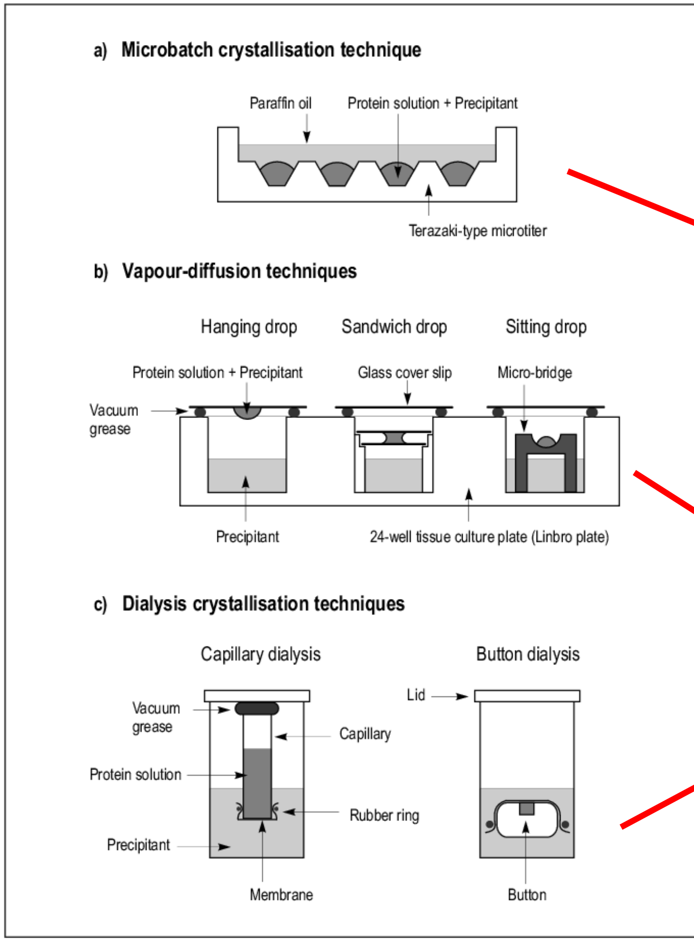
Crystallizing Macromolecules



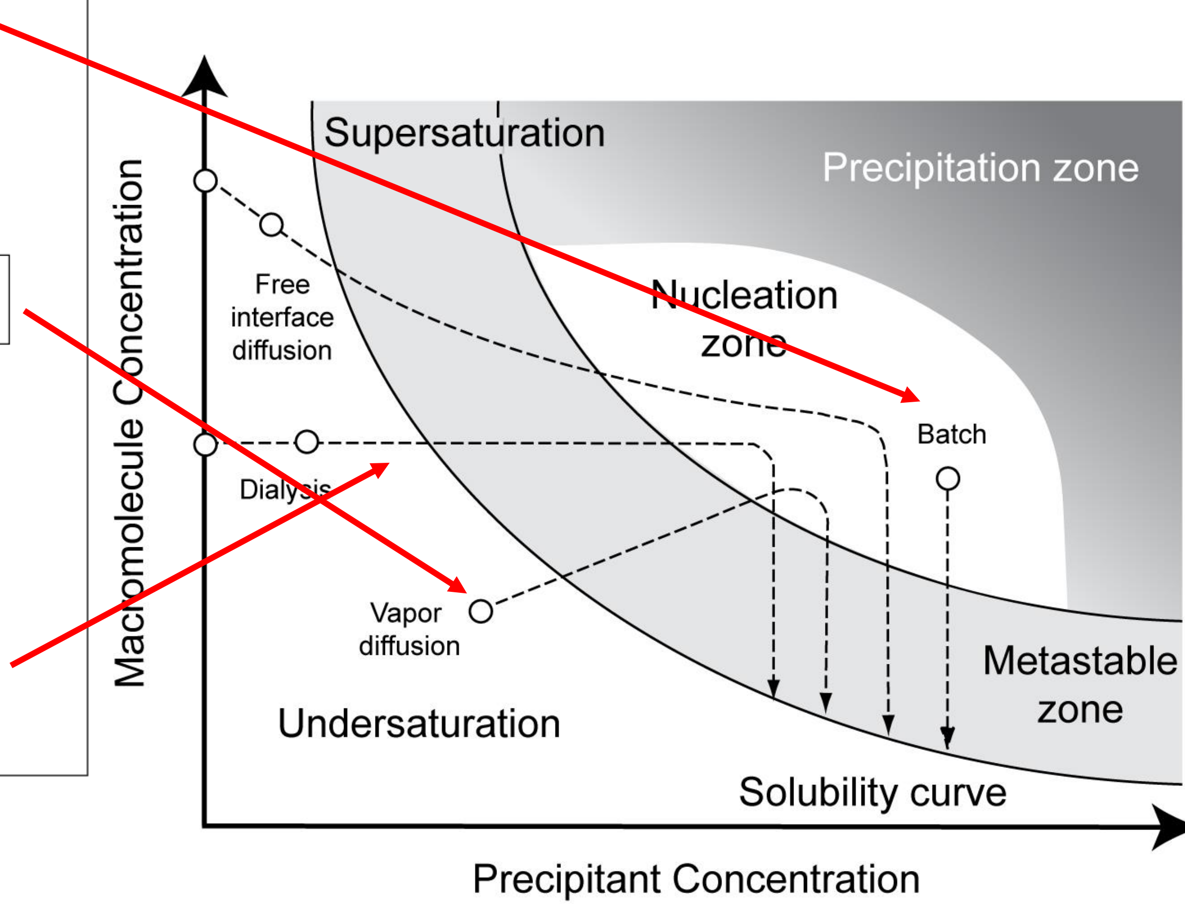
Many different methods but they all have things in common:

- They are designed to traverse the crystallization phase diagram.
- They use many different kinds of solutions to sample crystallization space at many points.

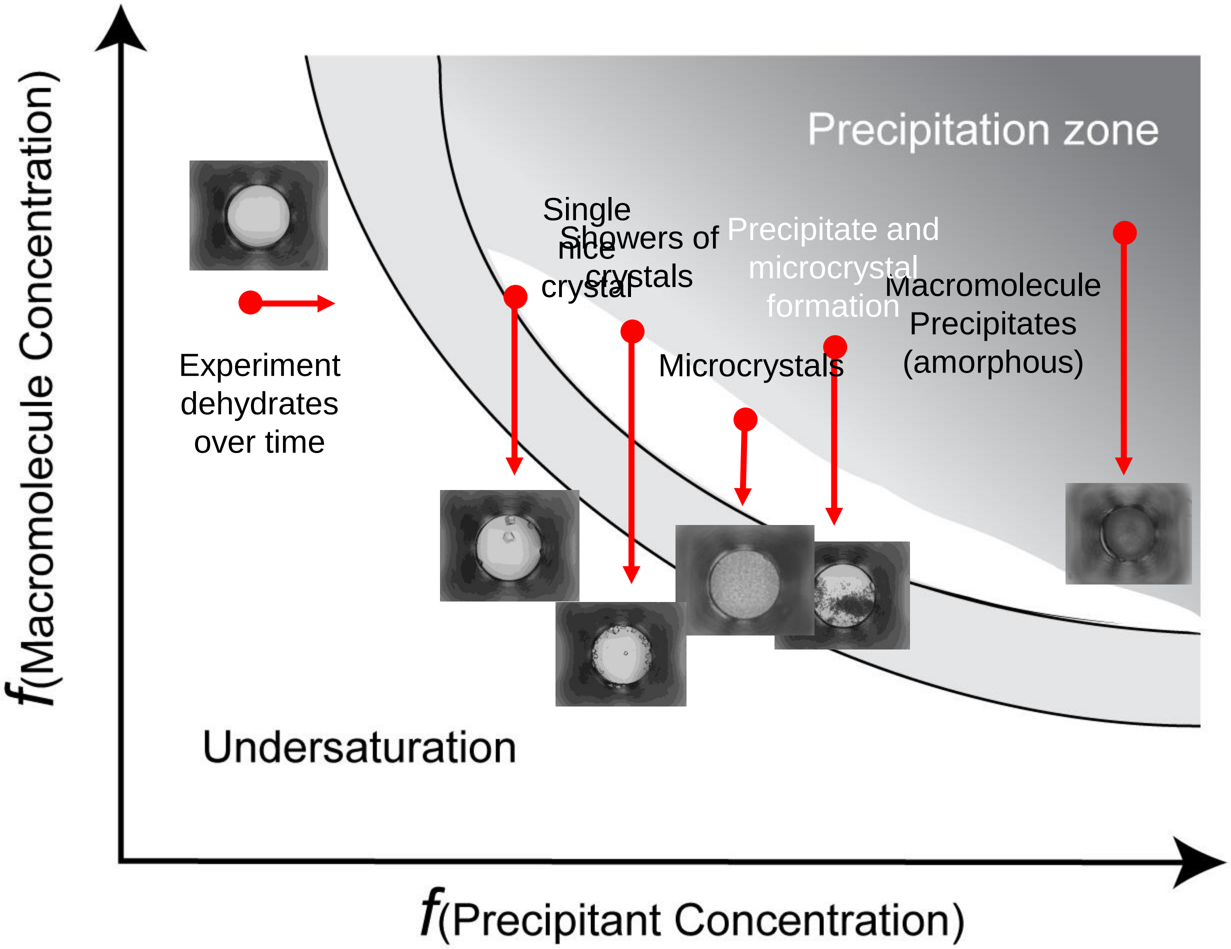
Simplified phase diagram for crystallization



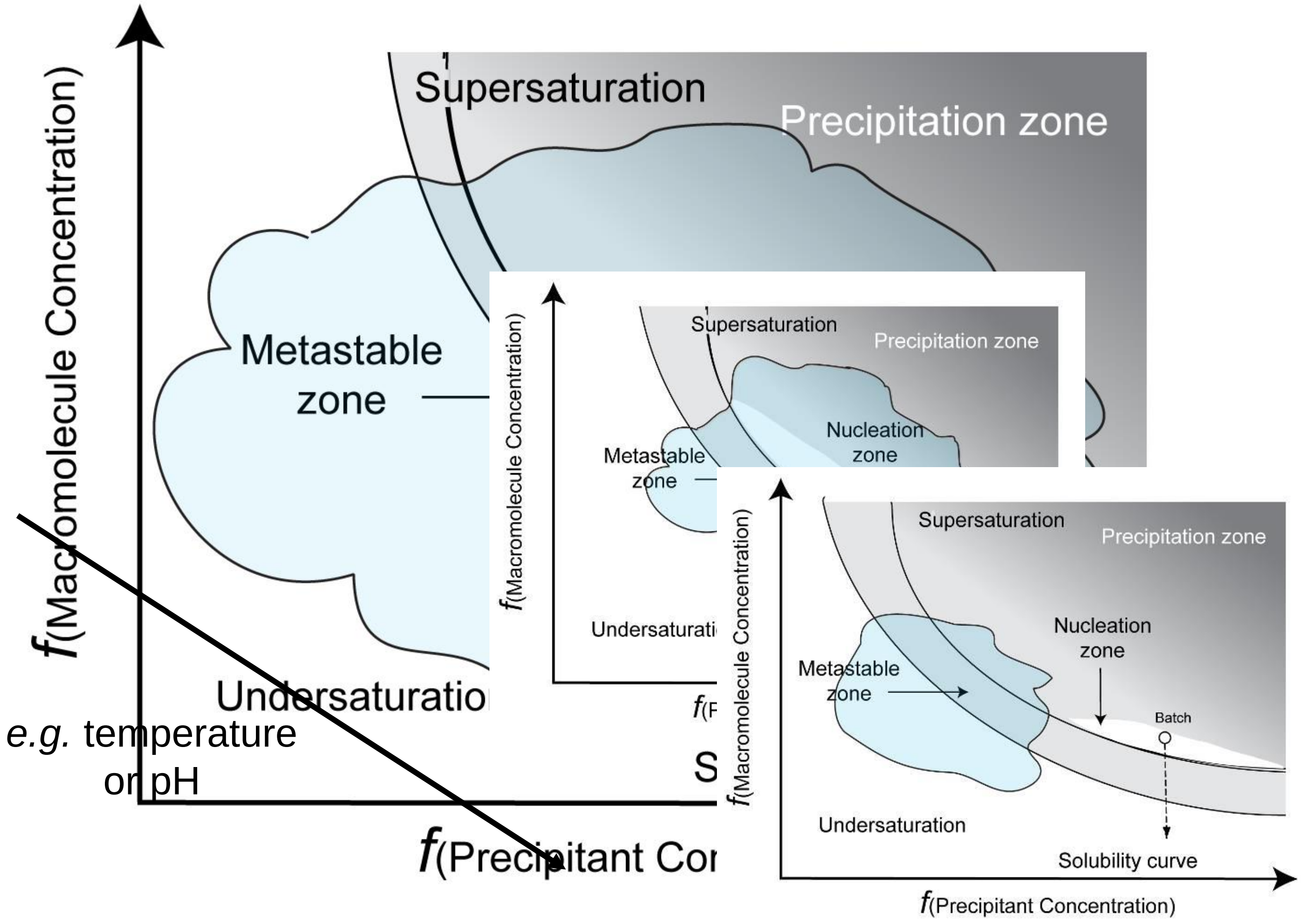
Different techniques traverse the crystallization space differently.

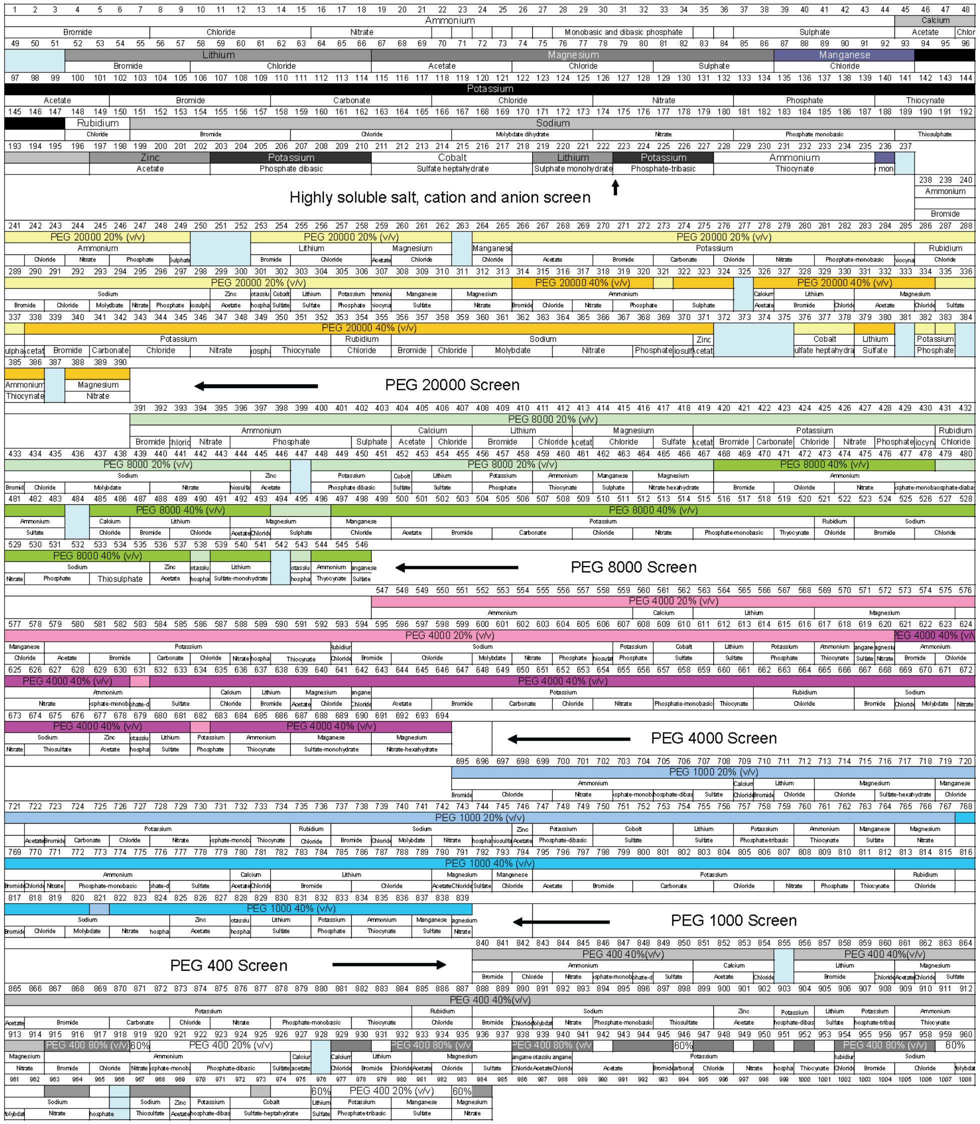


What results can we expect to see?



Typical situation, multidimensional area sampled

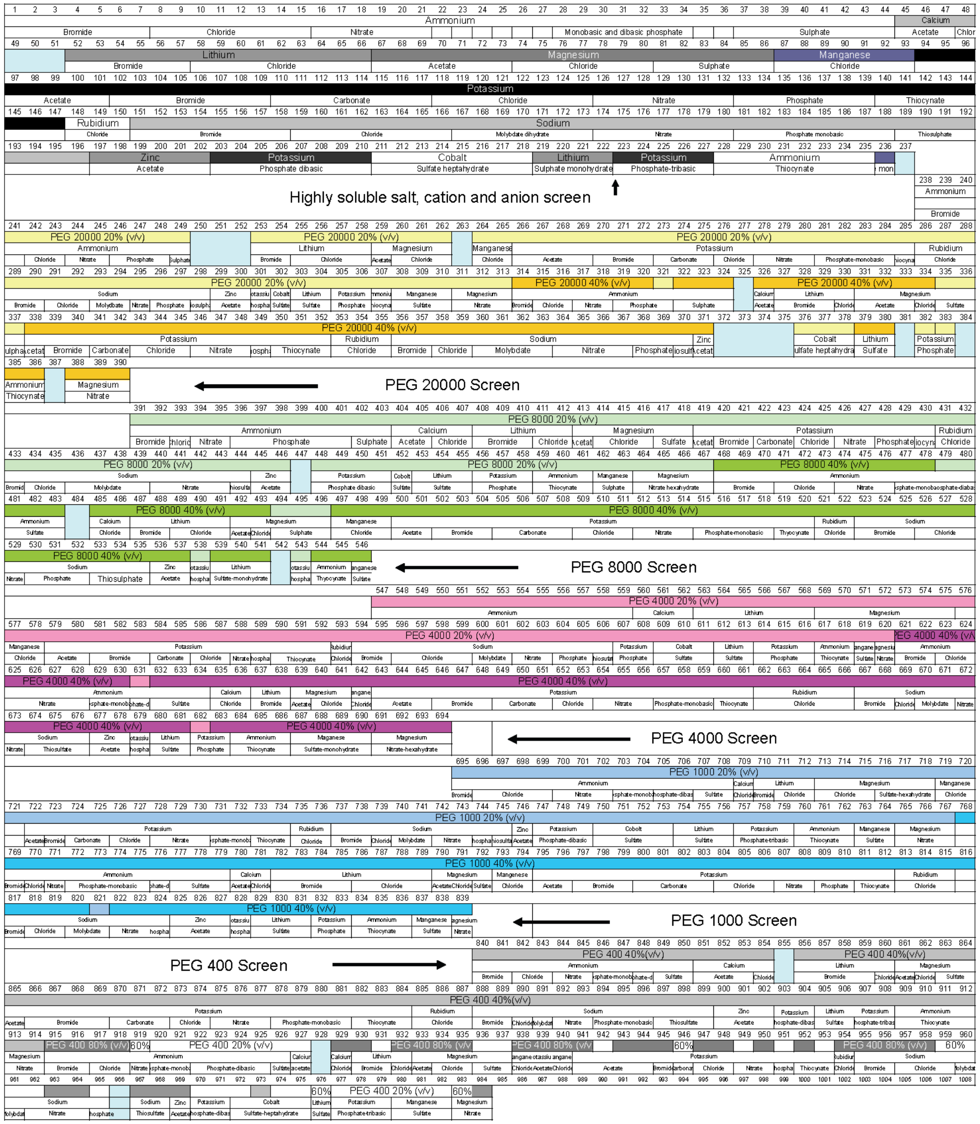




The HWI crystallization cocktail screen.

The 1536 diverse chemical cocktails (Luft et al., 2003). The 984 in-house conditions comprise an incomplete factorial sampling of 36 salts, eight buffers, and 5 different PEGs.

The remainder of 1536 cocktails are comprised of commercial screens available from Hampton Research. Specifically, in order of use; the Natrix Screen, Quick Screen, Nucleic Acid Screen, Sodium Malonate Grid, PEG/Ion, PEG 6000 Grid, Ammonium Sulfate Grid, Sodium Chloride Grid, HT Screen, Index and the SaltRx screen.



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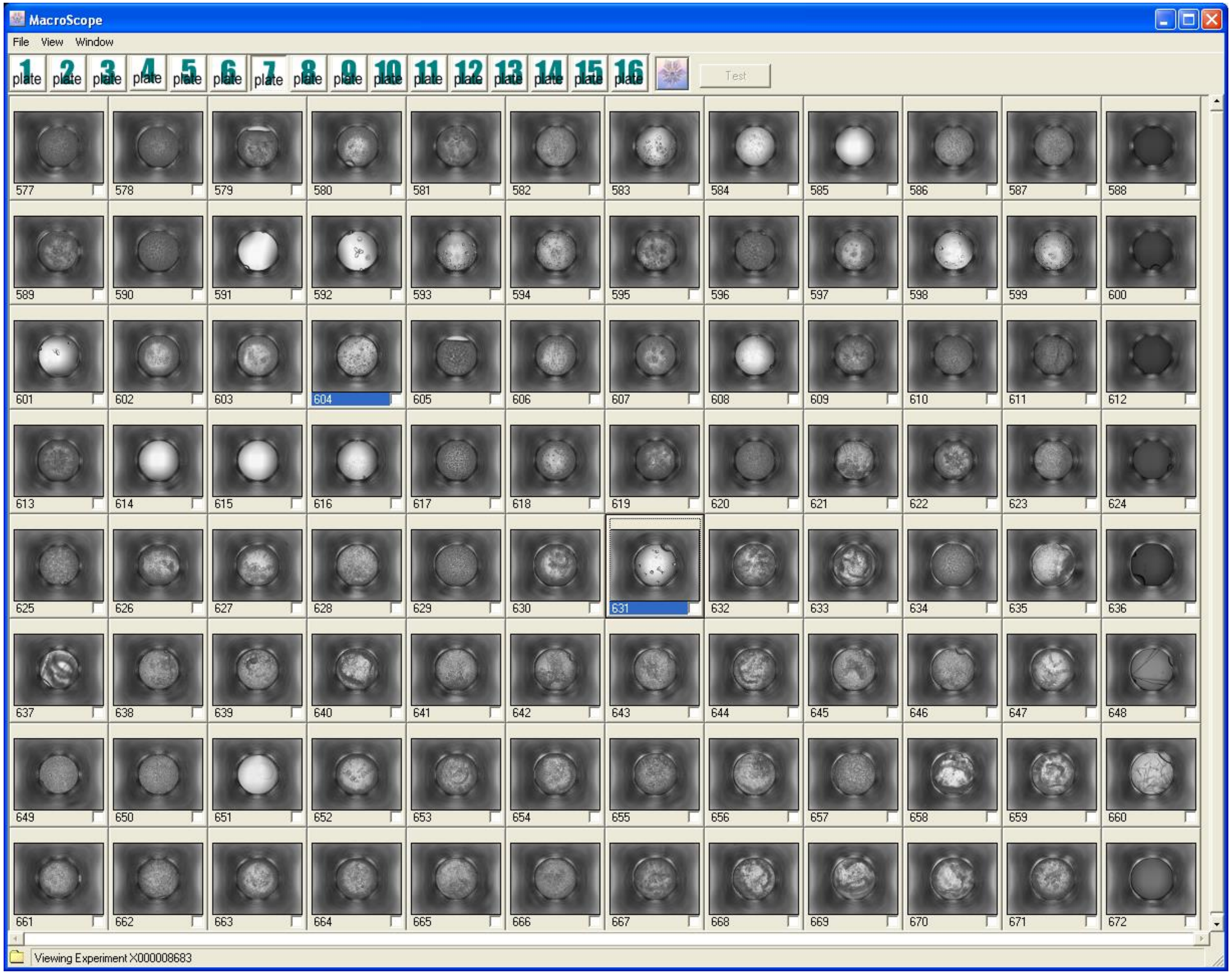
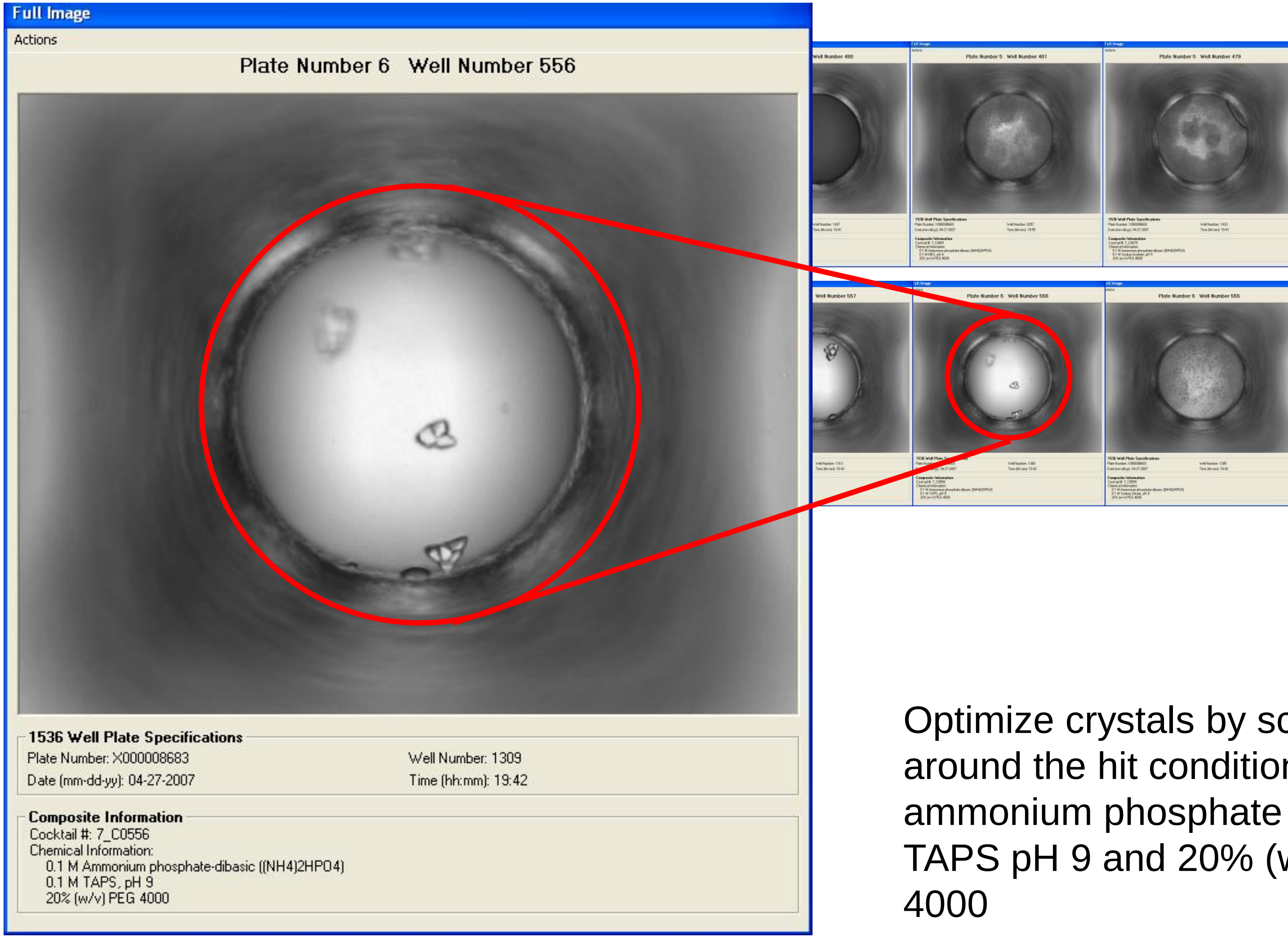
What do we see from the data?

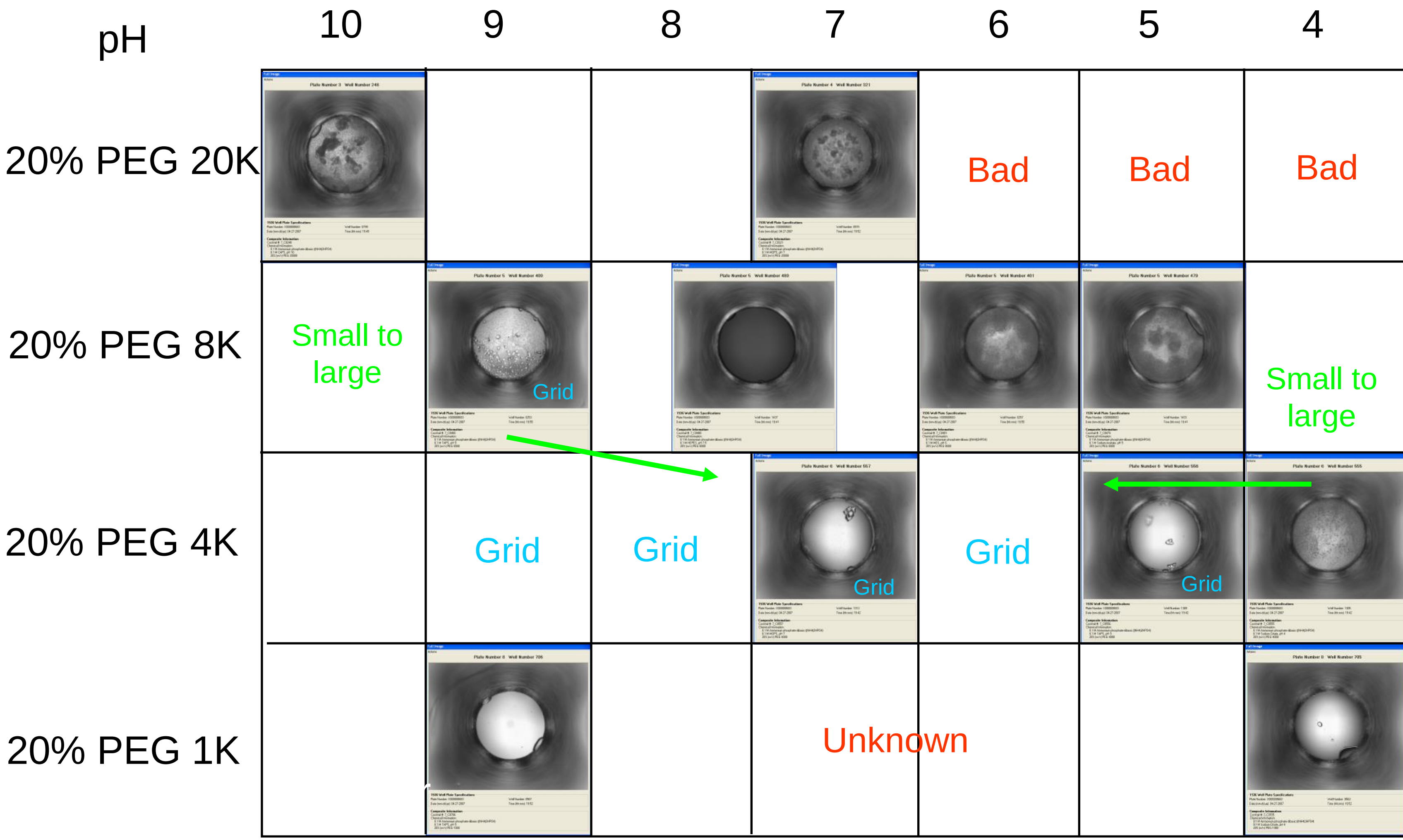


What do we actually see?



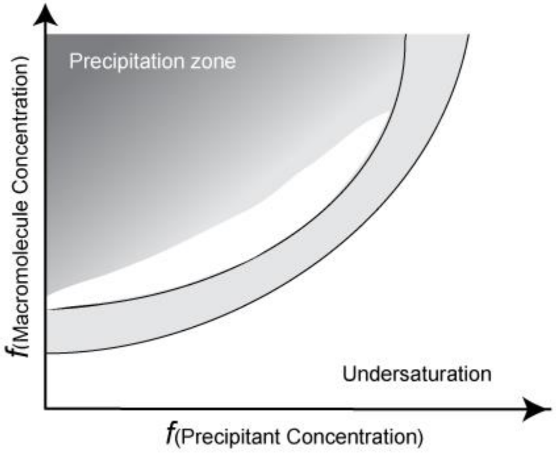
Optimize crystals by screening around the hit conditions, *i.e.* 0.1 M ammonium phosphate dibasic, 0.1 TAPS pH 9 and 20% (w/v) PEG 4000

If we plot the results in chemical space the road becomes clear

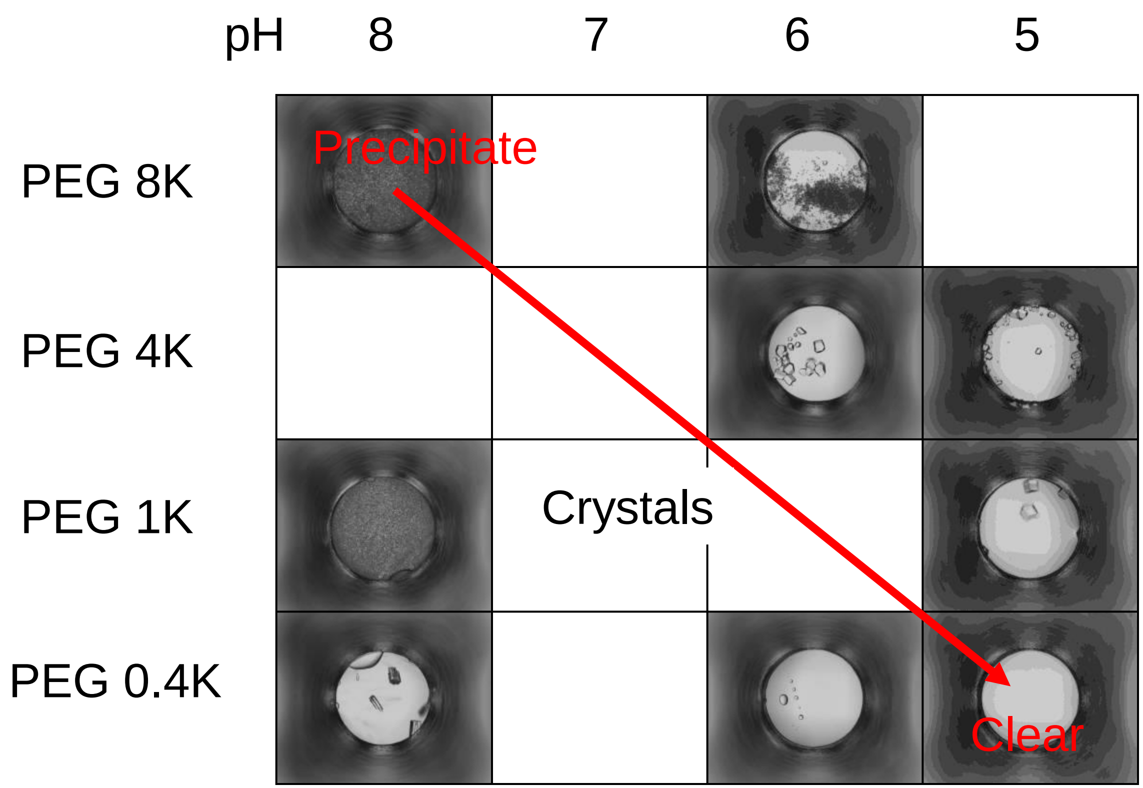


Chemical space provides a vector for optimization

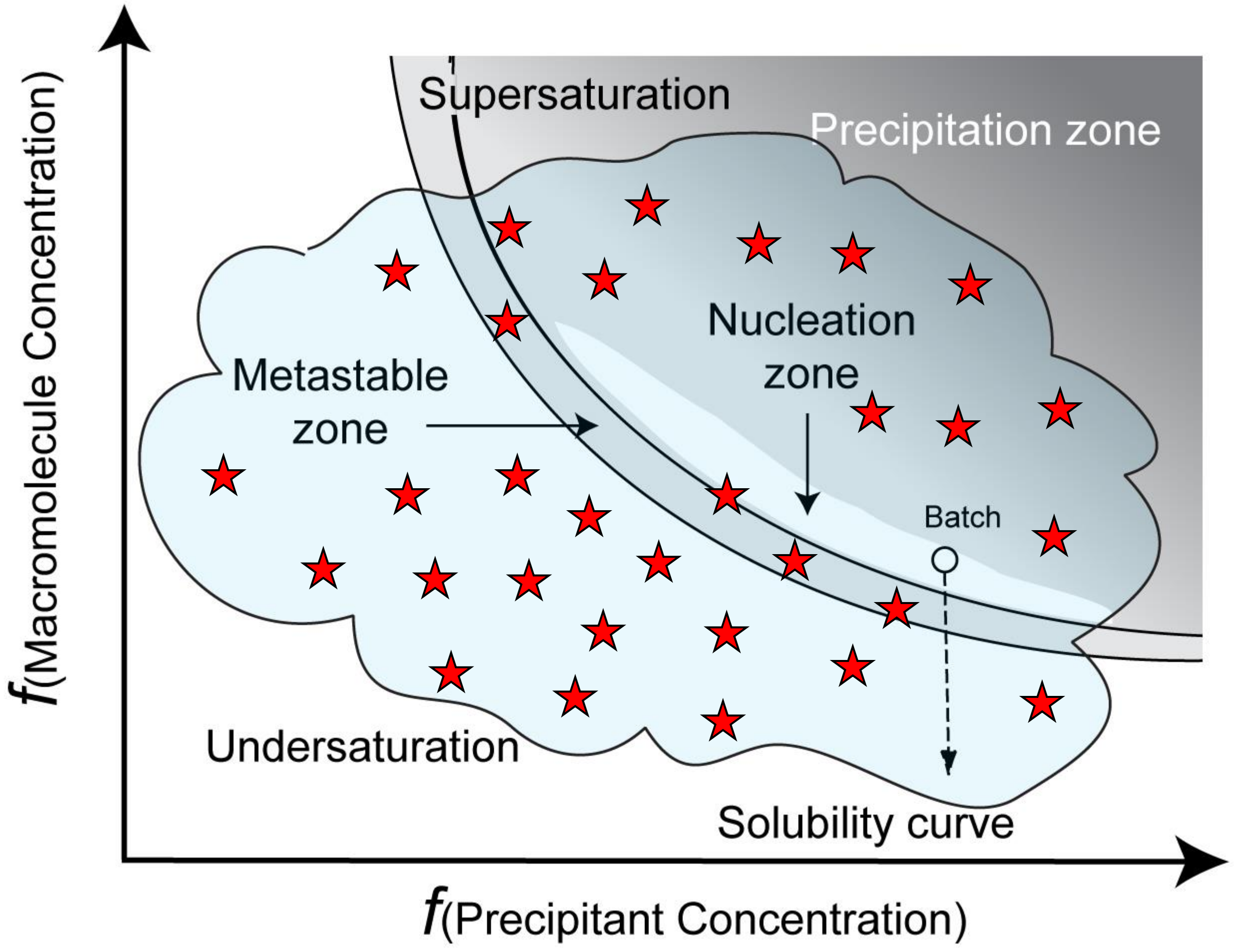
In this case the path from precipitate through crystals to clear is obvious. The phase diagram is reversed. Also clear are the number of chemical conditions that have not been sampled.



Ubiquitin, 40% PEG, 0.1M zinc acetate



It also illustrates the space we do <u>not</u> sample



We only sample discrete points within the sampling space

Numbers – the quantity of data

AutoSherlock ™

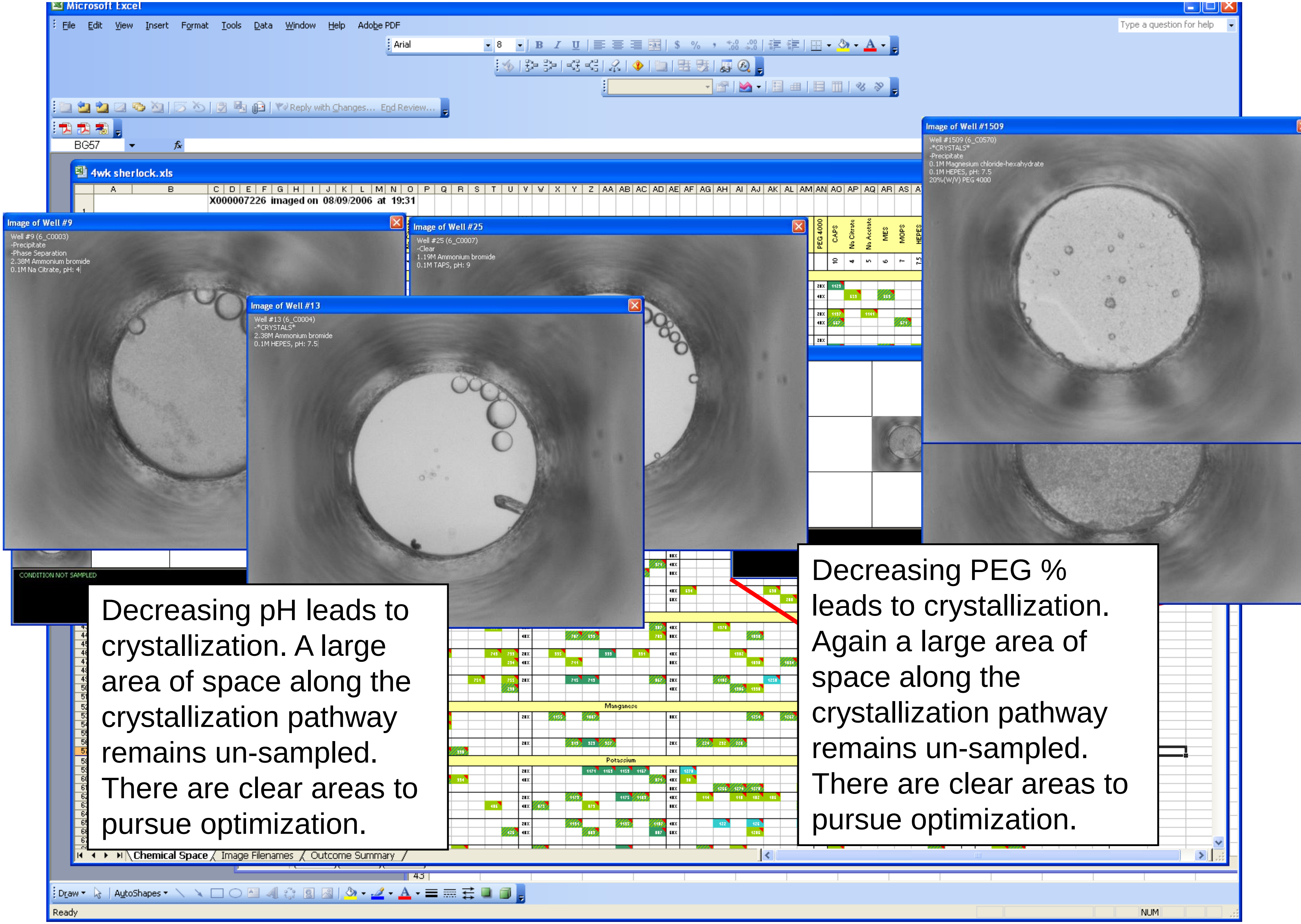
“Because scientists would rather spend less time organizing their data, and more time learning from it.”

As of the New Year:

Over 850 laboratories sending samples (multiple samples from PSI centers)
Over 9,500 different macromolecules to date
Over 14.5 million images
Over 3000 years of computing time spent analyzing 1% of those images

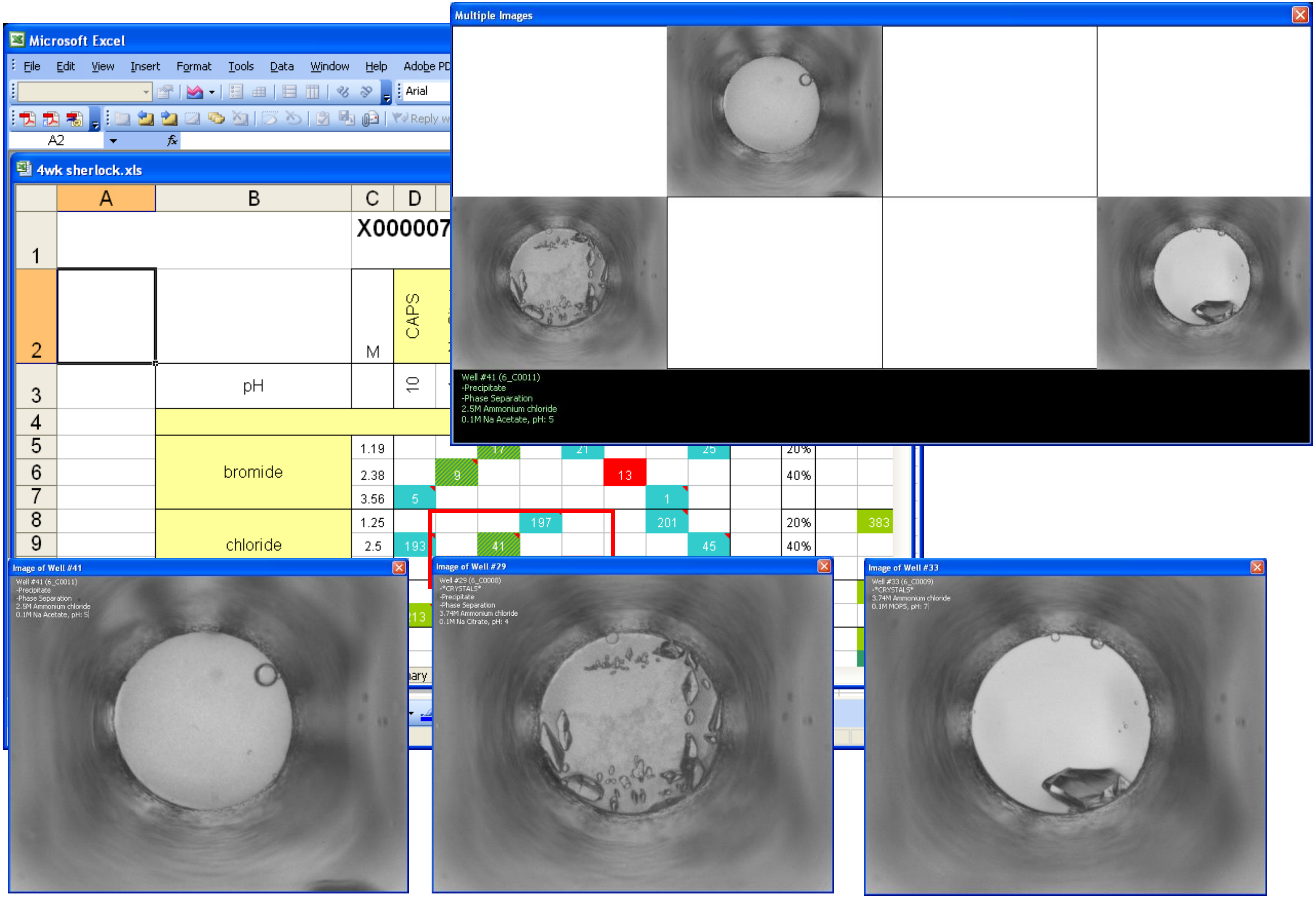
A difficult task to easily visualize results.

Develop automated procedures.



Decreasing pH leads to crystallization. A large area of space along the crystallization pathway remains un-sampled. There are clear areas to pursue optimization.

Decreasing PEG % leads to crystallization. Again a large area of space along the crystallization pathway remains un-sampled. There are clear areas to pursue optimization.


Microsoft Excel
File Edit View Insert Format Tools Data Window Help Adobe PD
Arial
Reply w
A2
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A B C D
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M
CAPS
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chloride
1.19
2.38
3.56
1.25
2.5
17
21
25
9
13
5
1
197
201
383
193
41
45
20%
40%
20%
40%
Multiple Images
Well #41 (6_C0011)
-Precipitate
-Phase Separation
2.5M Ammonium chloride
0.1M Na Acetate, pH: 5
Image of Well #41
Well #41 (6_C0011)
-Precipitate
-Phase Separation
2.5M Ammonium chloride
0.1M Na Acetate, pH: 5
Image of Well #29
Well #29 (6_C0008)
-*CRYSTALS*
-Precipitate
-Phase Separation
3.74M Ammonium chloride
0.1M Na Citrate, pH: 4
Image of Well #33
Well #33 (6_C0009)
-*CRYSTALS*
3.74M Ammonium chloride
0.1M MOPS, pH: 7

The Commercial Screens in the HWI crystallization cocktails

The commercial screens incorporate several distinct mechanisms of sampling the crystallization space. Examples are shown here.

The original Hampton Research 1+2 sample a set of conditions known to produce crystals in the past with the predominant variable being pH. Although described as a sparse matrix the number of samples is small and the distribution in chemical space wide therefore it is difficult to relate results from one condition to results from other conditions. This is the primary reason that crystallization today is target focused.

Salt RX

Magnesium				
Formate dihydrate	0.4M	B11	B12	C1
	0.7M		C2	
Sulfate hydrate	1.0M	G1	G2	G3
	1.8M	G4	G5	G6
Lithium				
Sulfate monohydrate	0.8M	F7	F8	F9
	1.0M		F11	
	1.5M	F10		F12
Potassium				
Sodium tartrate	0.6M		H1	H3
	1.2M		H2	H4
Thiocyanate	0.5M	H5	H6	H7
DL-Malic acid				
	1.2M		C9	
	2.2M		C10	
Succinic acid				
	0.5M		E11	
	1.0M		E12	
Tacsimate				
	35%		H11	
	60%		H12	
		pH		
		5	6.9	8.2
Sodium	1.0M	E1	E2	E3

The SaltRx screen samples 22 crystallization salts with varying concentration and pH. [...] true sparse matrix whe[...] can be related in term[s] [...] chemical space.

None, A2, A3, C6, C7, C8, C9, D6, D7, D8		D11		C1, C4, D9, D10	C5, C11, D2, D3, D5	C12, D12
E1, E2, E3, E4, E5, E6, E7, E8, G4		E9, E10, E11, E12, F1	F2, F3, F4, F5, F6, F7	F8, F9, F10, F11, F12, G1, G2, G3	G4, G5, G6, G7, G8, G9, G10, G11, G12, H1	H3, H4, H5, H6, H7, H8, H9

A number of Grid screens are incorporated, in this case Sodium Chloride. These provide a fine sampling of a small subset of individual conditions and serve to indicate the sensitivity (or lack of it) to small changes in precipitant conditions.

Sodium Chloride						
Conc	pH					
(M)	4	5	6	7	8	9
1	A1	A2	A3	A4	A5	A6
2	B1	B2	B3	B4	B5	B6
3	C1	C2	C3	C4	C5	C6
4	D1	D2	D3	D4	D5	D6

A special case – The Hampton Research Index Screen

Hampton Research Index Screen

Note, the HT screen is not a convential screen as such. It is designed to sample a range of reagents and provide an indication of the appropiate chemical area and variables that would be appropiate for crystallization and should be used in this manner.

pH	Ammonium Sulfate 2.0M	Sodium chloride 3.0M		Magnesium formate dihydrate			Sodium phosphate			Neutralized organic acids (ph 7.0)		High supersaturation salt and low polymer			Low ionic strength systems			Non-volatile organics	
pH				0.3M	0.5M		pH					pH			pH			pH	
3.5	A1	A7					5.6	B5		B9		5.5	C8		3.5	D4		5.5	D12
4.5	A2	A8					6.9	B6		B10		6.5	C6		4.5	D5			E2
5.5	A3	A9		B1			8.2	B7		B11		8.5	C7		5.5	D6		6.5	E1
6.5	A3	A10			B2					B12		7	C9		6.5	D7			E3
7.5	A5	A11		B3						C1			C10			D10			E6
8.5	A6	A12			B4					C2			C11			D11			E9
										C3			C12		7	D2			E10
	Classic salt versus pH									C4						D3		7.5	E4
										C5					7.5	D8			E7
	Hits here indicate that a variation of salt concentration and pH in a grid screen has a strong potential for crystallization														8.5	D9			E8
																			E11
																		8.5	E5
																			E12

PEGs and Salts as a function of pH								PEG 3350 and salts											
3.35K						10K	3.35K												
pH	Ammonium sulfate	Sodium chloride	Lithium sulfate monohydrate	Ammonium acetate	Magnesium Chloride hexahydrate	Ammonium acetate	Mixed chloridehydrates	%	Potassium sodium tartrate tetrahydrate	Sodium malonate pH 7.0	Ammonium citrate tribasic pH 7.0	Succinic acid pH 7.0	Sodium formate	DL-Malic acid pH 7.0	Magbesium formate dihydrate	Zinc acetate dihydrate	Sodium citrate tribasic dihydrate	Potassium thiocyanate	Potassium bromide
5.5	F6	F10	G2	G6	G10	F5		15				H5			H8				
6.5	F7	F11	G3	G7	G11			20	H2	H3	H4		H6	H7		H9	H10		
7.5	F8	F12	G4	G8	G12		F4	25											
8.5	F9	G1	G5	G9	H1			30										H11	H12

Coarse test for chemical conditions likely to produce crystallization

Automagic?

A process carried out automatically in such a clever way that the result appears to be magic

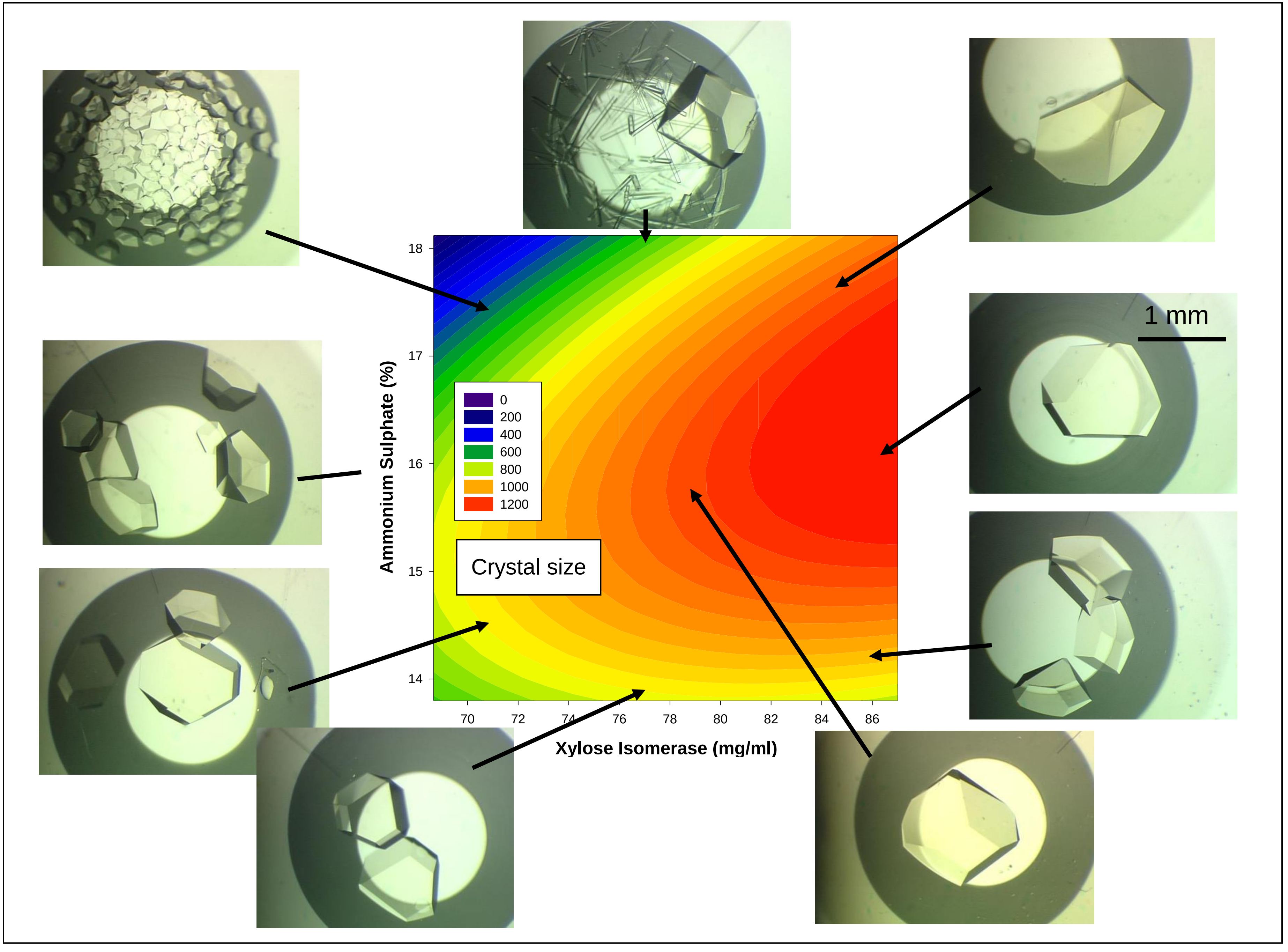
Not yet….

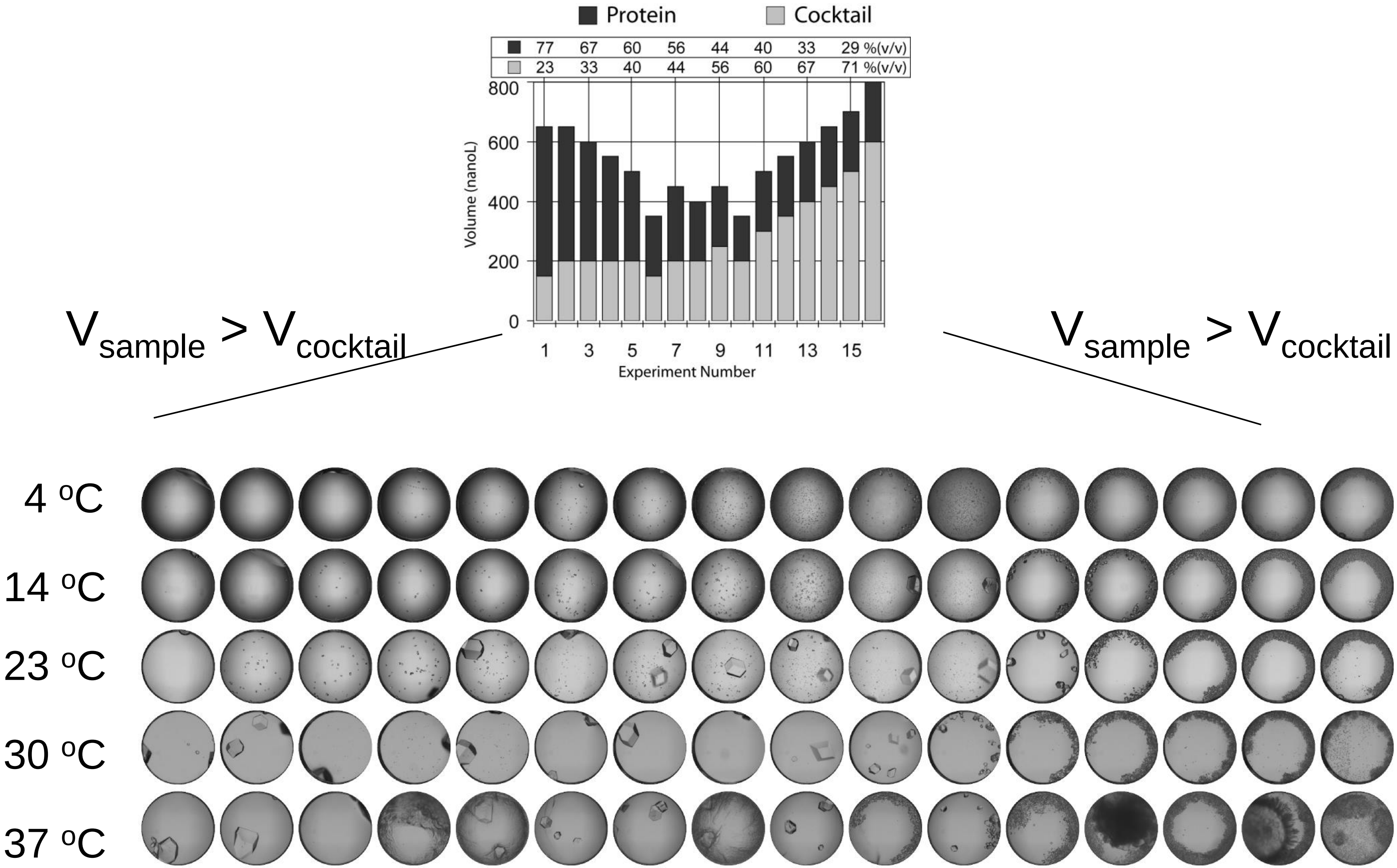
Rule 1: Think

- **Remember, Garbage In, Garbage Out**

- Crystallization is one percent inspiration and ninety-nine percent optimization – Unknown crystal grower.

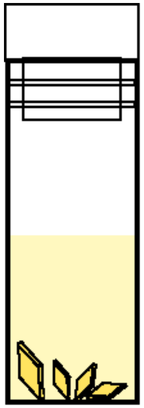
- **Rule 2 – Be an optimizer**


1 mm
Ammonium Sulphate (%)
Xylose Isomerase (mg/ml)
18
17
16
15
14
70
72
74
76
78
80
82
84
86
0
200
400
600
800
1000
1200
Crystal size

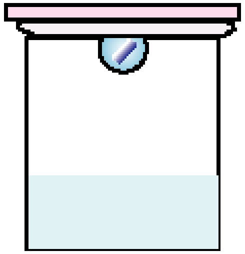


Protein
Cocktail
77 67 60 56 44 40 33 29 %(v/v)
23 33 40 44 56 60 67 71 %(v/v)
Volume (nanoL)
800
600
400
200
0
1 3 5 7 9 11 13 15
Experiment Number
$V_{sample} > V_{cocktail}$
$V_{sample} > V_{cocktail}$
4 oC
14 oC
23 oC
30 oC
37 oC

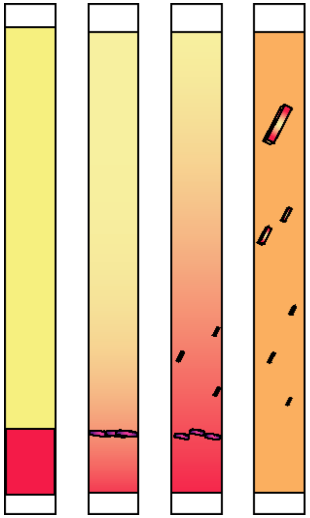
Three basic methods:
batch, vapor and liquid



Batch

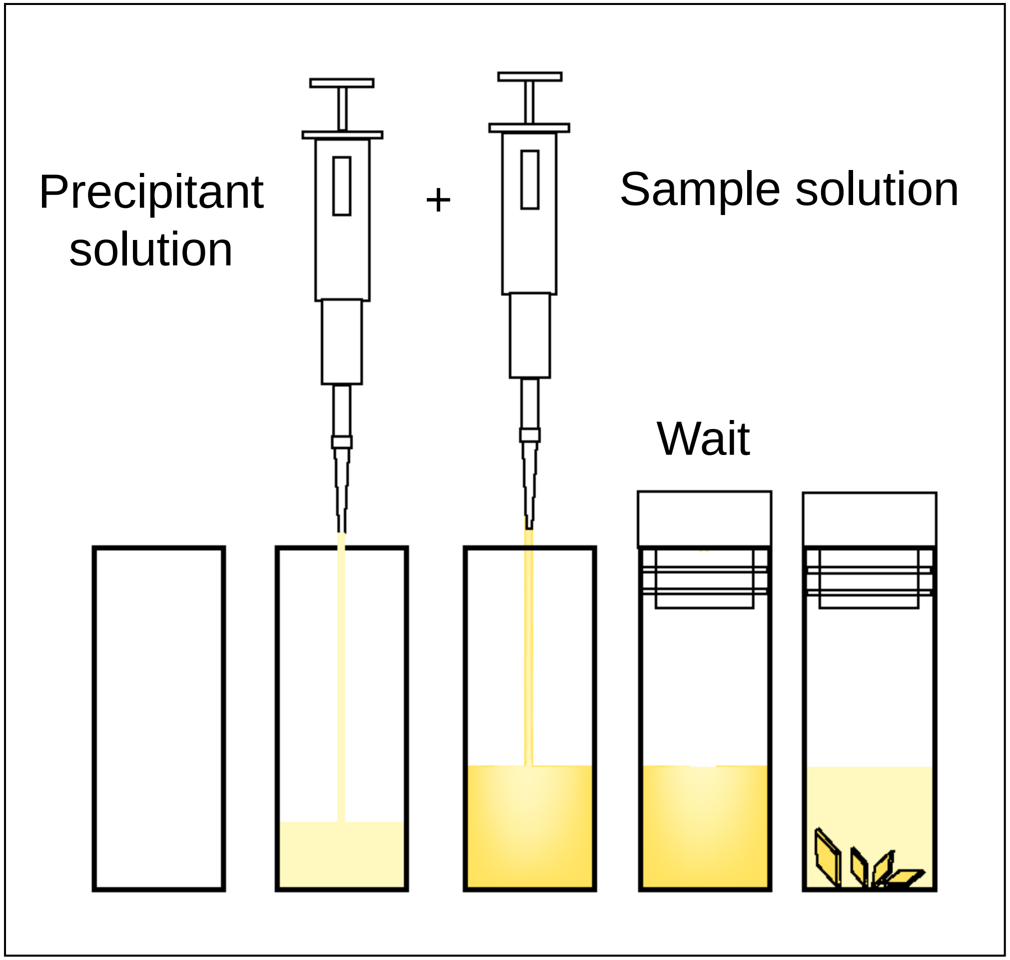


Vapor
(also known as vapour)



Liquid

Batch in a Vial: Set up

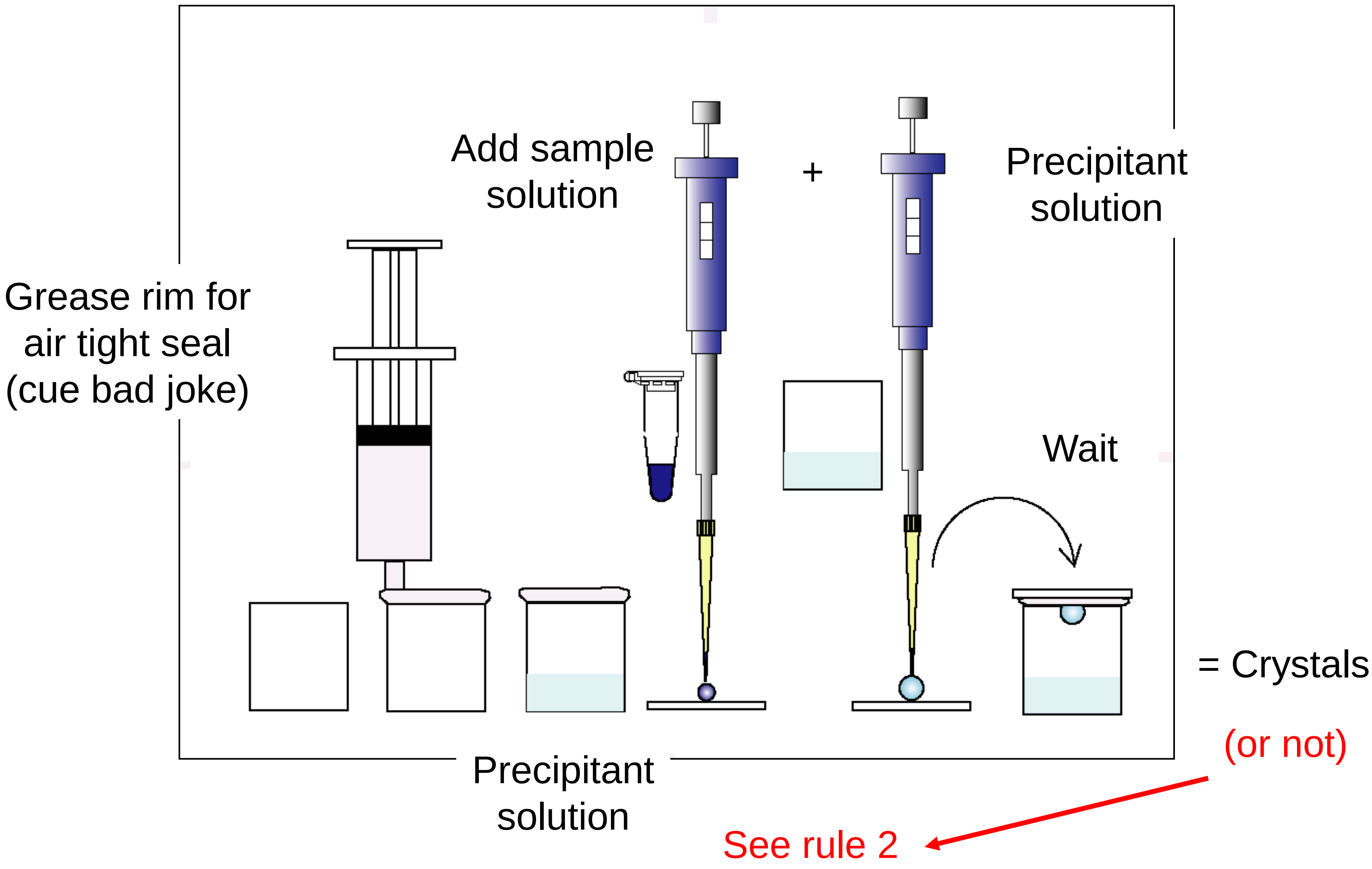


= Crystals

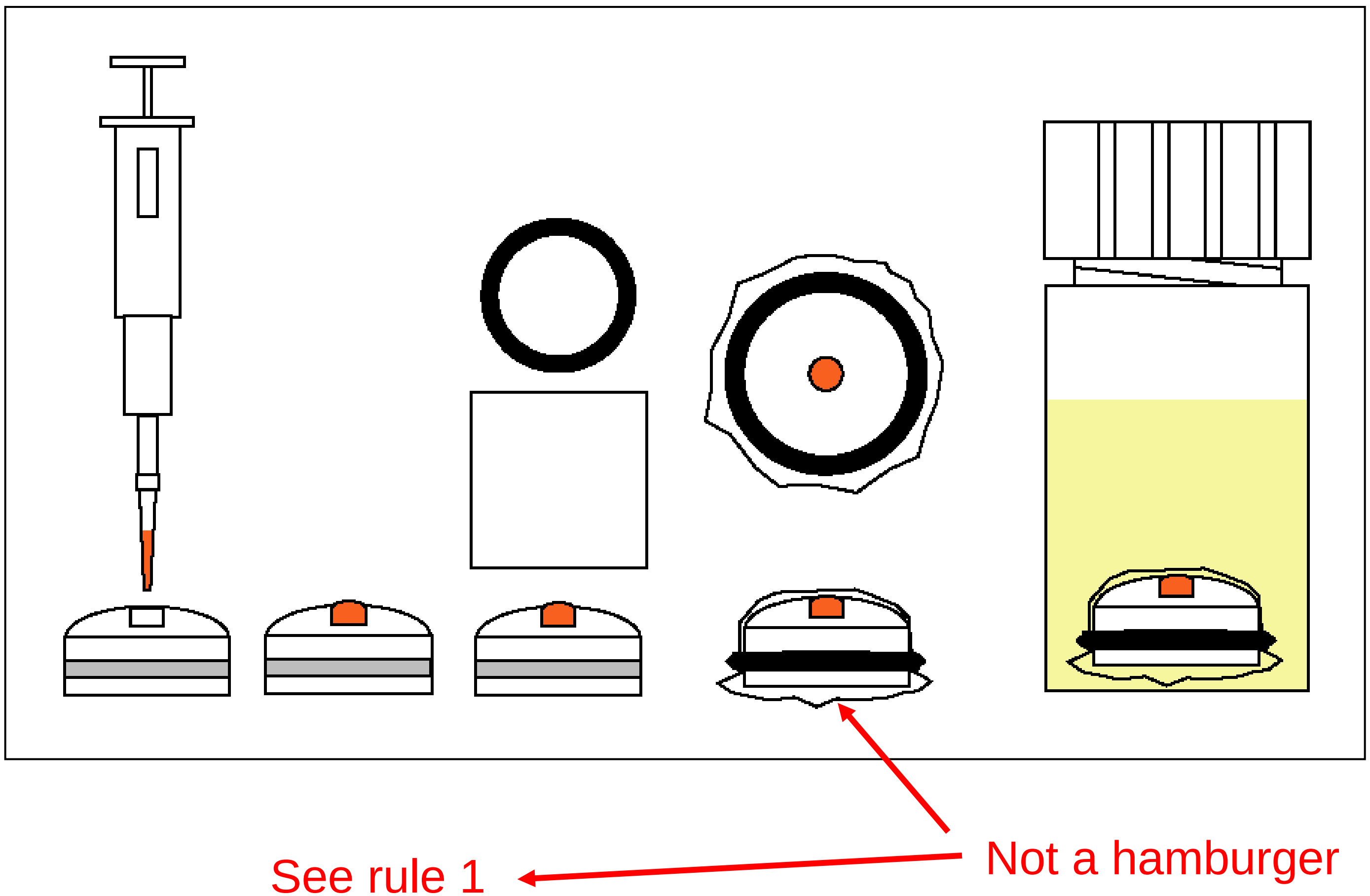
(or not)

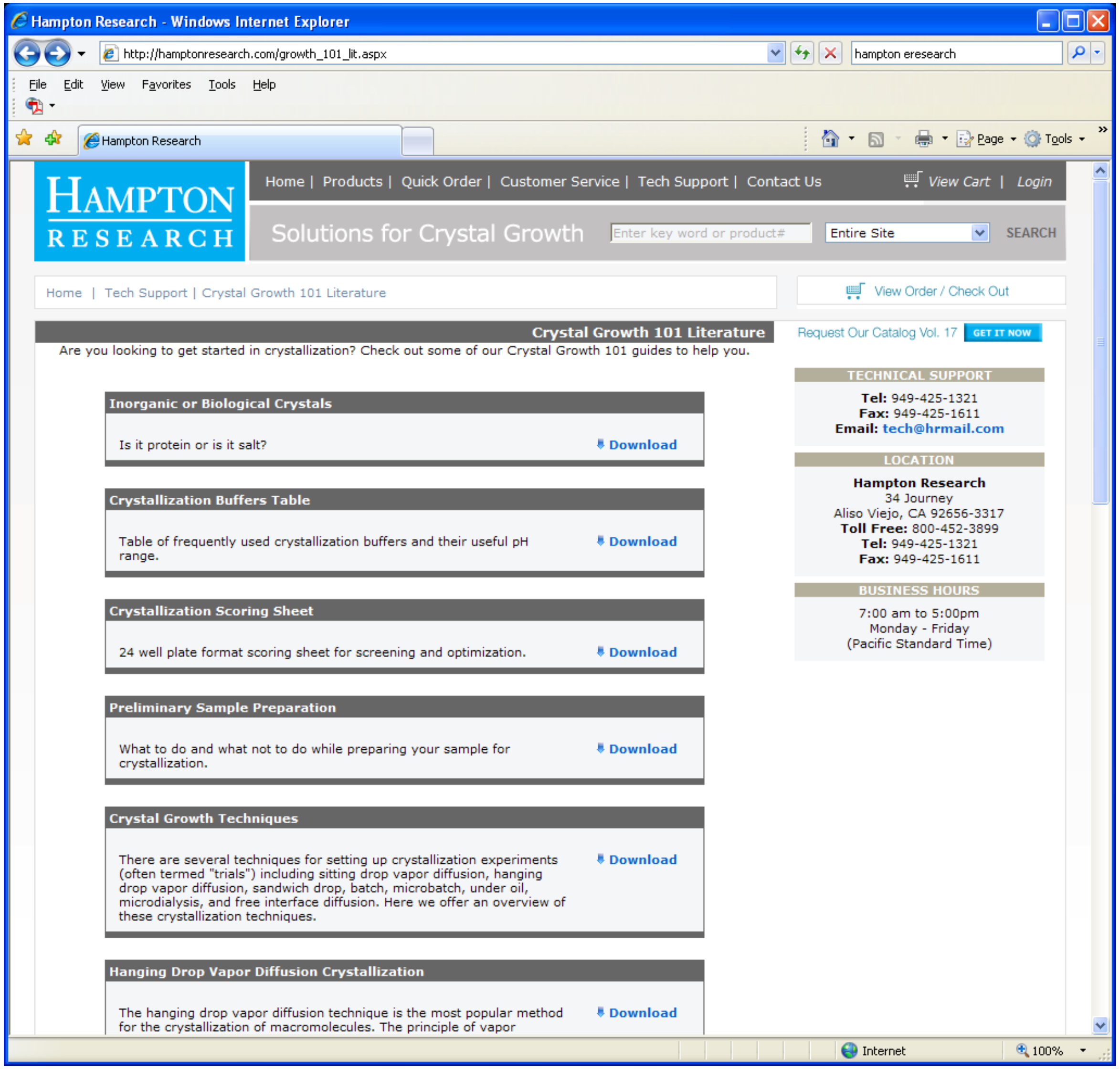
See rule 2

Vapor Diffusion Setup



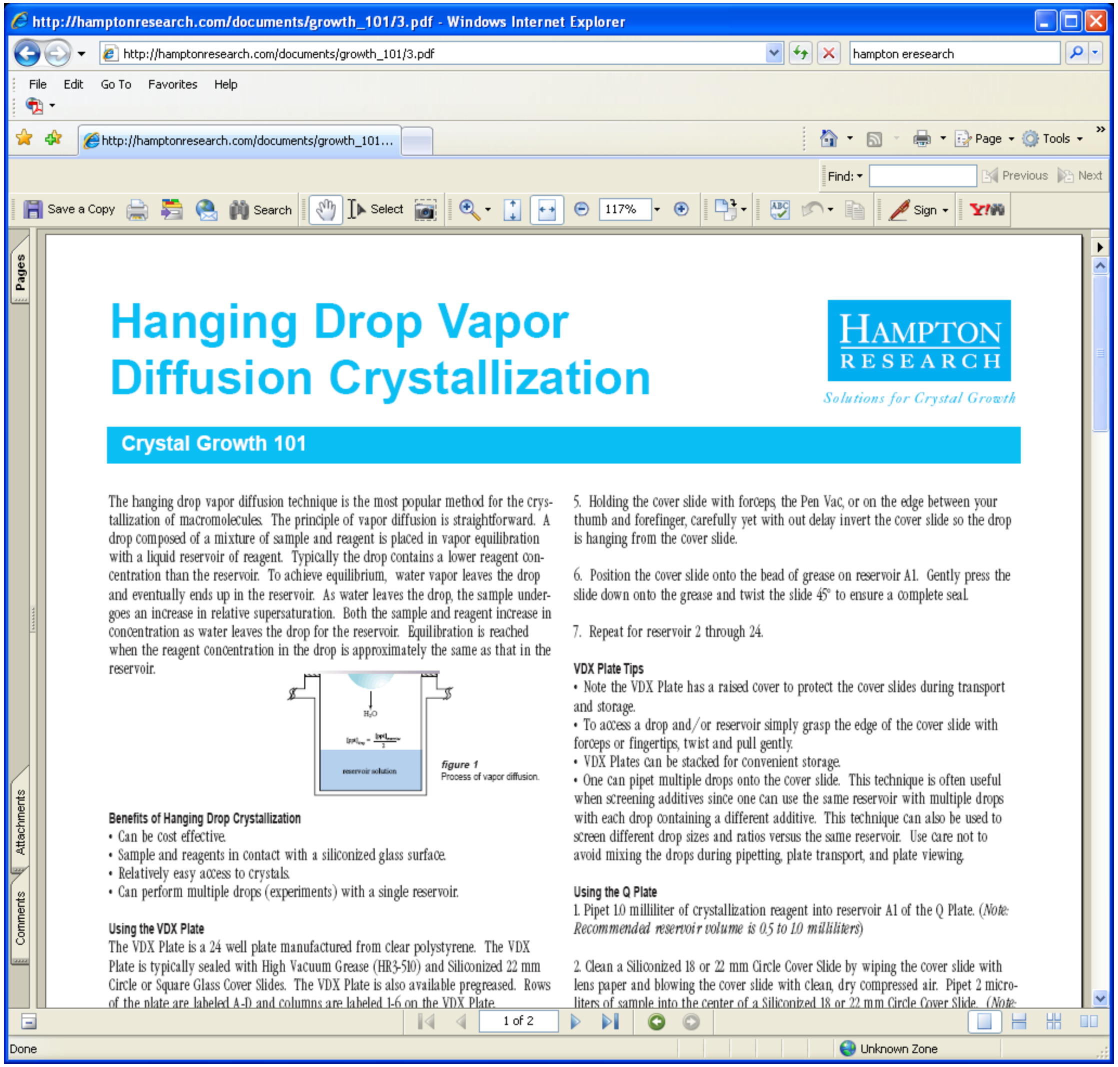
Dialysis Experiments





Useful websites:

www.hamptonresearch.com



Hanging Drop Vapor Diffusion Crystallization

HAMPTON RESEARCH

Solutions for Crystal Growth

Crystal Growth 101

The hanging drop vapor diffusion technique is the most popular method for the crystallization of macromolecules. The principle of vapor diffusion is straightforward. A drop composed of a mixture of sample and reagent is placed in vapor equilibration with a liquid reservoir of reagent. Typically the drop contains a lower reagent concentration than the reservoir. To achieve equilibrium, water vapor leaves the drop and eventually ends up in the reservoir. As water leaves the drop, the sample undergoes an increase in relative supersaturation. Both the sample and reagent increase in concentration as water leaves the drop for the reservoir. Equilibration is reached when the reagent concentration in the drop is approximately the same as that in the reservoir.

figure 1
Process of vapor diffusion.

Benefits of Hanging Drop Crystallization
- Can be cost effective.
- Sample and reagents in contact with a siliconized glass surface.
- Relatively easy access to crystals.
- Can perform multiple drops (experiments) with a single reservoir.

Using the VDX Plate
The VDX Plate is a 24 well plate manufactured from clear polystyrene. The VDX Plate is typically sealed with High Vacuum Grease (HR3-510) and Siliconized 22 mm Circle or Square Glass Cover Slides. The VDX Plate is also available pregreased. Rows of the plate are labeled A-D and columns are labeled 1-6 on the VDX Plate.

5. Holding the cover slide with forceps, the Pen Vac, or on the edge between your thumb and forefinger, carefully yet with out delay invert the cover slide so the drop is hanging from the cover slide.

6. Position the cover slide onto the bead of grease on reservoir A1. Gently press the slide down onto the grease and twist the slide 45° to ensure a complete seal.

7. Repeat for reservoir 2 through 24.

VDX Plate Tips
- Note the VDX Plate has a raised cover to protect the cover slides during transport and storage.
- To access a drop and/or reservoir simply grasp the edge of the cover slide with forceps or fingertips, twist and pull gently.
- VDX Plates can be stacked for convenient storage.
- One can pipet multiple drops onto the cover slide. This technique is often useful when screening additives since one can use the same reservoir with multiple drops with each drop containing a different additive. This technique can also be used to screen different drop sizes and ratios versus the same reservoir. Use care not to avoid mixing the drops during pipetting, plate transport, and plate viewing.

Using the Q Plate
1. Pipet 1.0 milliliter of crystallization reagent into reservoir A1 of the Q Plate. (*Note: Recommended reservoir volume is 0.5 to 1.0 milliliters*)

2. Clean a Siliconized 18 or 22 mm Circle Cover Slide by wiping the cover slide with lens paper and blowing the cover slide with clean, dry compressed air. Pipet 2 microliters of sample into the center of a Siliconized 18 or 22 mm Circle Cover Slide. (*Note:*

Nice 101 technical notes on many basic techniques.

Other sites include:

http://www.moleculardimensions.com/

http://www.emeraldbiosystems.com/

Rules of thumb to convert vapor diffusion to batch:

http://www.douglas.co.uk/convert.htm

Choosing a starting point for pH

http://www.ruppweb.org/cryspred/default.html

General crystallization phenomena

http://xray.bmc.uu.se/~terese/crystallization/library.html

Bad news on X-rays

Ionizing radiation can remove an electron from water:

$$H_2O^+ + H_2O \longrightarrow H_3O^+ + OH$$

And the ejected electron

$$e- + H_2O \longrightarrow OH^- + OH$$

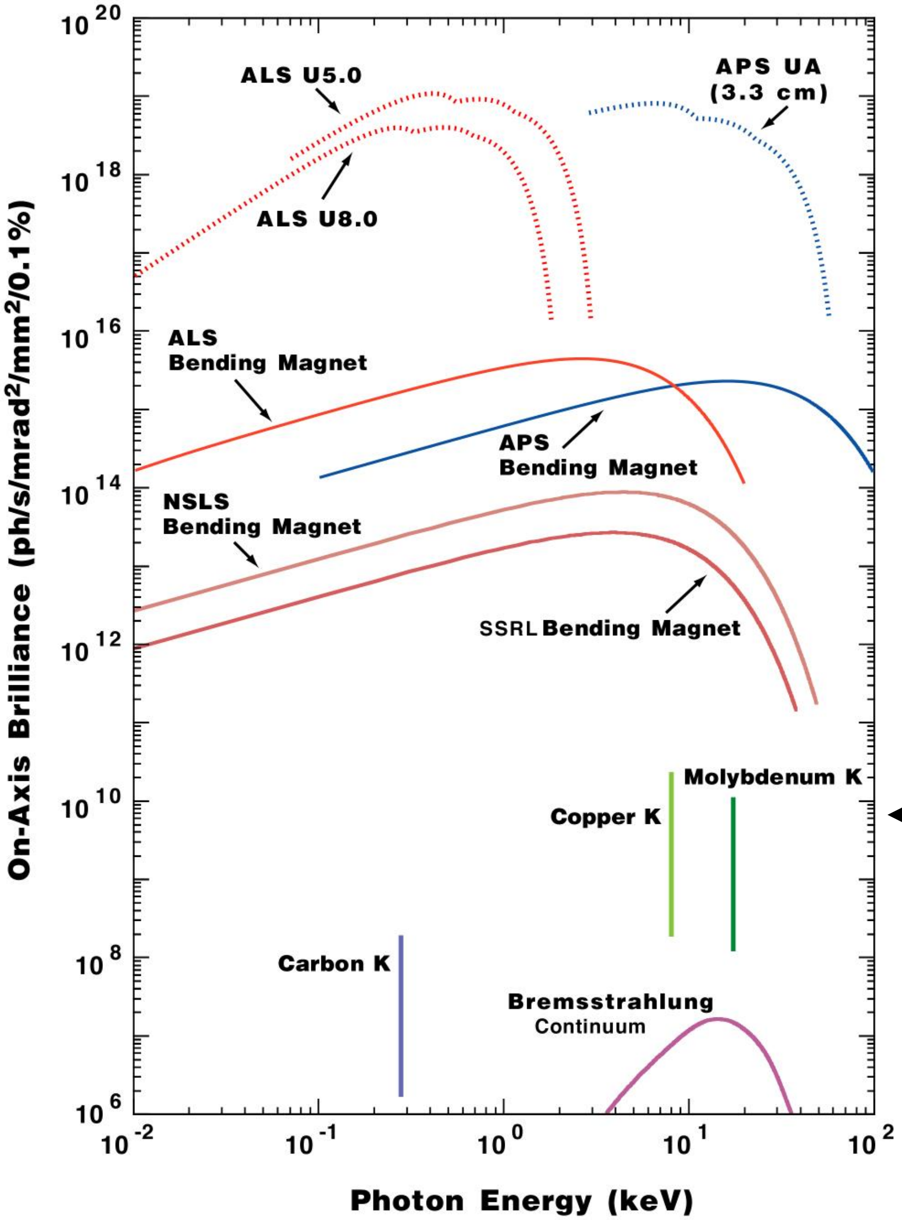
The simultaneous formation of H and OH free radicals gives further reactions

$$H + OH \longrightarrow H_2O$$

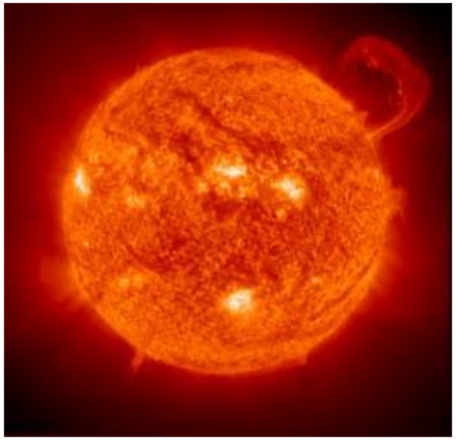
$$H + H \longrightarrow H_2$$

$$OH + OH \longrightarrow H_2O_2$$

Your body, cells, protein crystals contain water, the body ~66%, crystals 30-70%.



Synchrotron radiation is 10^9 times
More brilliant than the sun
and about 100 million miles closer



Processes of radiation damage

Primary, secondary, direct and indirect radiation-damage events in a protein crystal.

The incoming X-ray photons cause primary damage events, represented by darker stars. The paths of secondary radicals are shown by dotted arrows, and the damage events they induce are represented by lighter stars. Direct events occur on the protein molecules, and indirect events occur in the solvent region.

Primary effects are a fact of life, we cannot prevent them. Secondary effects can be reduced by cryocooling.

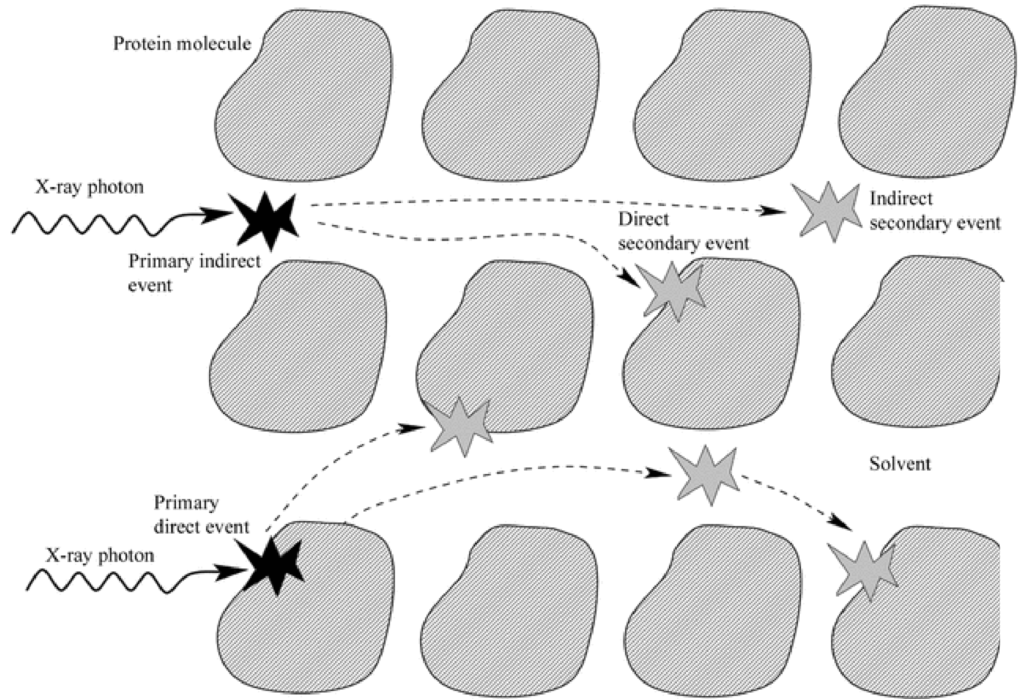
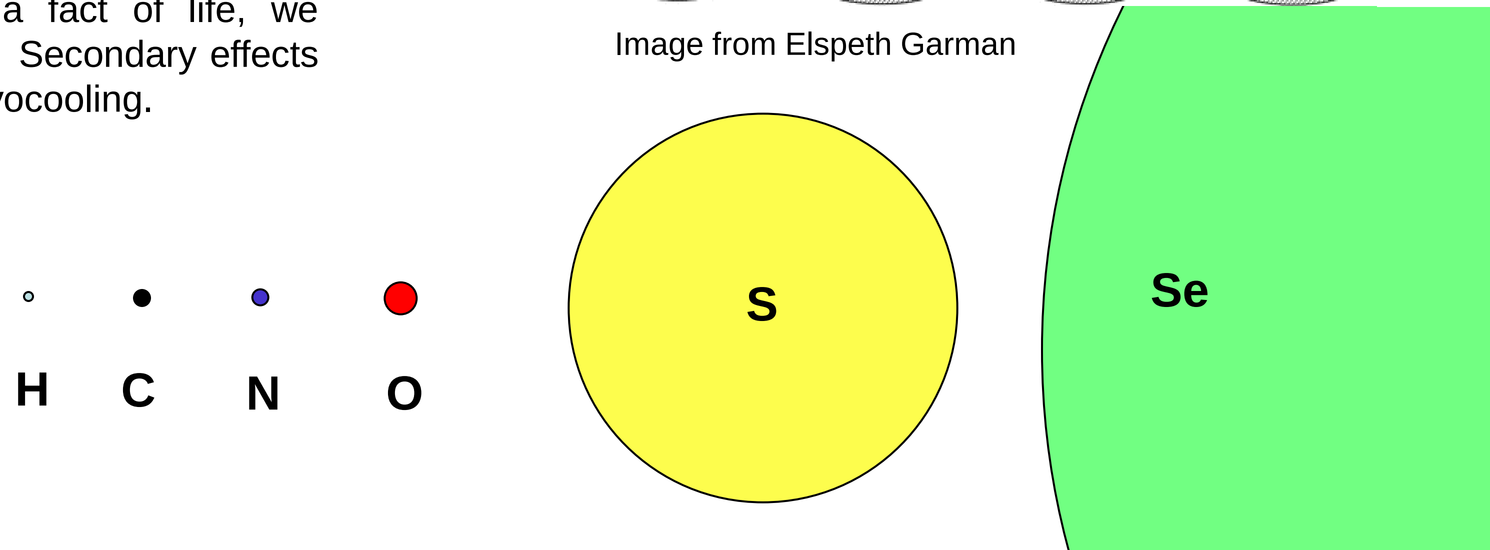
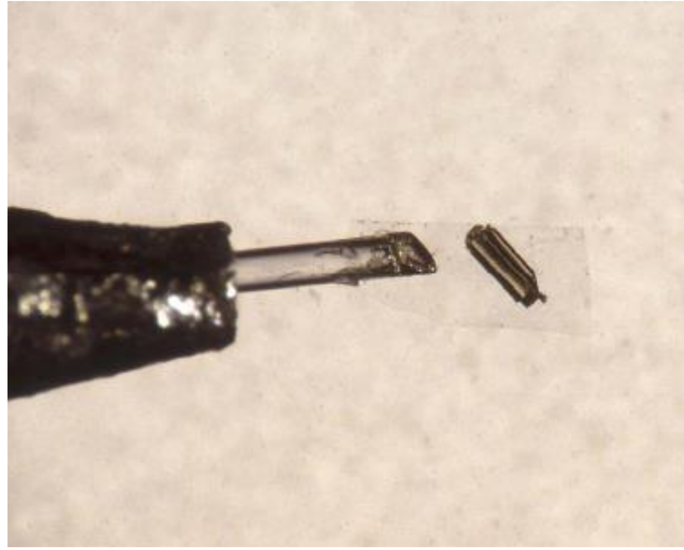


Image from Elspeth Garman

Effect on Other atoms



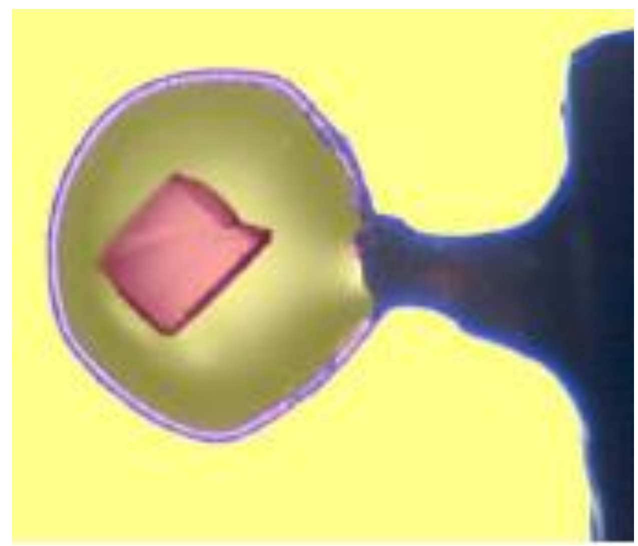
Development of cryocooling

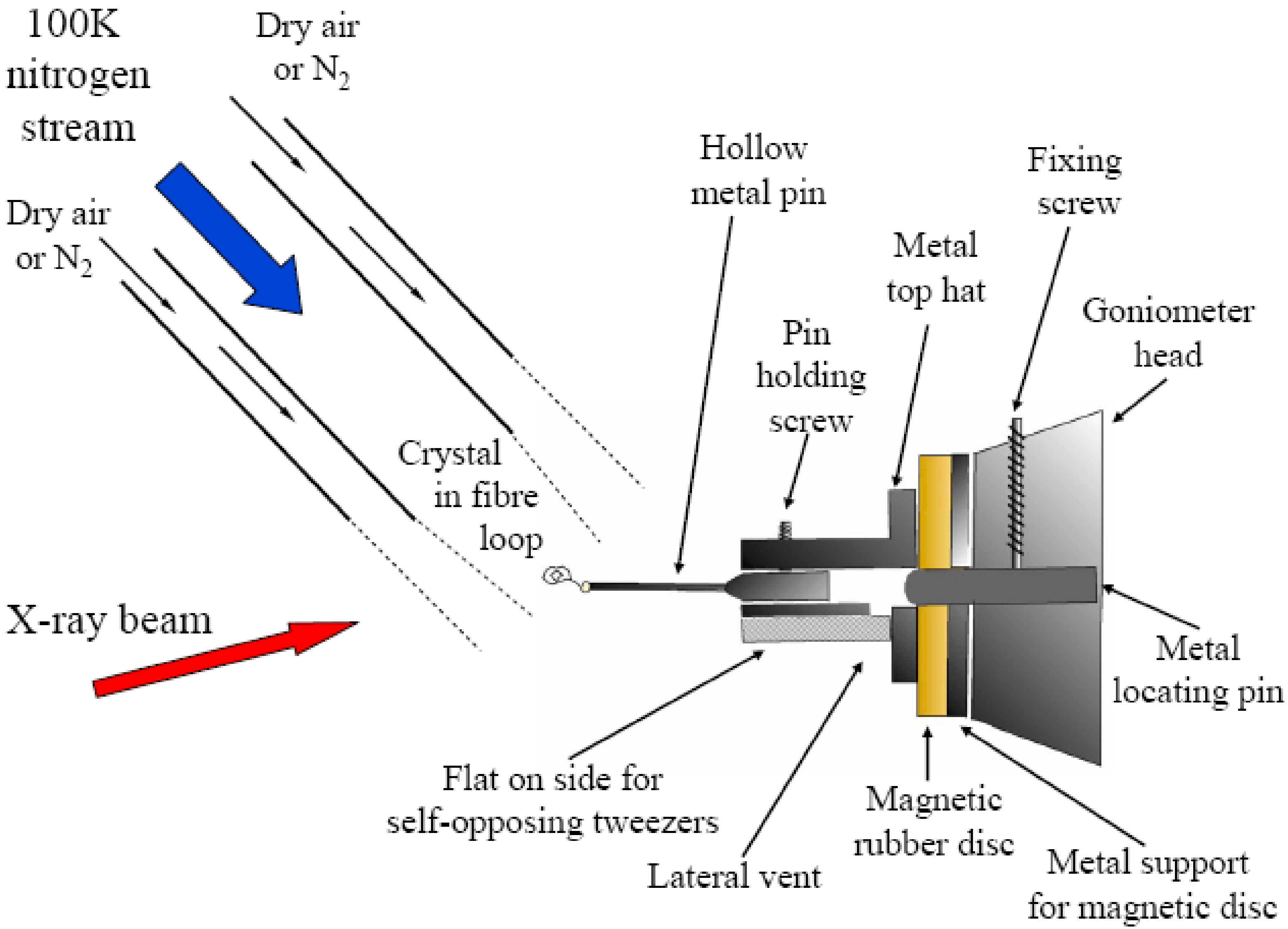


Hope Acta Cryst. B44, 22-26 (1988) at 130K with oil and spatulas.

Loop mounting, Teng J. Appl. Cryst. 23, 387-391 (1990) first introduced a metal loop which is now the nylon loop we know today.

Cryocooling with loops is younger than everyone that uses it!

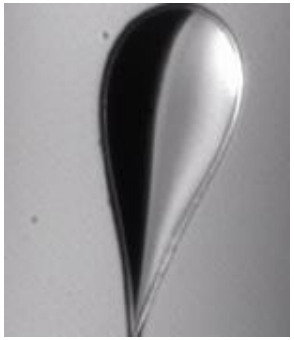




Courtesy of Elspeth Garman

Cryocooling – How?

- Cool the crystal fast enough so that amorphous ice rather than crystalline ice is formed (vitrification).
- To vitrify water cooling has to occur in 10^{-8} s.
- Cryoprotectants extend this time to 1-2 s.
- A cryobuffer is the buffer the crystal is grown in with the cryoprotectant added.
- The cryoprotectant replaces water, it does not dilute the solutions.
- Visually clear is usually a good indication of a good cryoprotectant condition.
- Collect data below 130 K, preferably as low as possible but never above 140K.



Good cryobuffer

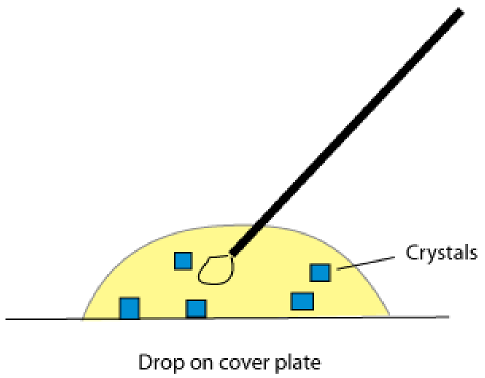
Bad cryobuffer

Read any of the papers by Elspeth Garman listed at http://biop.ox.ac.uk/www/garman/publications.html

Cryo-buffers (make your own)

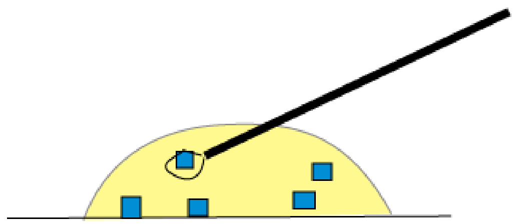
- Look for similar crystallization conditions in other publications.
- PEG < 4K – increase PEG, add small PEGs
- PEG > 4K – add small PEGs
- 30% of cases – add 15-25% glycerol
- MPD – increase MPD concentration
- Salt – add MPD and/or ethylene glycol or glycerol
- Salt – increase concentration/add salt
- Salt – exchange salts
- Note low salt concentrations need greater concentration of cryoprotectant than higher salt concentrations.
- Other cryoprotectants, DMSO, propanediol etc.
- Butanediol is very effective but expensive.
- (with thanks to Elspeth Garman for many tips).

Getting the crystal



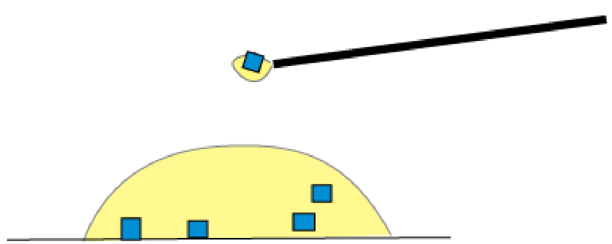
Ideally minimize the amount of liquid around the crystal while maintaining the crystal covered by the liquid

The loop size should be slightly larger than the crystal and used perpendicular to the liquid.



The loop should be brought up from below the crystal to capture the crystal.

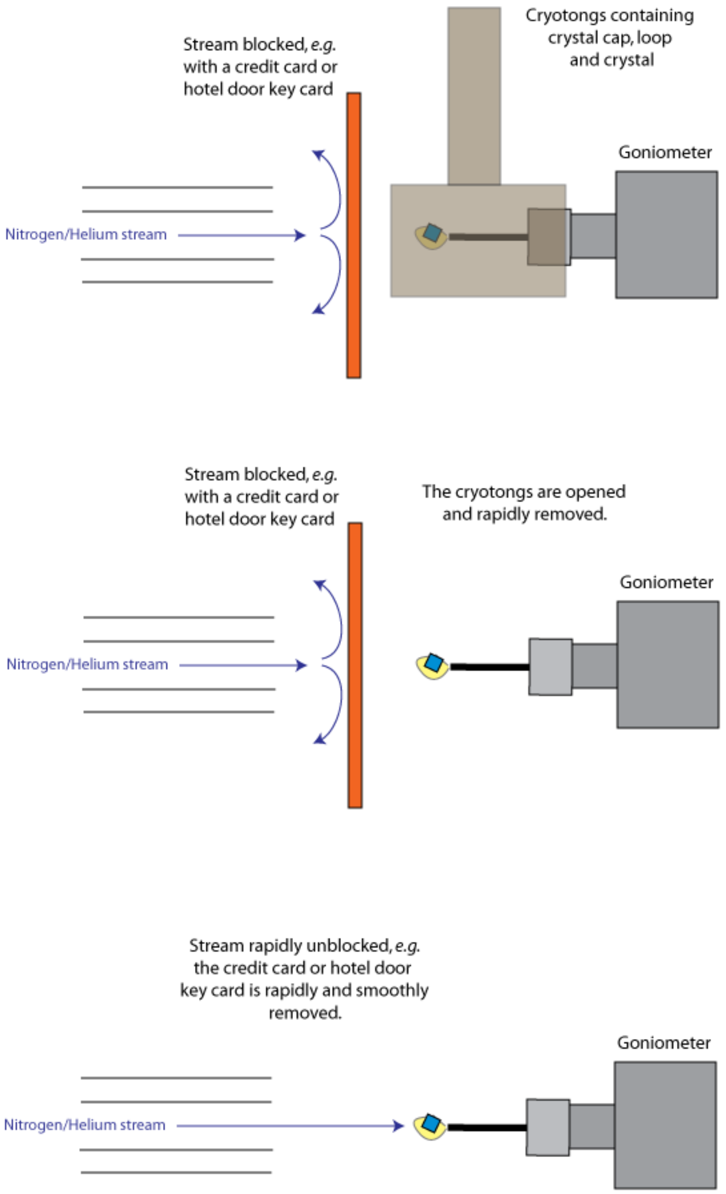
The loop containing the crystal is rapidly raised and as quickly as possible transferred to the cold stream or plunged into liquid nitrogen/propane.



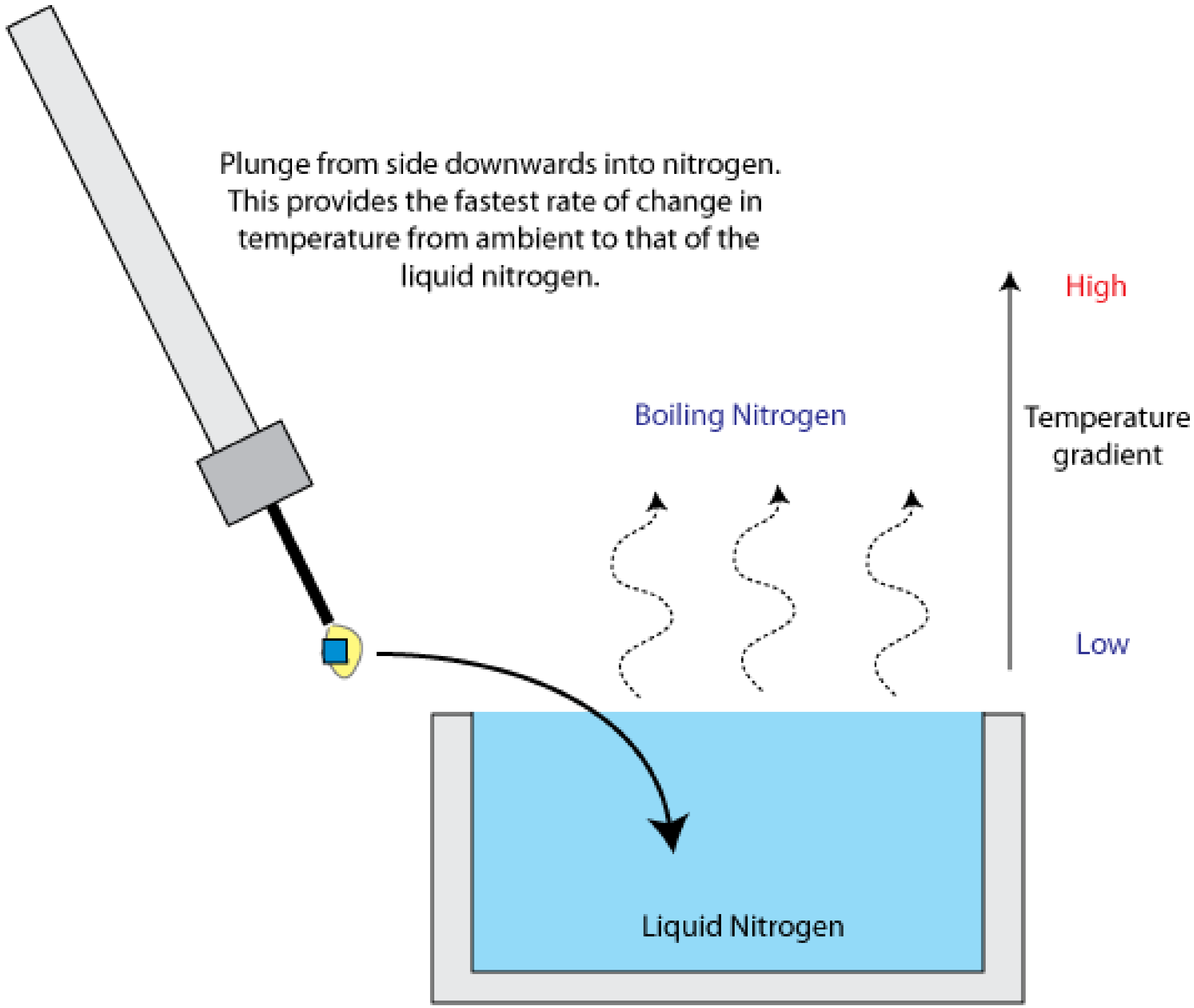
If more than one crystal is available more than one should be mounted and saved.

Note: Every step must be as rapid and smooth as possible to prevent turbulance

Flash cooling in the gas stream



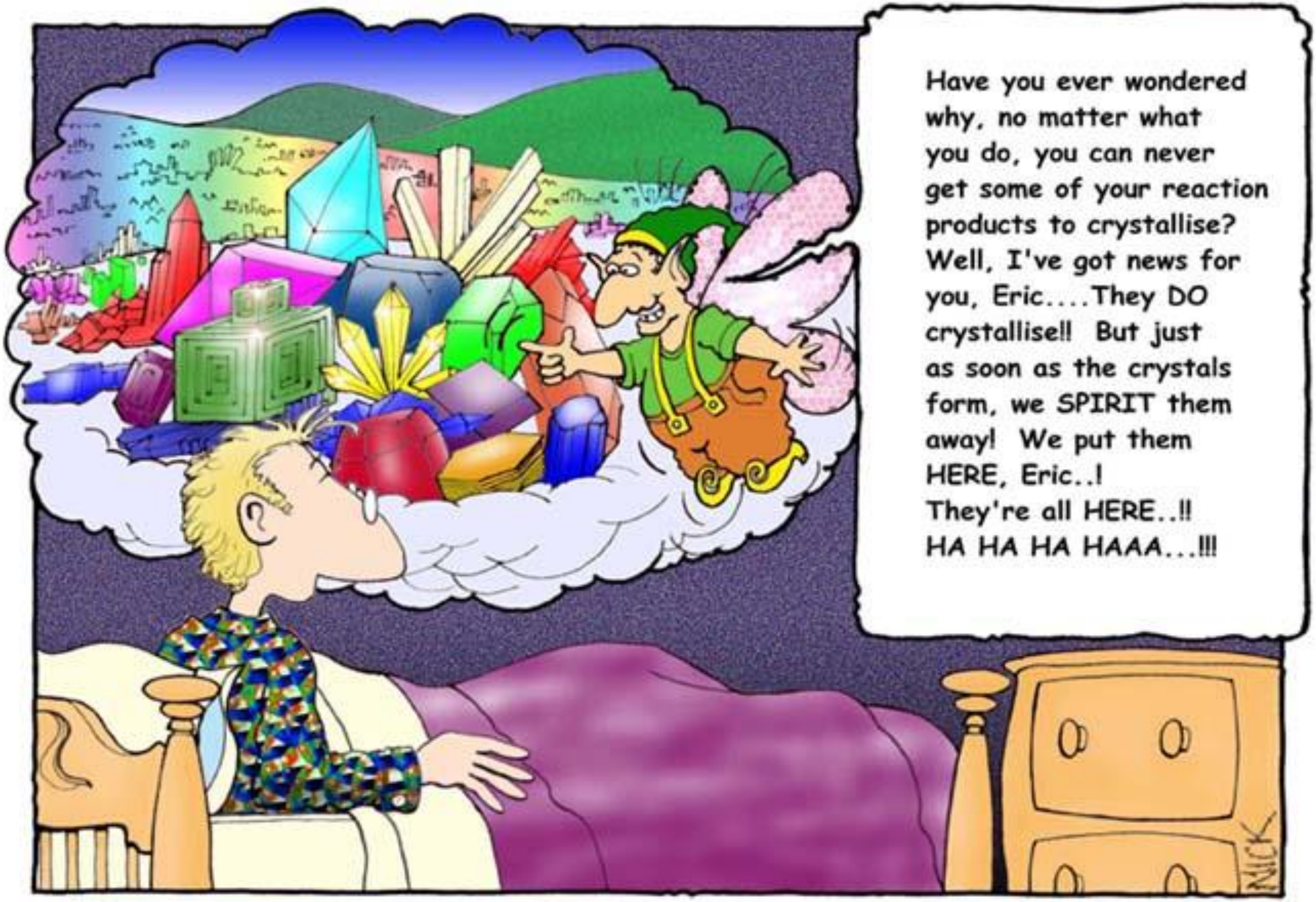
Flash cooling by plunging



Summary

- Rule 1: Think
- Rule 2: Be an optimist (or an optimizer)
- Rule 3: Write everything down
- Rule 4: Know the phase diagram by heart.

Most of all, try. If you get a crystal and get stuck at that point there are plenty of willing hands to help out and many other research opportunities open up.



THE EVIL PIXIE OF REACTION MIXTURES TAUNTS ANOTHER CHEMIST