The importance of nucleation and seeding in protein crystallization, case studies using microseed matrix seeding

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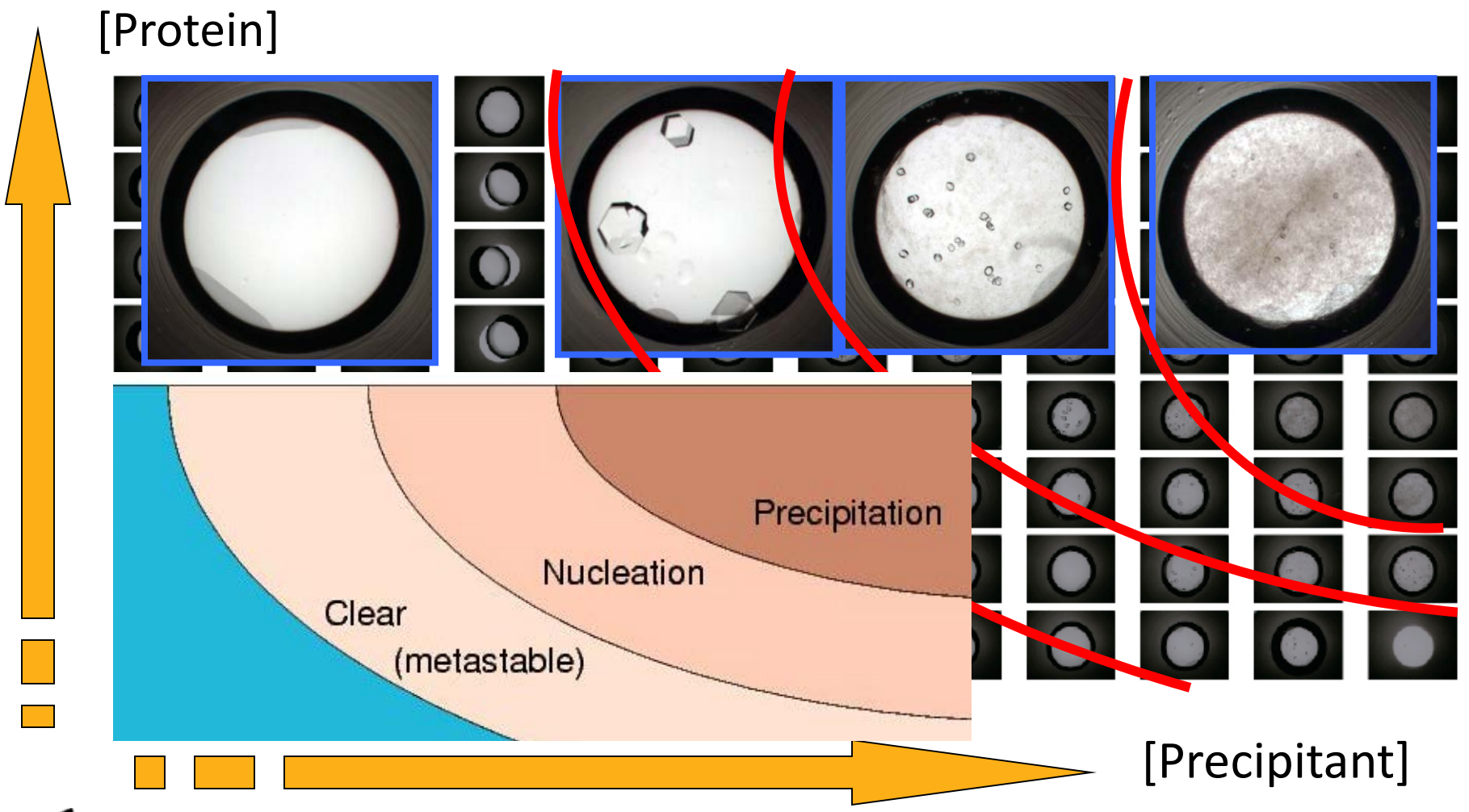
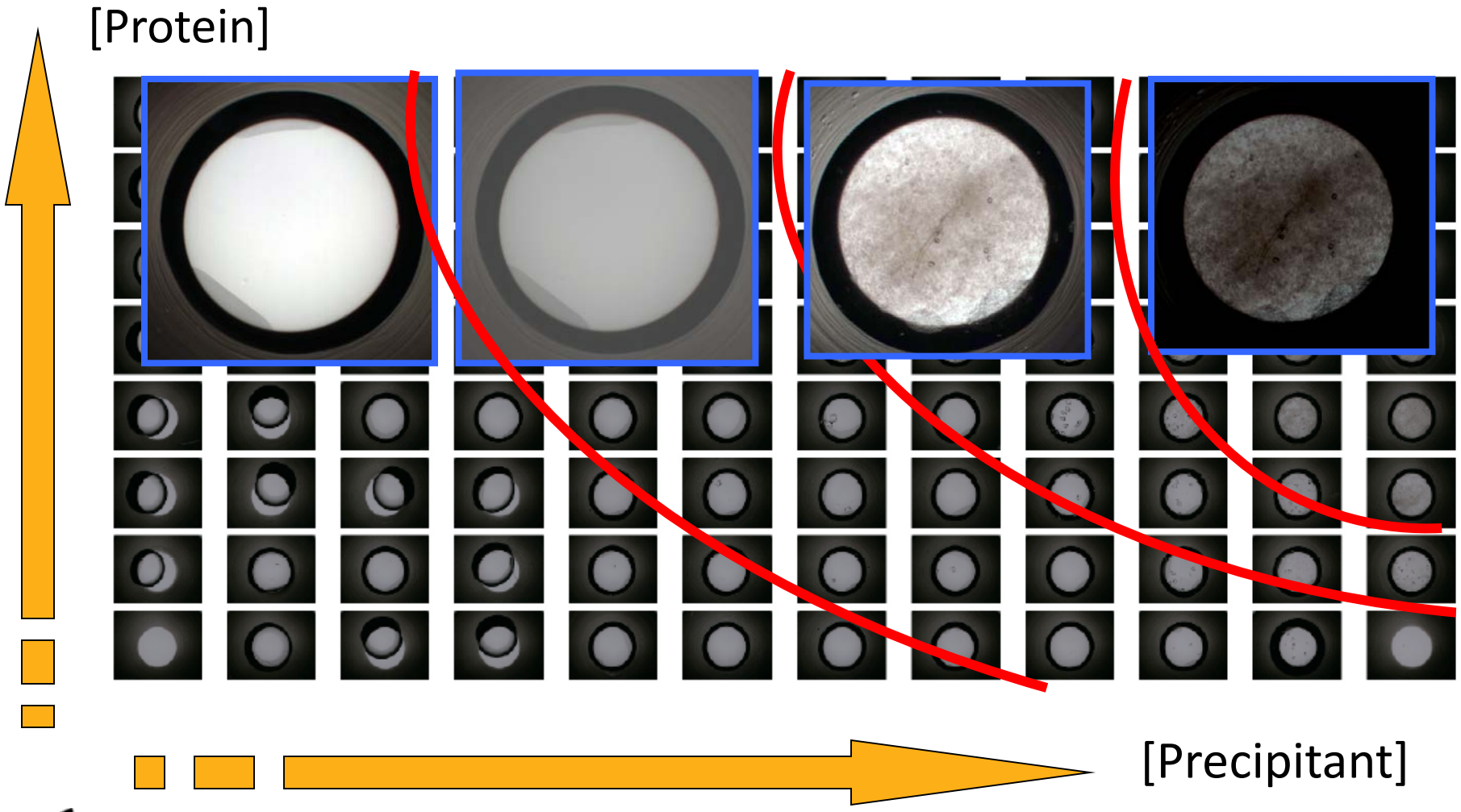
Nucleation

- The growth of protein crystals requires 2 sequential processes and optimum conditions for these processes differ
  - Nucleation (high concentrations of protein and/or precipitant)
  - 3D growth (lower concentrations of protein and/or precipitant)
- In screens there are often too many clear drops because spontaneous nucleation cannot occur
- Have we ignored the nucleation event?

The perfect phase diagram

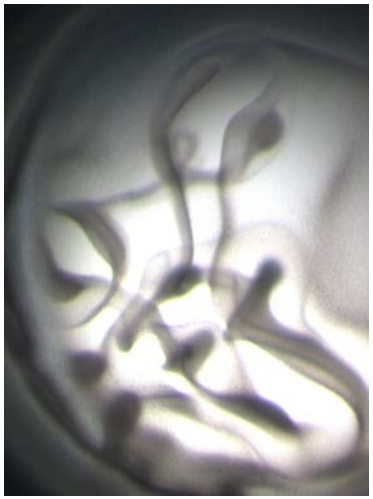


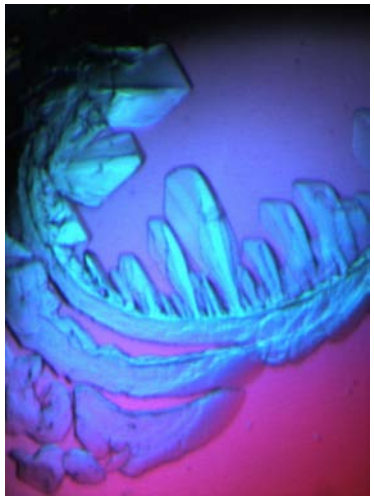
The not so perfect phase diagram



What effects nucleation?

- Sample concentration(1-100mg/ml)
- Sample preparation, filtered?
- Drop set up (shaken or stirred?)
- Drop size
- Surfaces





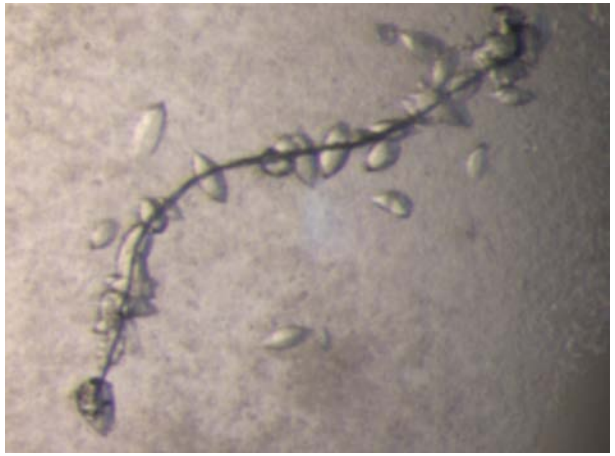
Drop size nanodrops better?

- **D. G. Georgieva, M. E. Kuil, T. H. Oosterkamp, H. W. Zandbergen and J. P. *Abrahams* et al.** *Acta Cryst.* (2007). D**63**, 564-570

Nanocrystallization is not a simple miniaturization of a protein-crystallization experiment and that one cannot reduce the crystallization volume without paying a penalty. In nanovolumes, surface-tension forces become more prominent and might have effects on the nucleation event. Furthermore, Bodenstaff et al. (2002) showed that the mean number of nuclei formed per unit volume is linearly proportional to the volume of the mother liquor. Moreover, when working in a nanolitre regime, the time before the first nuclei are formed increases dramatically.

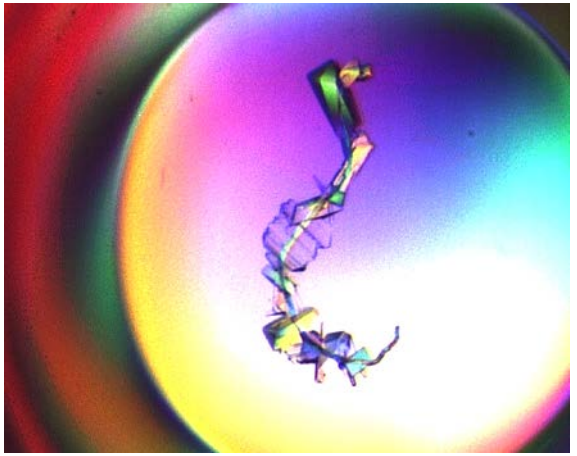
Surfaces

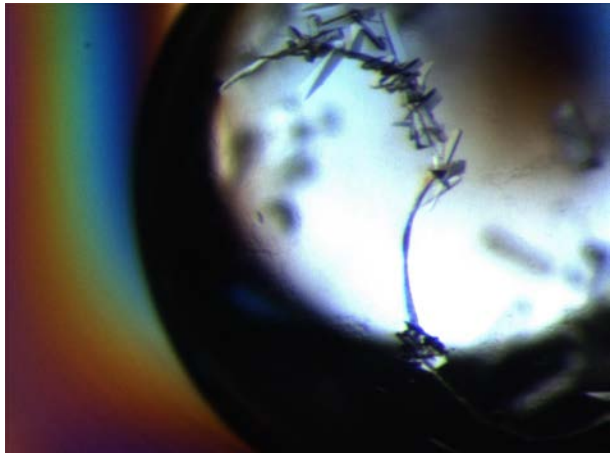
- Viral serine protease

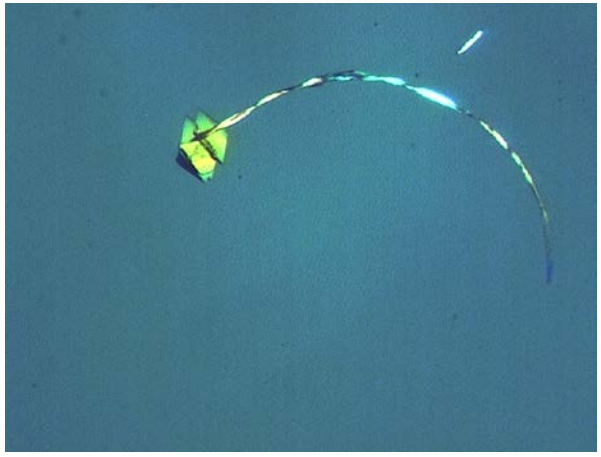


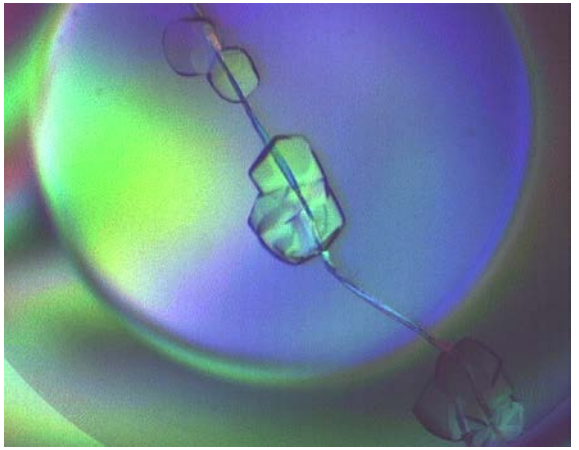
- Cysteine protease

Serine protease









- Cysteine protease

Tryptase

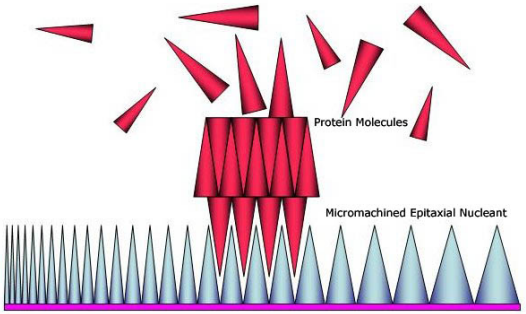
Elastase

The first artifial snow crystals

- *1954* – Ukichiro Nakaya
- Nakaya's real triumph, came from growing artificial snow crystals in the laboratory under controlled conditions.
- This success was apparently due to rabbit hairs falling from his parka hood into the experiment!!!

Can we induce epitaxial nucleation?

- Many have tried
- McPherson and Schlichta 1987: Crushed mineral materials
- Punzi et al.1991: Polyvinylidene Difluoride
- Chayen et al. 2001: Porous silica
- Rong et al. 2004: Porous silica
- Pechkova et al. 2001: 2002 Langmuir–Blodgett technique
- Fermani et al. 2001: Polymeric films.
- Haushalter and McPherson 2002: Nanoengineered Surfaces



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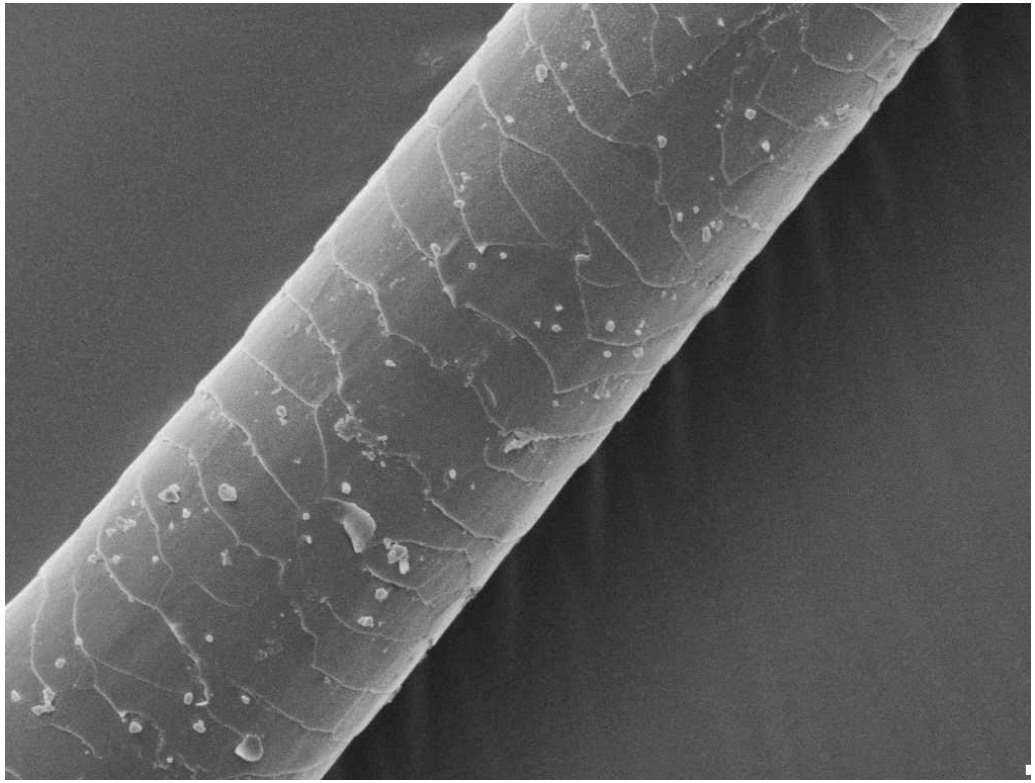
Horse hairs have structured surfaces



“We had a good supply of hairs for the experiments”

Tail hair

Mane hair

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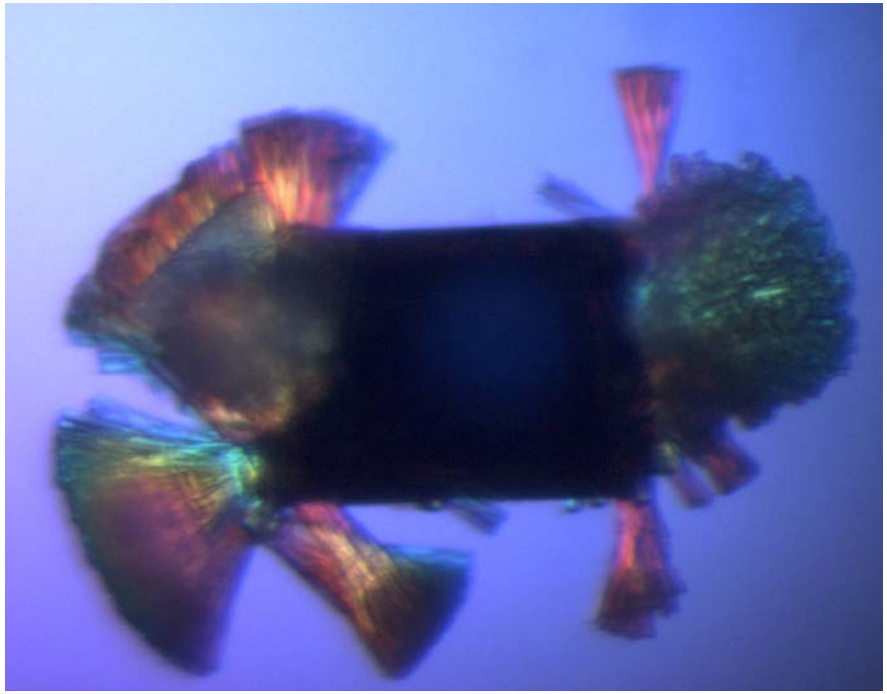
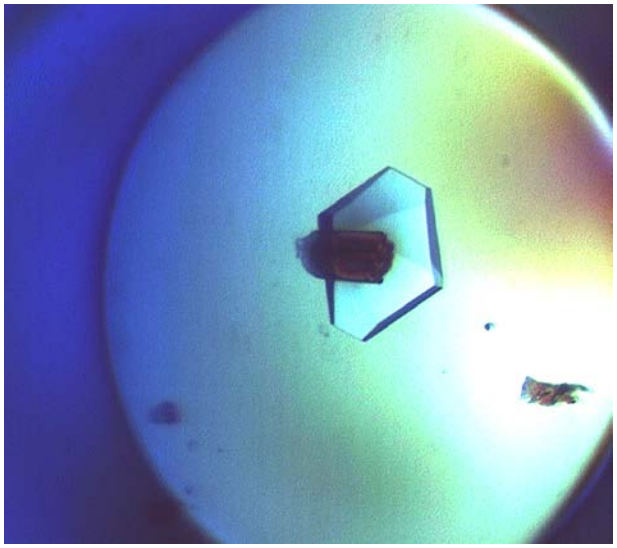
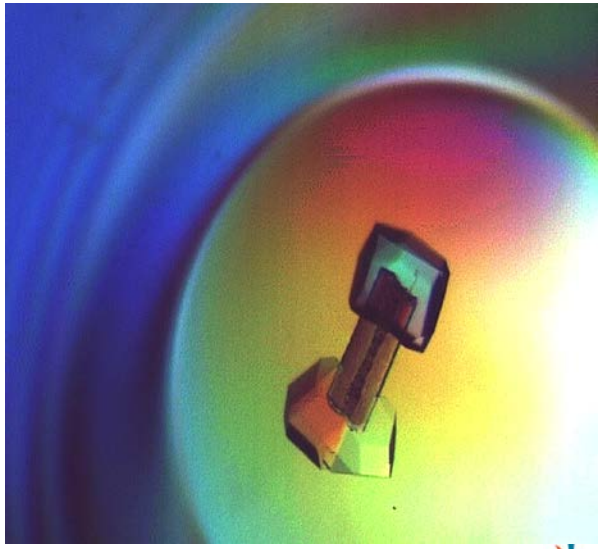
Protein crystal nucleation on horse hair

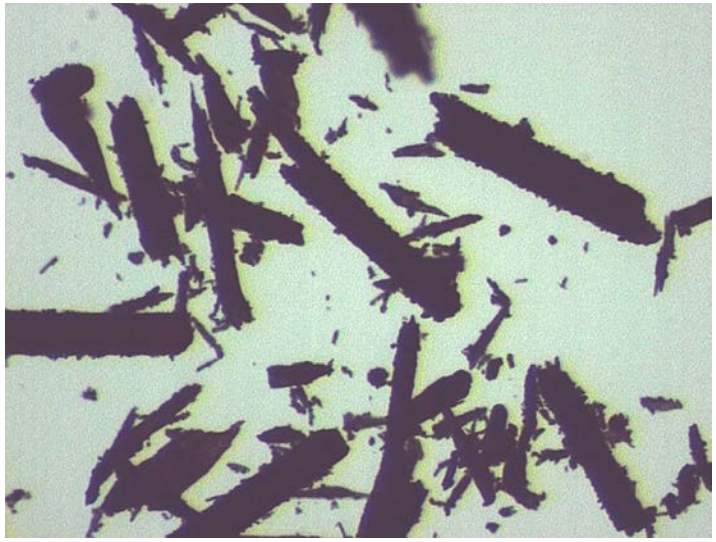




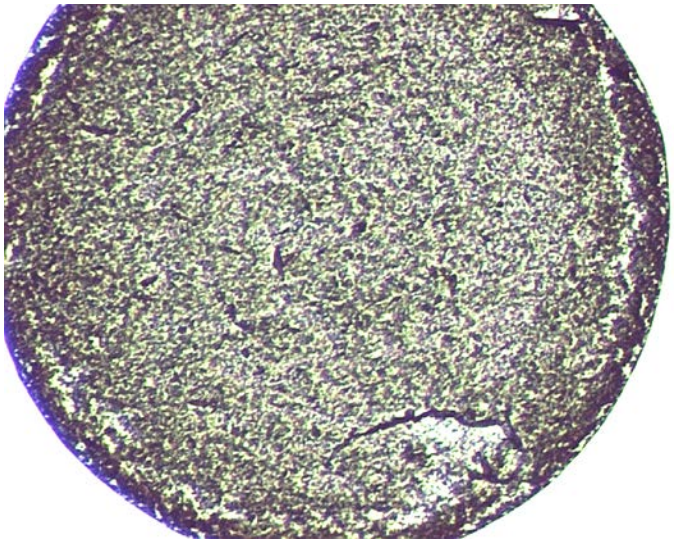


Making a hair seed suspension

Hairs after freezing in liquid nitrogen and mechanical crushing



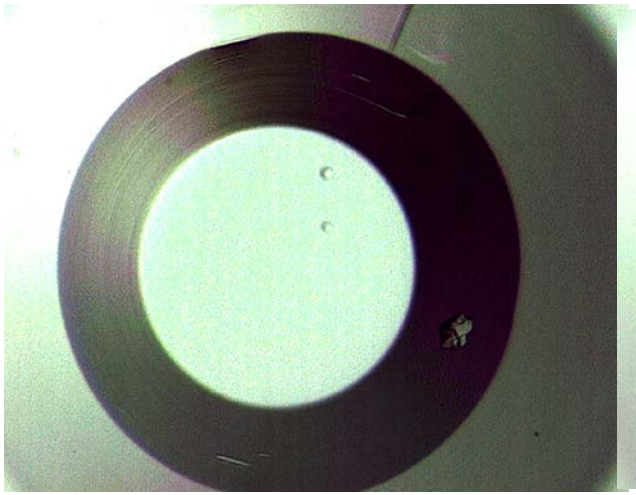
Hairs after re-suspension and sonification



Cause and effect/proof of concept

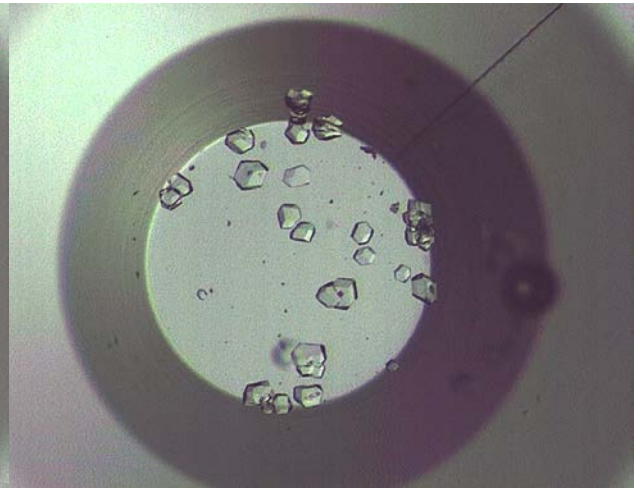
- Horse hair is responsible for nucleation

Control: no hair added

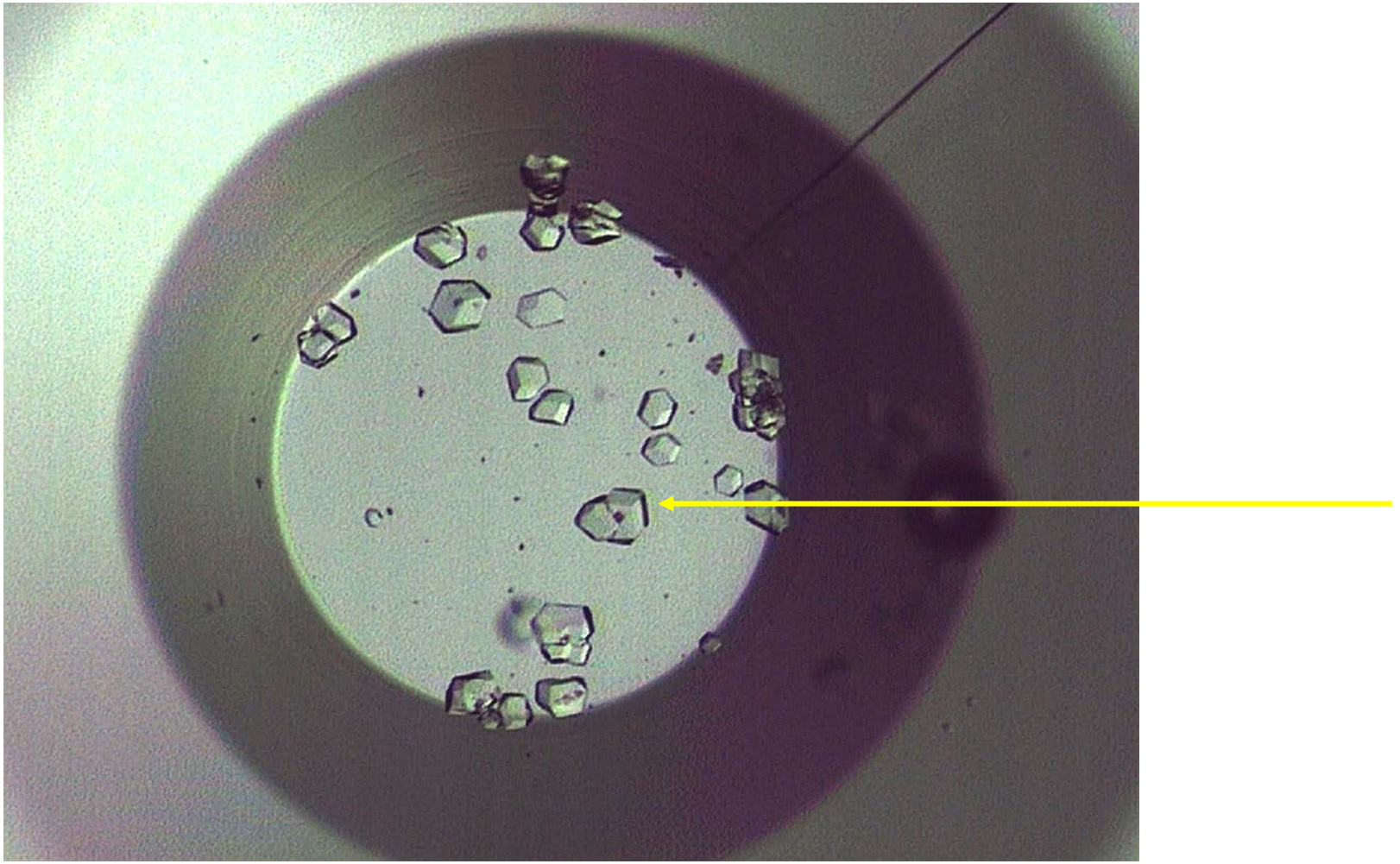


Concentrated hair added

Diluted hair added

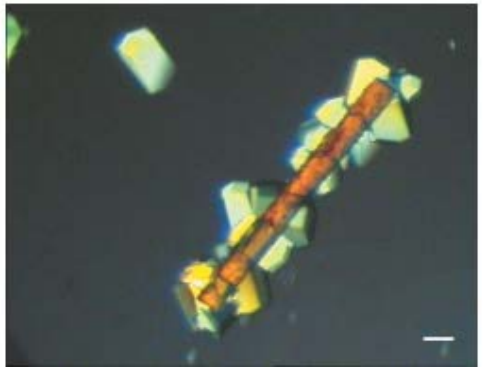
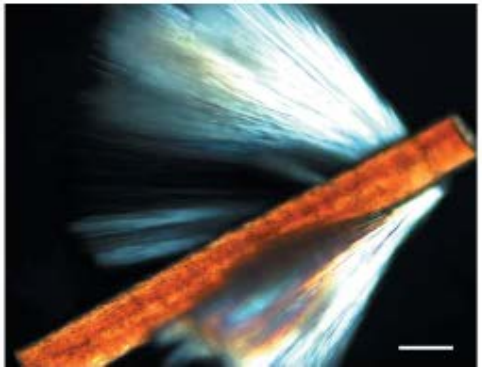
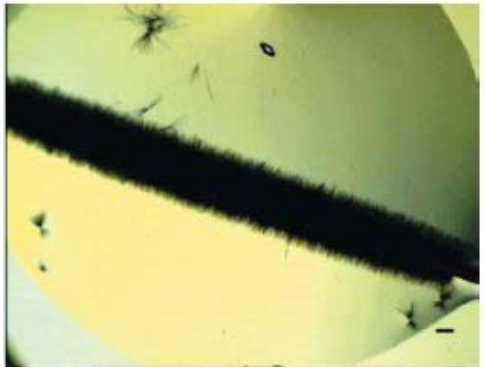


Hair is visible within crystal



Human hair has also been recently cited as a successful nucleant

***Acta Cryst.* (2007). D63, 564-570**
Heterogeneous nucleation of three-dimensional protein nanocrystals
D. G. Georgieva, M. E. Kuil, T. H. Oosterkamp, H. W. Zandbergen and J. P. Abrahams

Good starting point

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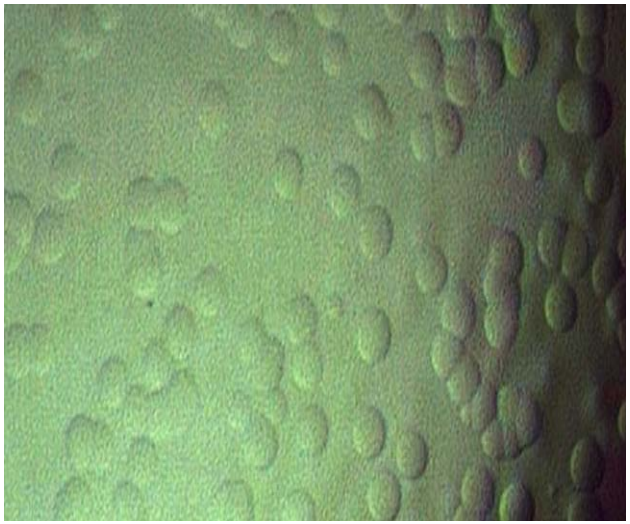
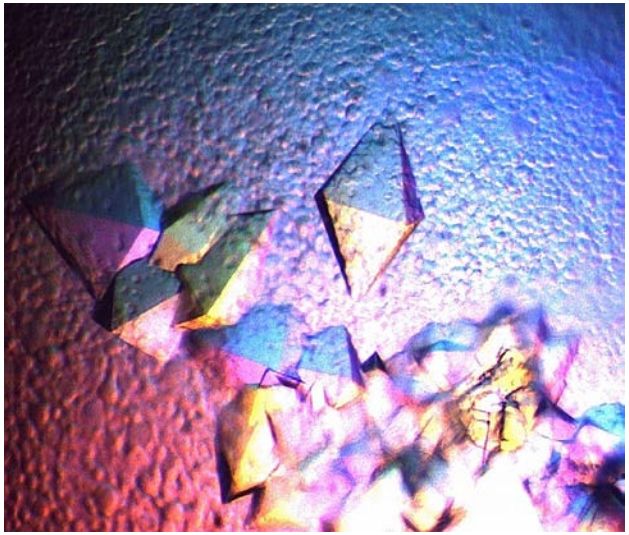
Specific and non specific nucleation

Seed anything that might be crystalline”
(Terese Bergfors)
http://xray.bmc.uu.se/~terese/crystallization/library.html





- A major breakthrough with the introduction of”

“Microseed Matrix Seeding

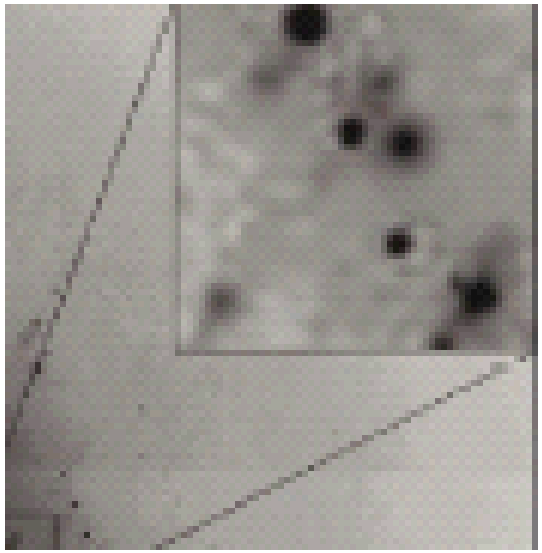
Matrix Seeding Background

- C.G. Ireton and B. Stoddard (2004) Microseed matrix screening to improve crystals of yeast cytosine deaminase Acta Cryst. D**60**, 601-605
- Seeding was carried out into different but similar conditions to improve crystal quality
- Technique termed ‘Microseed matrix seeding’
- Resulted in a dramatic increase in crystal quality and resolution
- We have expanded upon this method by automating the procedure and seeding directly into crystallization screens

Structure can now be solved

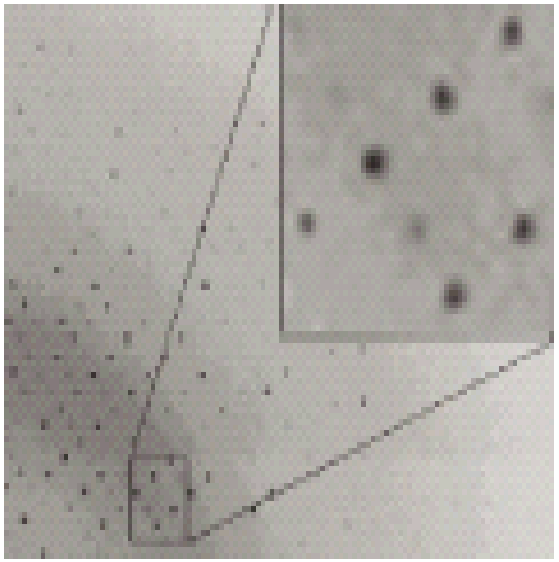
(~28% PEG 8000, 0.1 M sodium cacodylate pH 6.5)

- No Calcium



25% PEG 8000, 0.1 M
calcium acetate, 0.1 M
sodium cacodylate pH 6.5

- + Ca



All attempts to grow the new crystal form under these conditions by de novo nucleation (without transfer of microseeds) were unsuccessful.

Matrix Seeding -Method

- **Seed stock preparation:**
  - **Select best crystals possible**
  - **Crush crystals in their reservoir solution using Hampton tools**
  - **Transfer to Hampton seed bead and vortex for at least 3 minutes or place on Thermoshaker 10“ on 2“ off for 10-30 mins**
  - **Store at -80°C**
- **Screen Setup**
  - **Use Oryx robot with seeding function**
  - **Dispensed drops 0.6µl drops containing 0.3µl protein**

    0.2µl reservoir solution

    0.1µl seed stock

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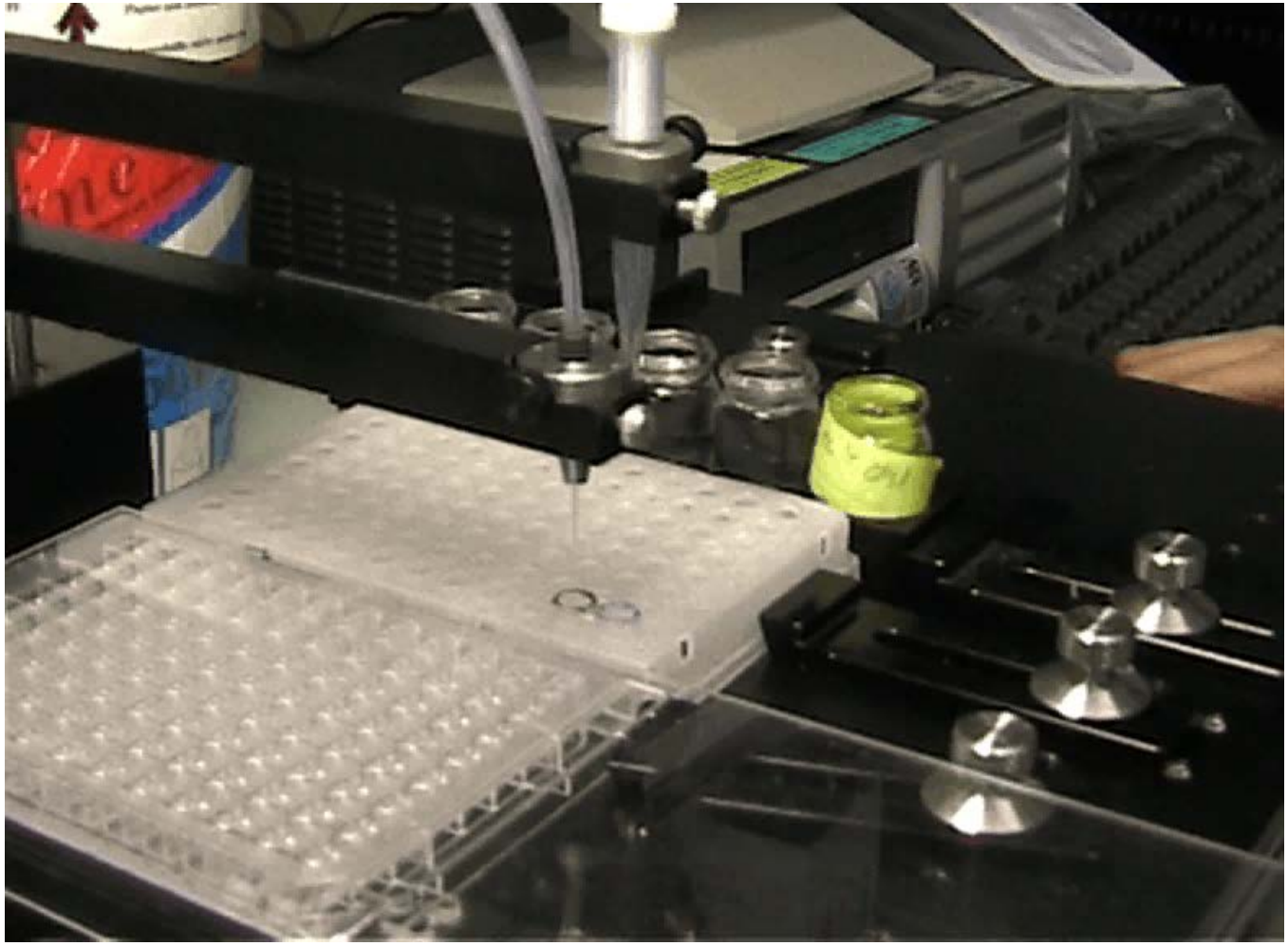
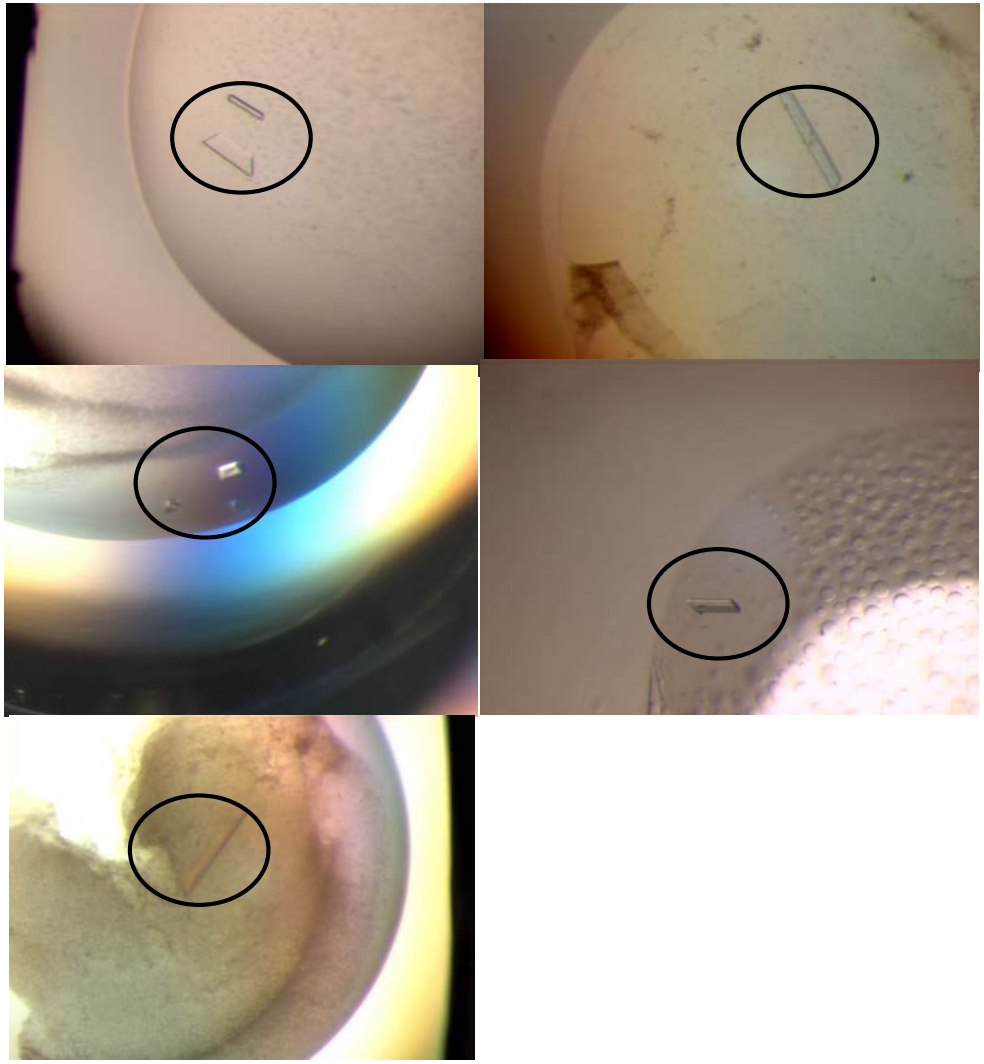
Seeds are stable!!!!

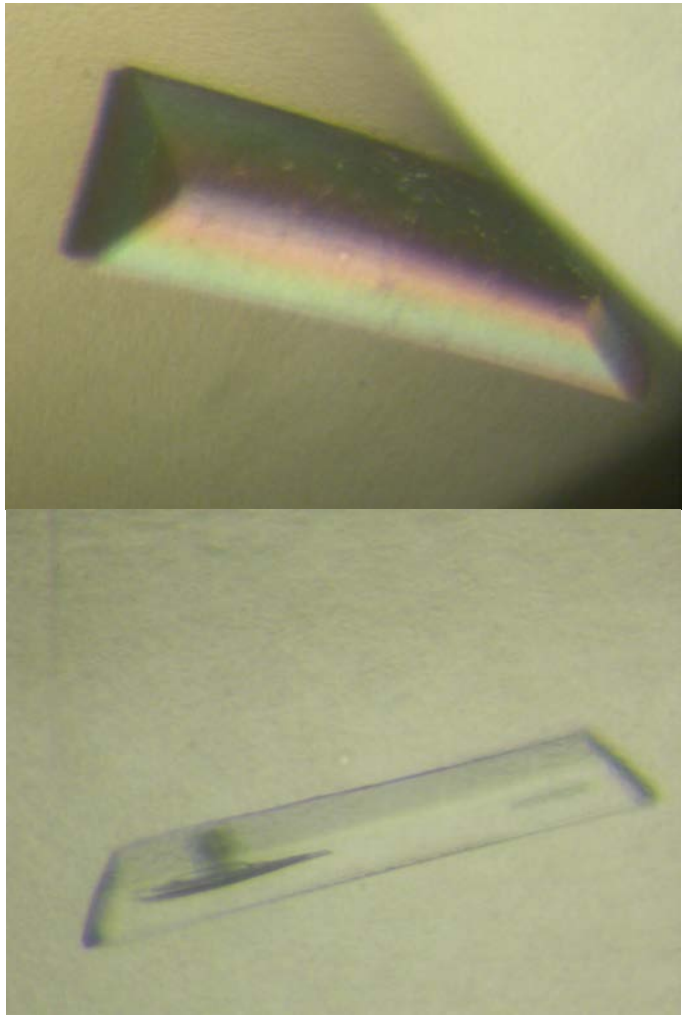
Once the correct seed dilution has been determined the seed stocks can be frozen at- 80ºC, thawed and refrozen many times

The experiment

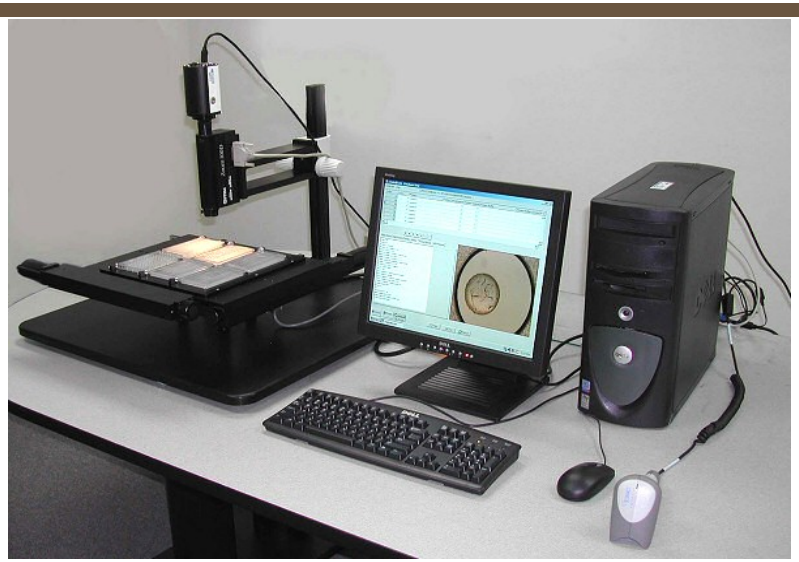


Identifying false positives: protein or salt?





Imaging and hit validation



Filter 1Transmission from 260 – 395 nm
Filter 2Transmission from 270 - 320 nm

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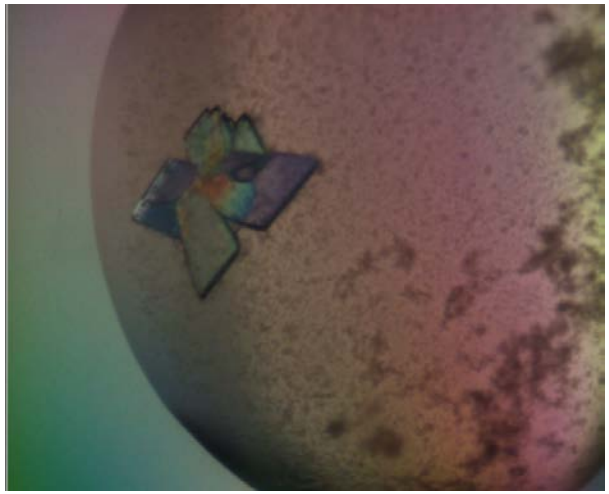
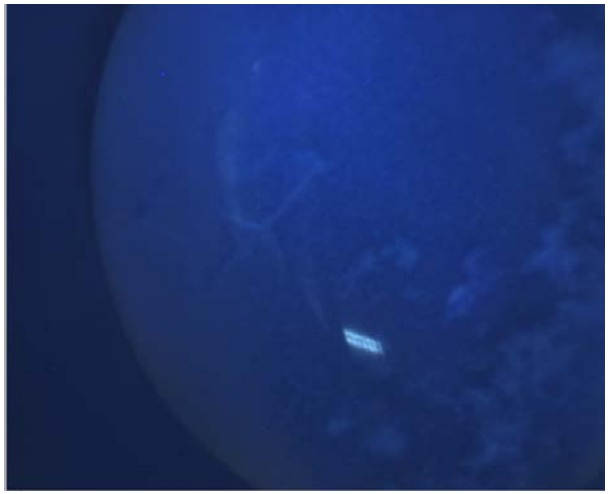
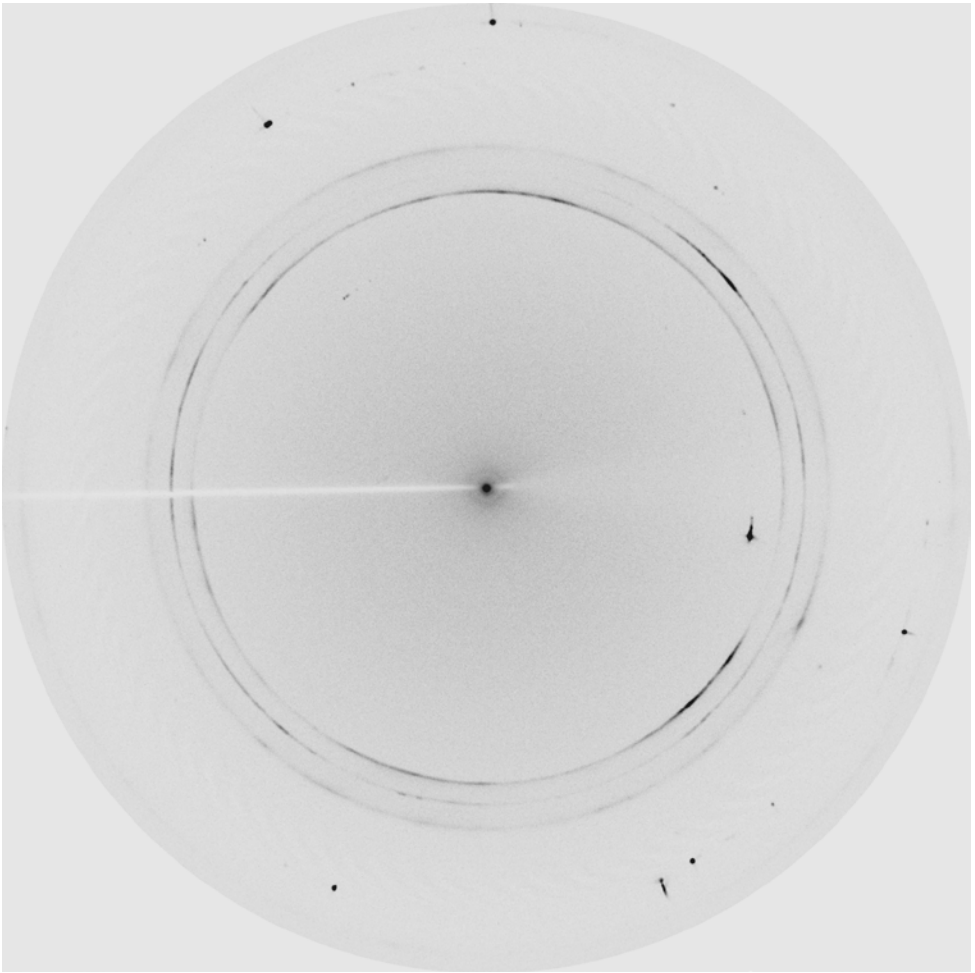
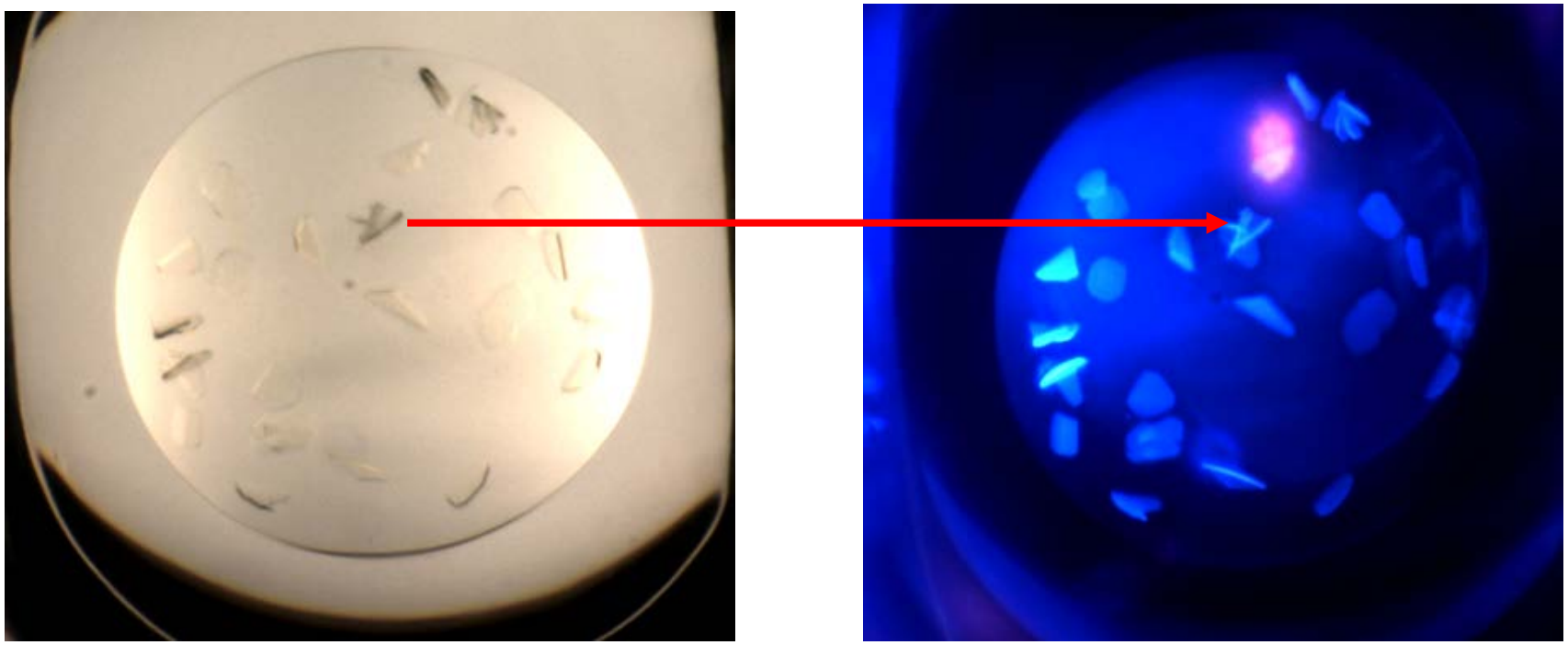
Salt crystals







Example of confirmed hit

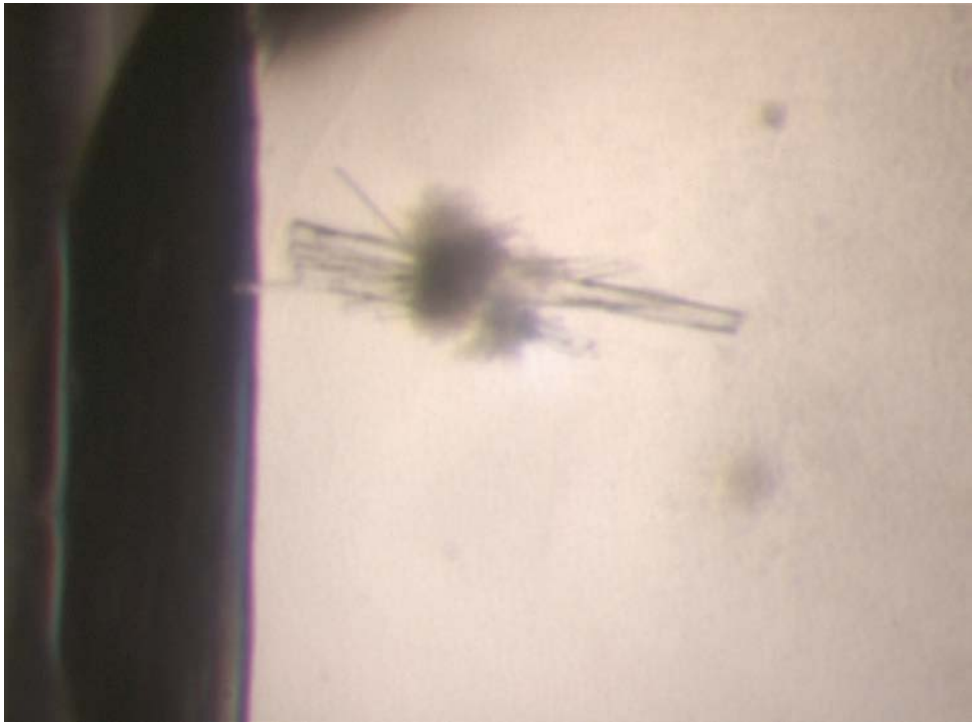


High throughput matrix seeding

Initial Results

Bi-functional viral protein

Original hit in Peg screen



Matrix seeding in new screen

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	0	0	0	0	0	0	0
B	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0
D	0	0	0	0	0	0	0	0	0	0	0	0
E	0	0	0	0	0	0	0	0	0	0	0	0
F	0	0	0	0	0	0	0	0	0	0	0	0
G	0	0	0	0	0	0	0	0	6	0	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0

- 1 hit

No seeding

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	0	6	6	6	6	0	6
B	0	6	6	6	0	6	0	6	0	6	6	6
C	0	0	0	0	0	0	6	6	6	6	6	6
D	6	0	6	6	6	6	0	0	6	6	6	6
E	6	0	6	6	0	6	6	6	6	6	0	6
F	6	0	0	0	6	6	6	6	6	6	6	0
G	6	0	6	6	6	6	6	6	6	6	6	0
H	6	6	0	6	6	6	6	6	0	6	0	0

- 64 hits

+ seeding

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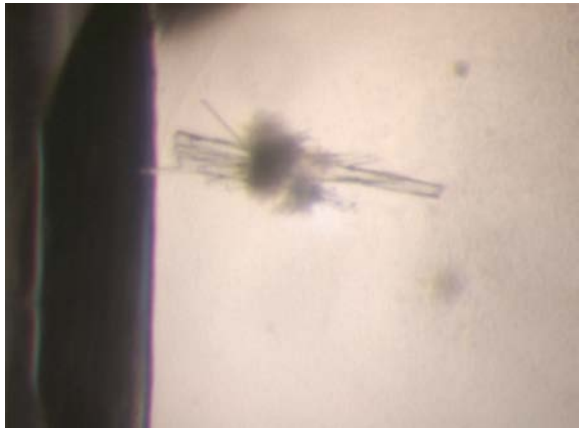
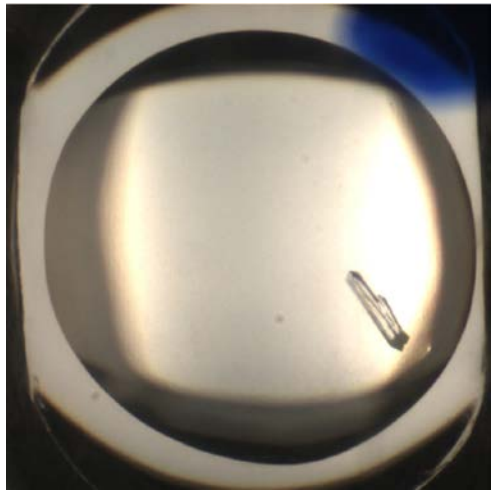
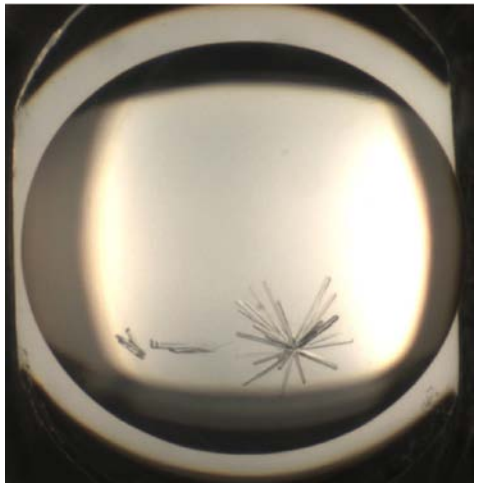
Homing in on the best hits

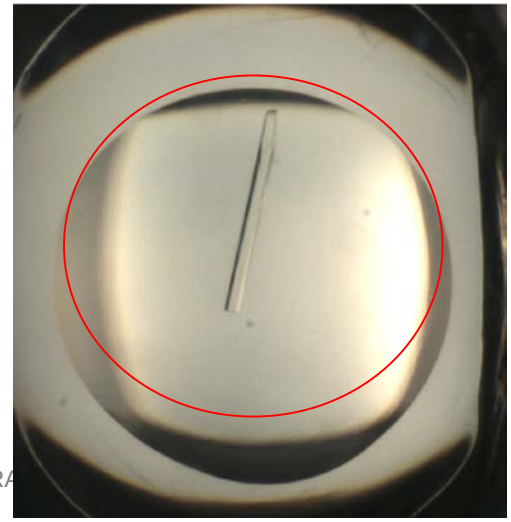
- Starting seeds

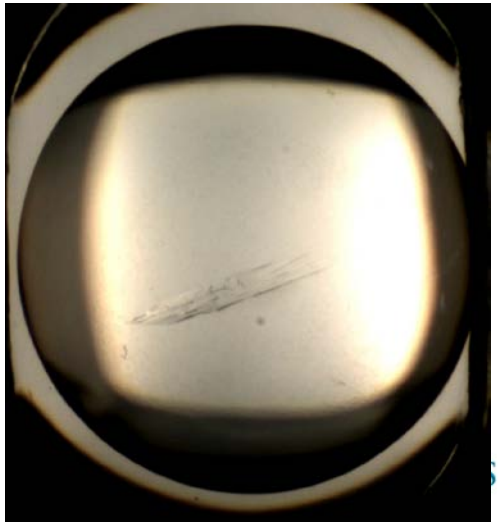


- Hits from seeded screen

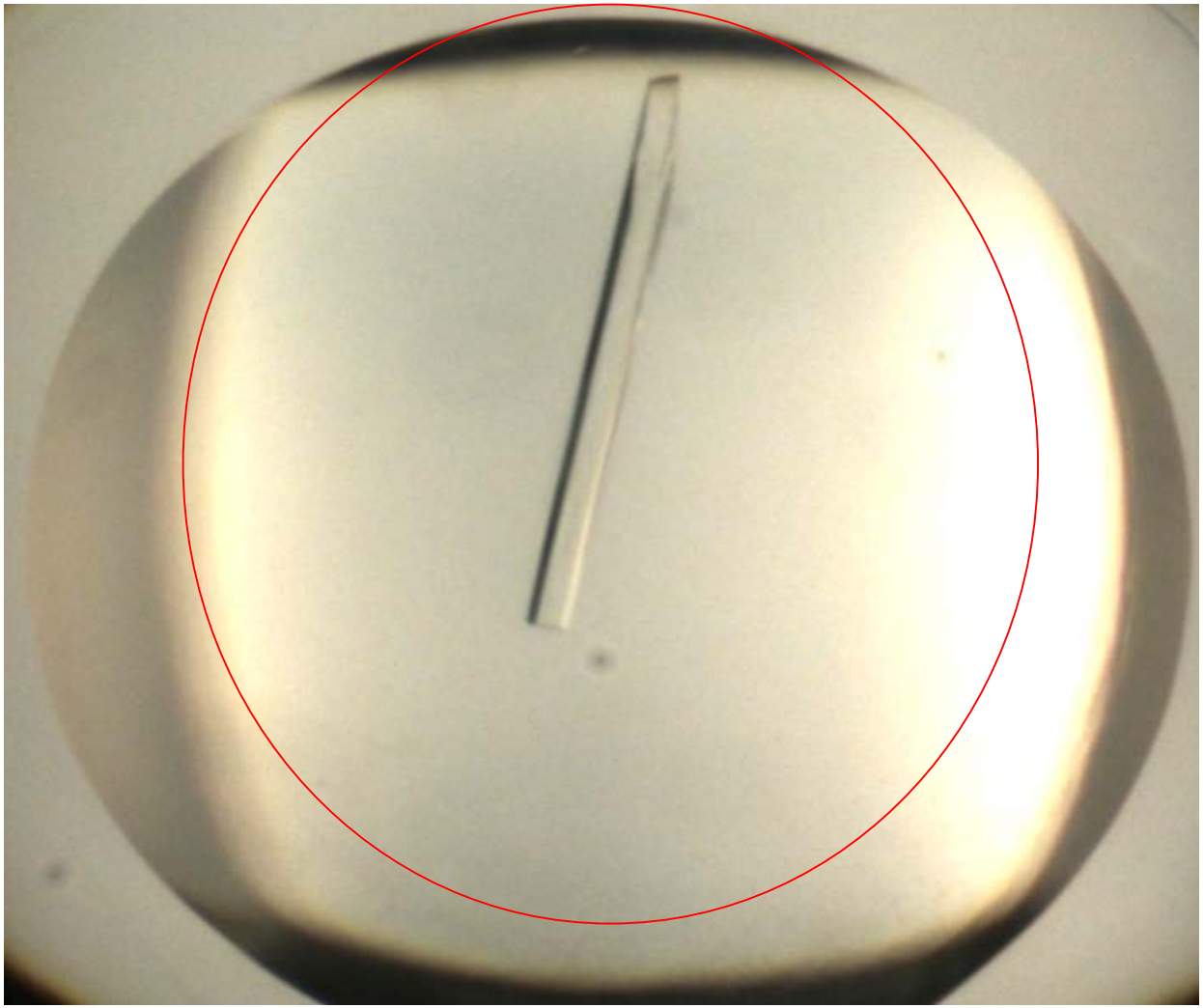






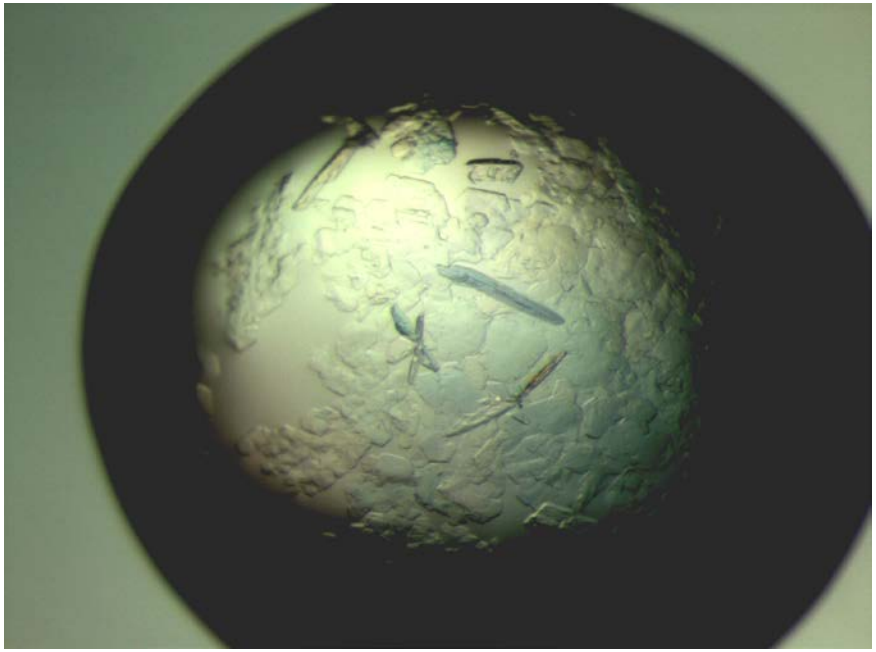


Clean single crystals



Cys protease

- Starting crystals using published conditions



Matrix seeding in new screen

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	0	0	0	0	0	0	0
B	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	6	0	0	0	0	0	0
D	0	0	0	0	0	0	0	0	0	0	0	0
E	0	0	0	0	0	0	0	0	0	0	0	0
F	0	0	0	0	0	0	0	0	0	0	0	0
G	0	0	6	0	6	0	0	0	0	0	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0

- 3 hits

No seeding

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	0	6	6	6	6	6	6
B	6	6	6	6	6	6	0	0	0	6	6	6
C	0	0	0	0	0	0	6	6	6	6	6	0
D	6	6	6	6	6	0	6	0	0	6	6	0
E	6	0	6	6	6	6	0	6	6	6	6	6
F	0	0	6	6	6	6	6	6	6	6	6	6
G	0	0	6	6	6	6	0	0	0	0	0	0
H	0	0	0	6	0	0	0	0	0	0	0	0

- 53 hits

+ seeding

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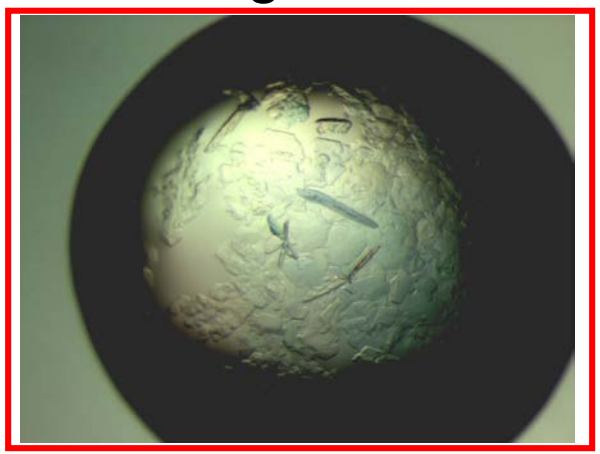
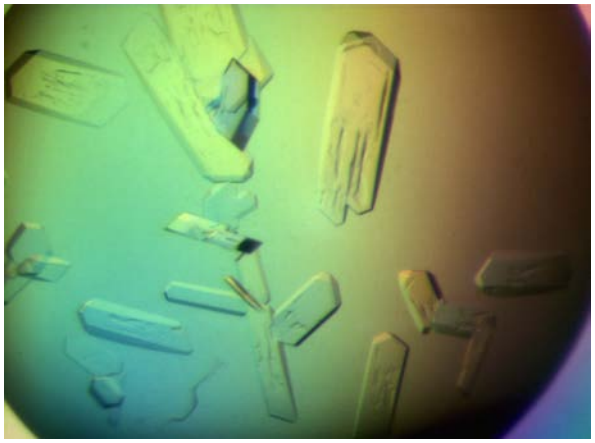
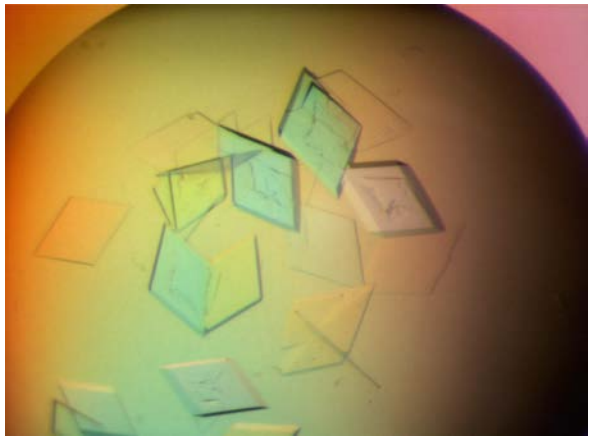
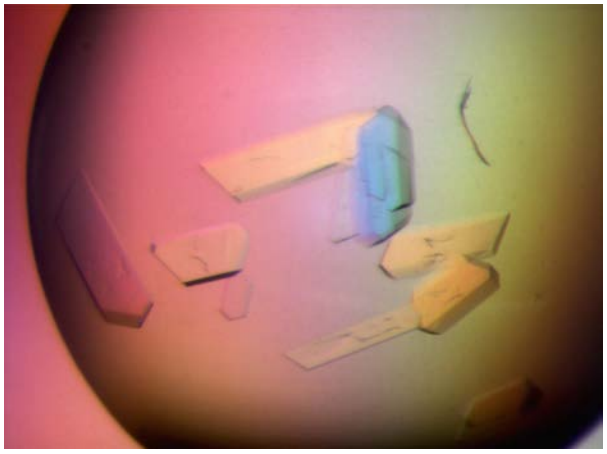
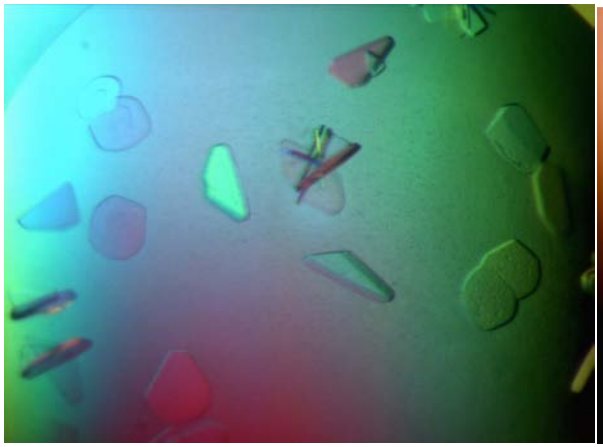
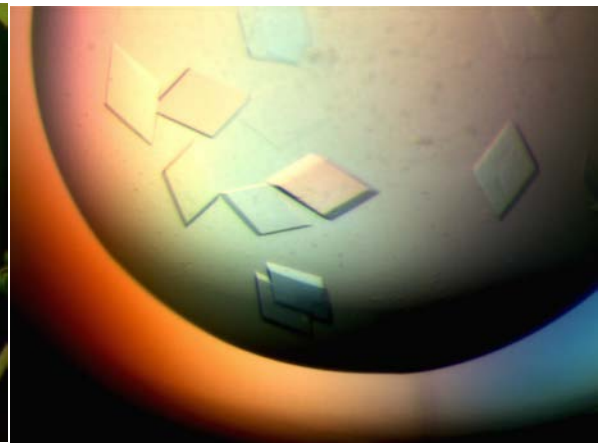
Hits from seeded screen

- Starting seeds

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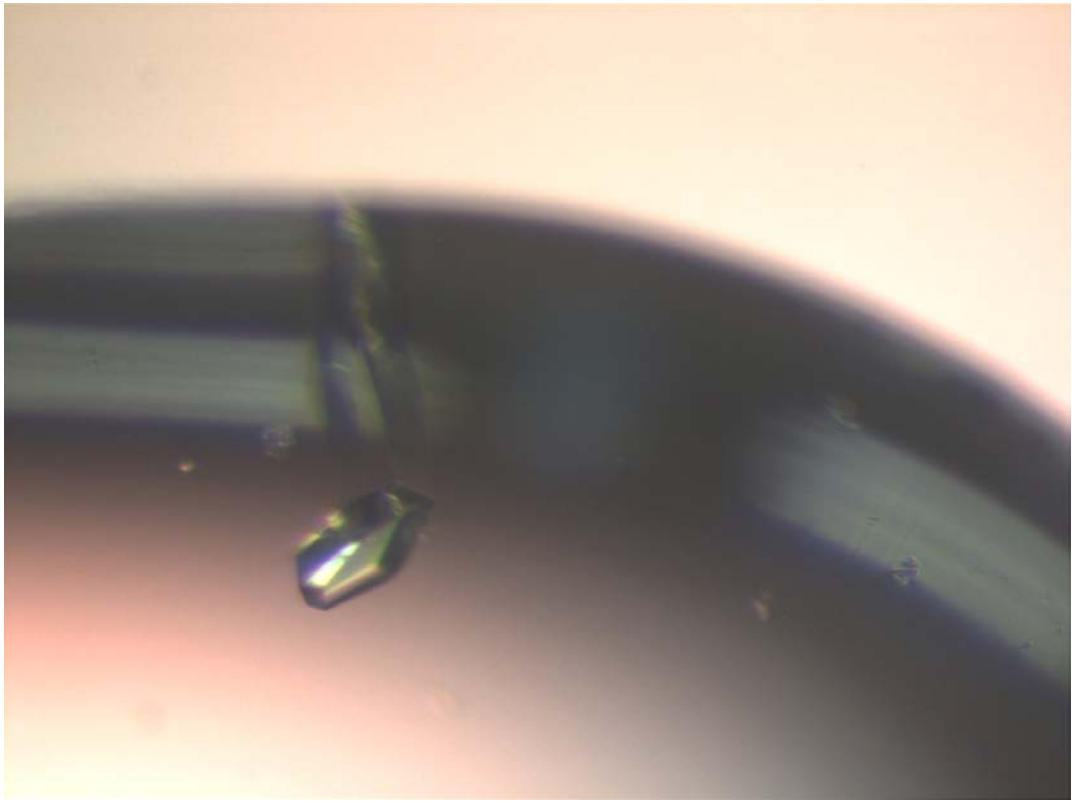
Metallo protease

- Only one crystal grew spontaneously in published conditions



Matrix seeding in new screen

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	0	0	0	0	0	0	0
B	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0
D	0	0	0	0	0	0	0	0	0	0	0	0
E	0	0	0	0	0	0	0	0	0	0	0	0
F	0	0	0	0	0	0	0	0	0	0	0	0
G	0	0	0	0	0	0	0	0	0	0	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0

- No hits

No seeding

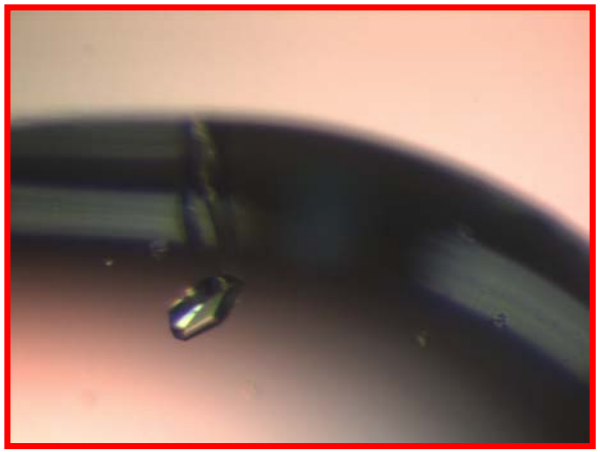
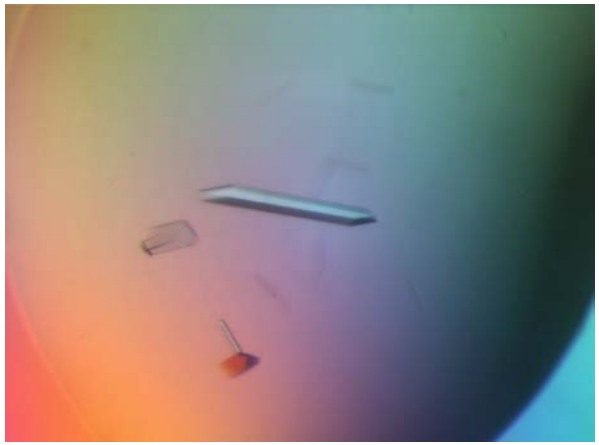
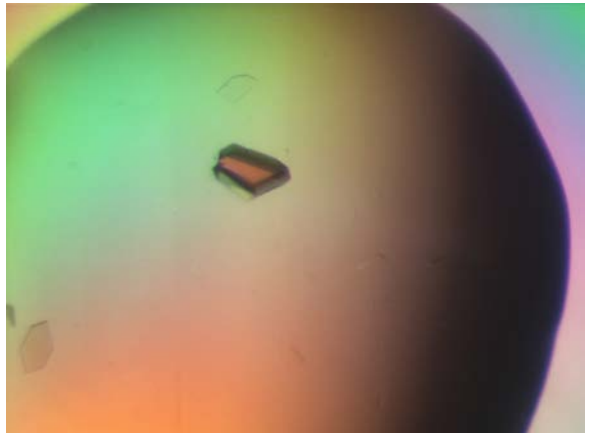
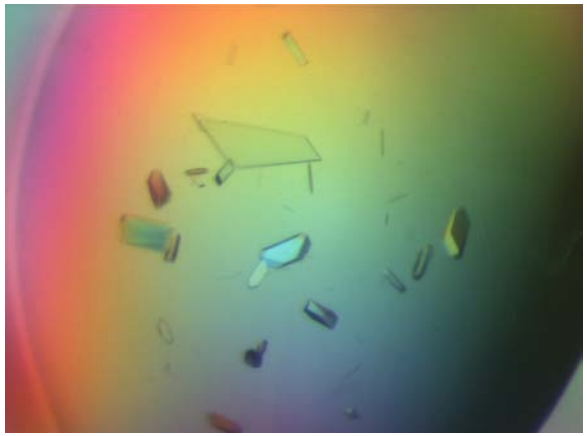
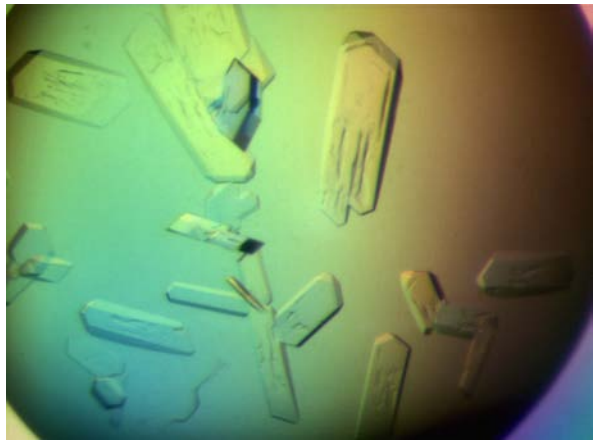
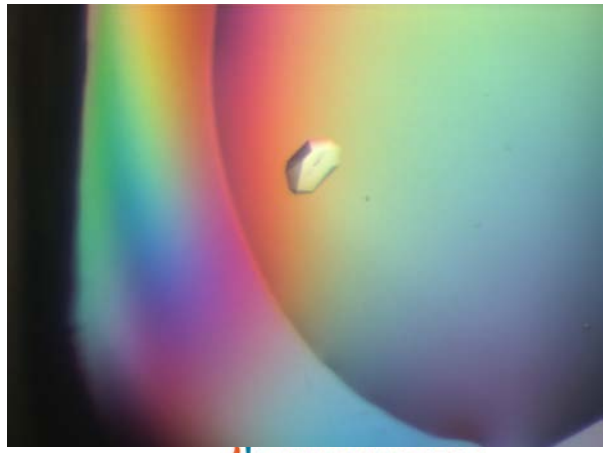
	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	0	0	0	0	0	0	0
B	0	0	0	0	6	6	0	0	0	0	0	0
C	0	0	0	0	0	0	6	6	6	6	6	0
D	6	6	6	6	6	6	6	6	6	6	0	0
E	0	0	0	6	6	6	6	6	0	6	0	0
F	6	6	6	6	6	6	6	6	0	0	0	0
G	6	0	6	6	6	0	6	6	6	6	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0

- 39 hits

+ seeding

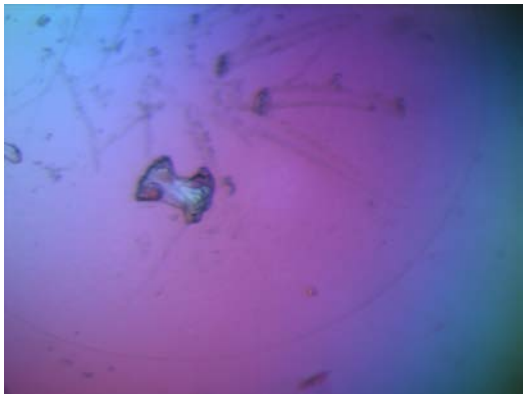
Hits from seeded screen

- Starting seeds

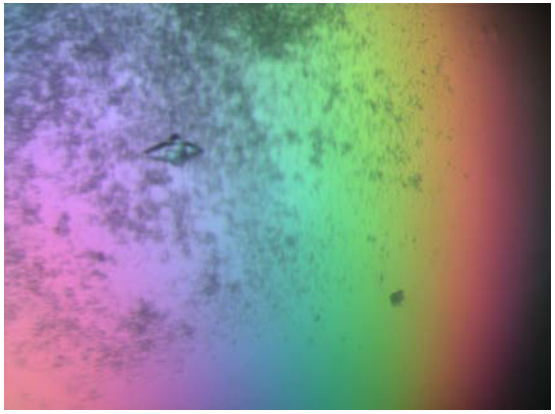







Solving some real problems

0.1 M Succinic Acid pH 7.0, 15% w/v PEG 3350



0.2 M Mg Chloride, 0.1 M HEPES pH 7.5, 25% w/v PEG 3350

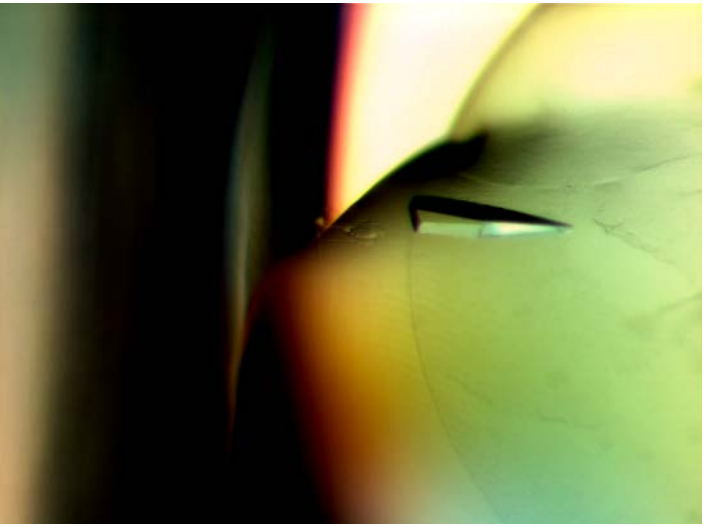


Initial hits

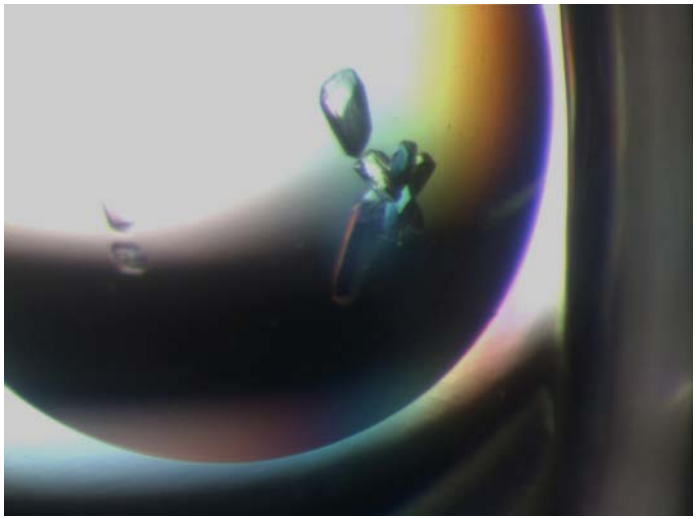
- Used for matrix seeding

Best crystal from seeded screen

Hits in 100mM Mg Formate 15% peg 3350



Seeded into same conditions



Used for new matrix seeding screen

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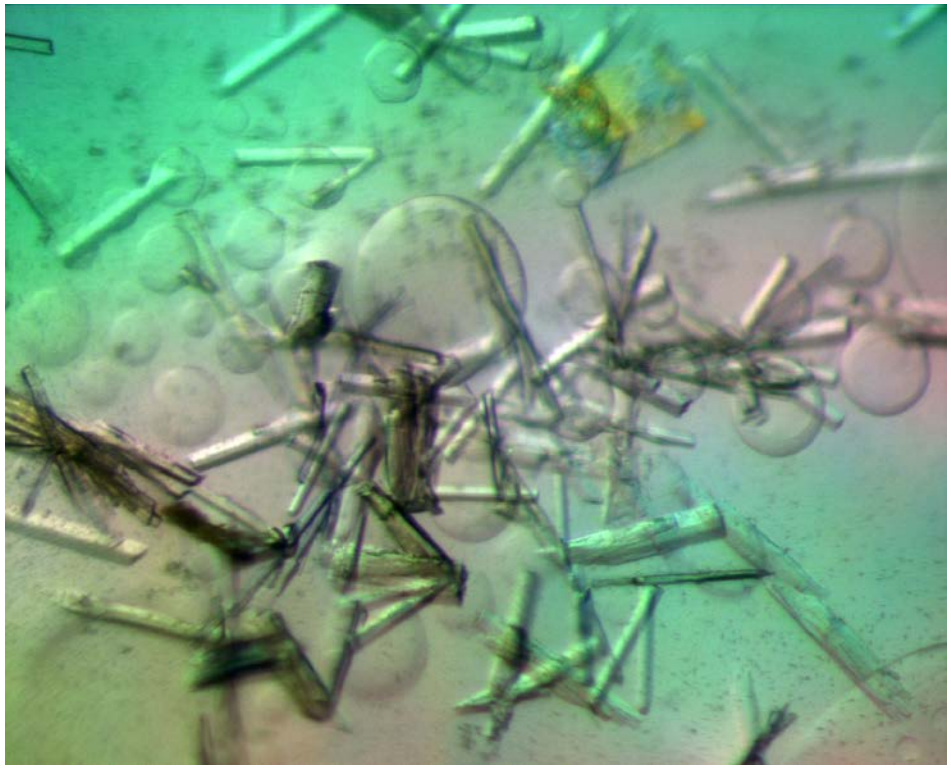
Reproducible system, structure solved

- New crystals with wild type protein

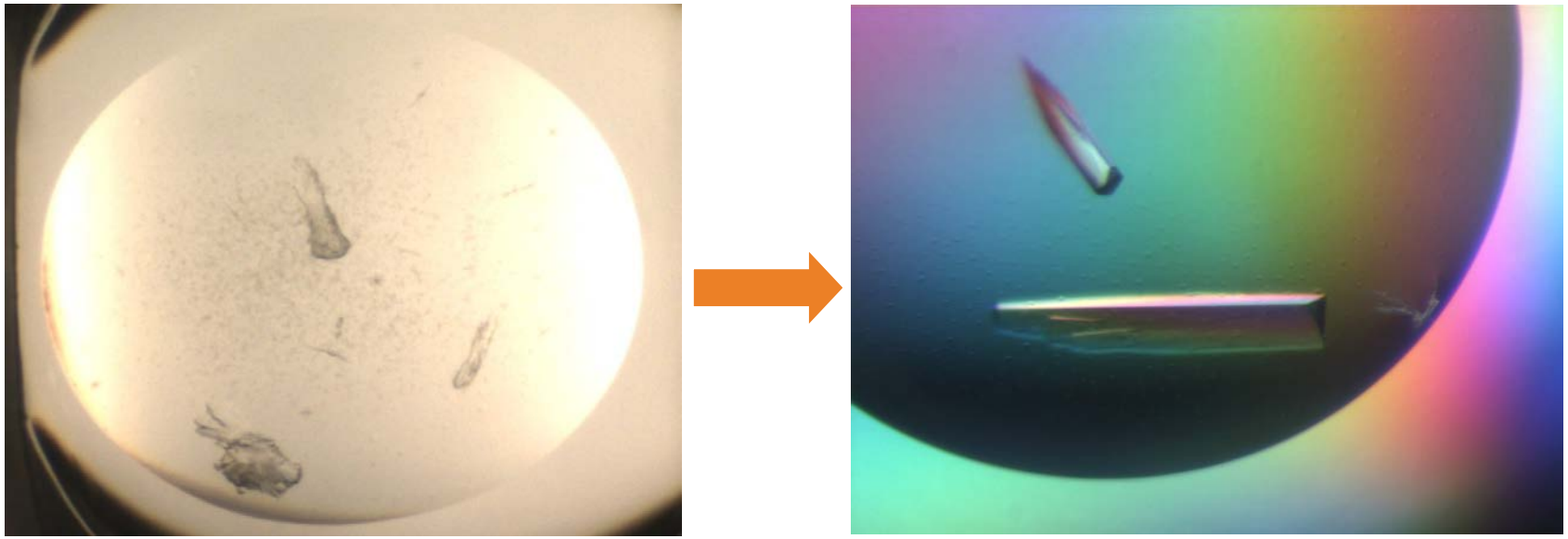


- Note the conditions

1.1M di ammonium tartrate
100mm bis tris pH 6.0/7.0

General trend

- 27 proteins tested
- 25 showed improvement (increased hit rate, better morphology or better diffraction)
- 92.6%



Labs having success with MMS: http://www.douglas.co.uk/mms.htm

Novartis (2007) reported that the average number of hits obtained for 5 target proteins increased by a factor of 7. Other users have reported similar improvements. For example

Lesley Haire (NIMR, UK) obtained 6 hits in a screen, all poorly formed. After using MMS, around 30 hits were obtained, including several well-formed crystals.

Jens-Christian Navaro Poulsen (University of Copenhagen) obtained 1 hit in 288 wells. After using MMS, 10 hits were found in 96 wells. Then, using one of those crystals for a second-generation MMS experiment, a further 10 hits were found in another 96 wells.

Laura Cendron (University of Padova) used the same approach with a protein that crystallized easily, but tended to give small crystals. The best crystals obtained without microseeding are shown on the left. After microseeding, several good crystals were obtained (right).

Terses Bergfors (University of Uppsala)
Increased hit rates, statistics in preparation

Experimental Goals

- Demonstrate general application of method 
- Show success on different classes of proteins? 
- Find new Space Groups? 
- Improve diffraction quality? 
- Fine useful hits when there were none before? 
- Demonstrate cross seeding between different proteins?

Acknowledgements

Frederic Villard

May Marsh

James Smith

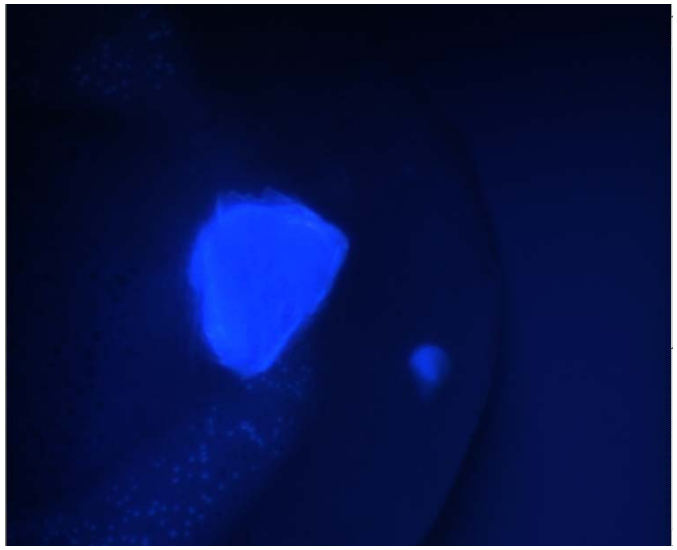
Terese Bergfors

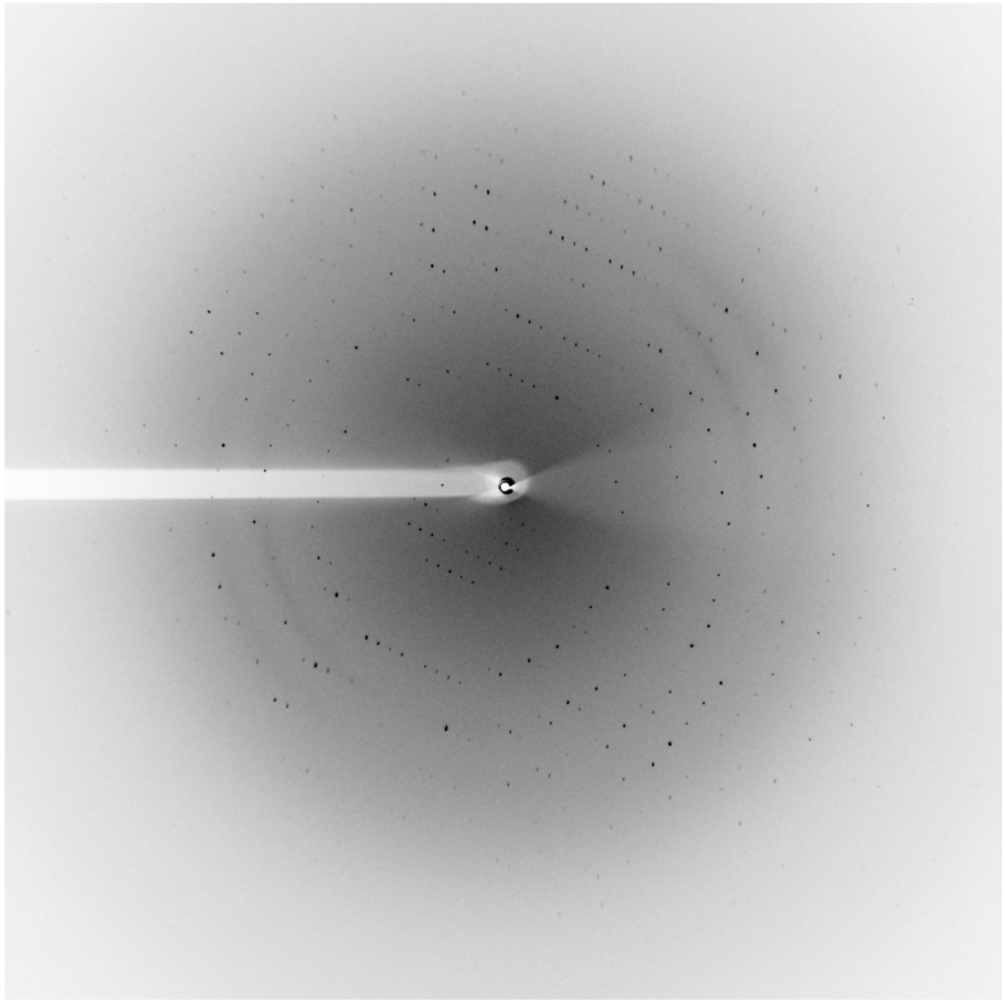
Title

Subtitle

NOVARTIS

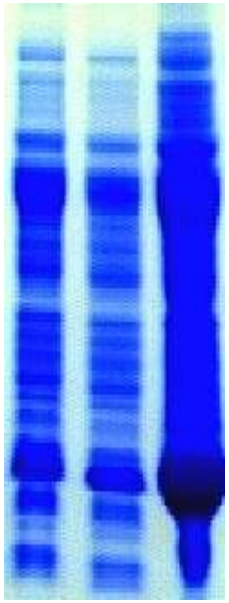
Protein crystals





Different types of protein
(some do some don't)

- Proteins that cannot crystallize → Wrongly folded or aggregation during purification
- Proteins that are easy to crystallize → Purity not critical, give hits in many condition
- Proteins that are difficult to crystallize → High levels of purity required, few hits in screen



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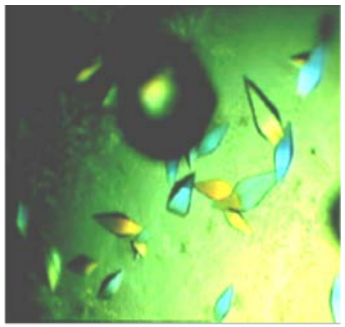

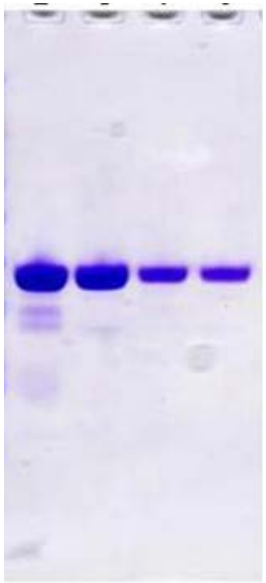

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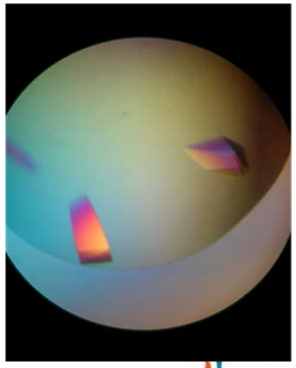
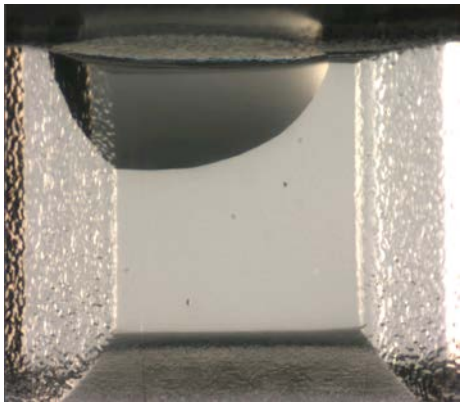
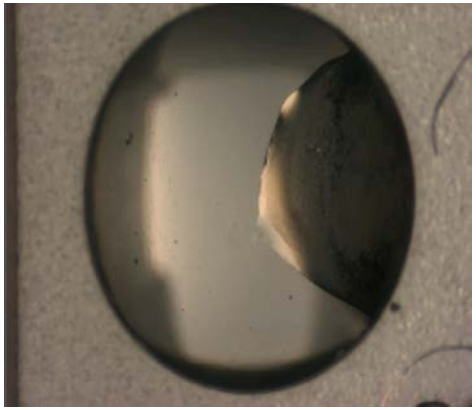
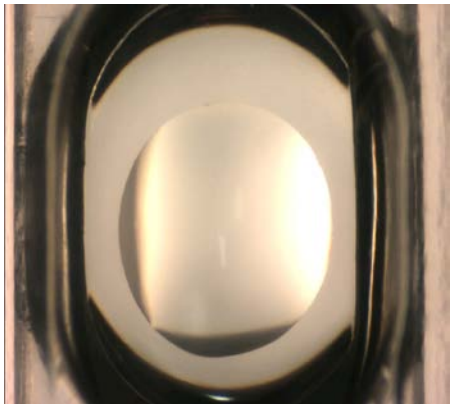


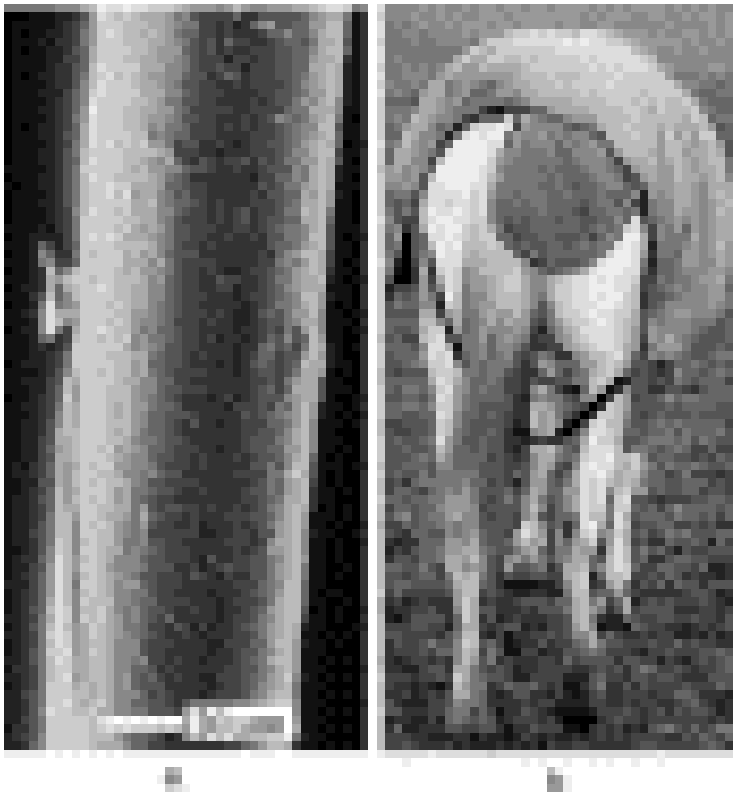
High throughput screening

- Reproducibility, plates….expensive robots, poor plates, missing drops

- Is high throughput high output?

Bringing innovation into crystallization



Bergfors suggests using horse hair to help nuclation

- **Bergfors, T. (2003). J. Struct. Biol. 142, 66–76.**
- **Editor A. McPherson!!**

Title
Matrix Seeding -Method
Subtitle

- Seed stock preparation:
  - Select best crystals possible
  - Crush crystals using Hampton tools
  - Transfer to Hampton seed bead and vortex for at least 3 minutes
  - Store at -80°C
- Screen Setup
  - Use Oryx robot with seeding function
  - Dispensed drops 0.6µl drops
    containing 0.3µl protein
    0.2µl reservoir solution
    0.1µl seed stock

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Nucleation

•The nucleation event in protein crystallization is a parameter that is poorly controlled.

•The protein should be in the metastable phase for crystal growth, but for nucleation higher levels of saturation are needed.

In many crystallization experiments sufficiently high levels of saturation are not reached and this critical nucleation event cannot occur.

•If an environment can be created that favours a higher local concentration of macromolecules the energy barrier for nucleation may be lowered.

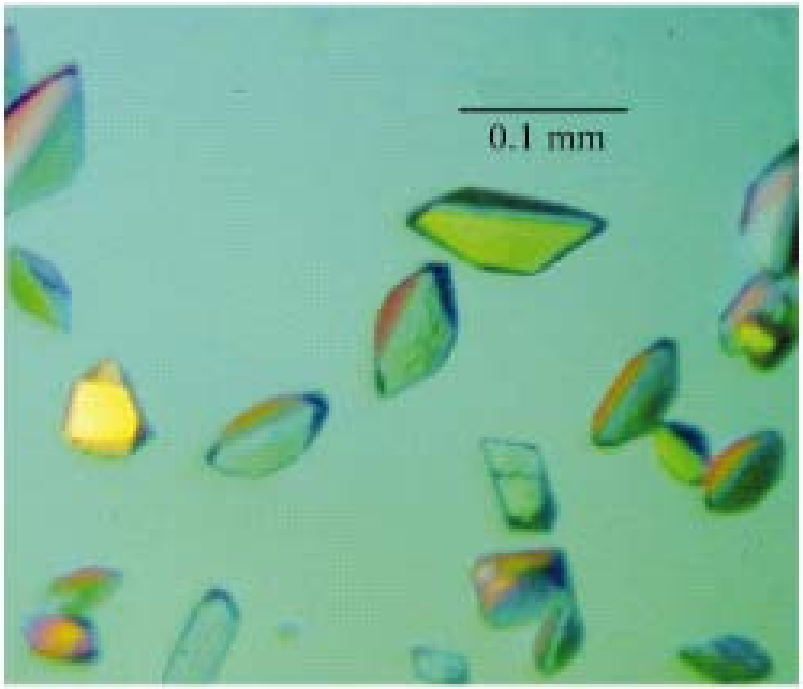
Microseed matrix seeding

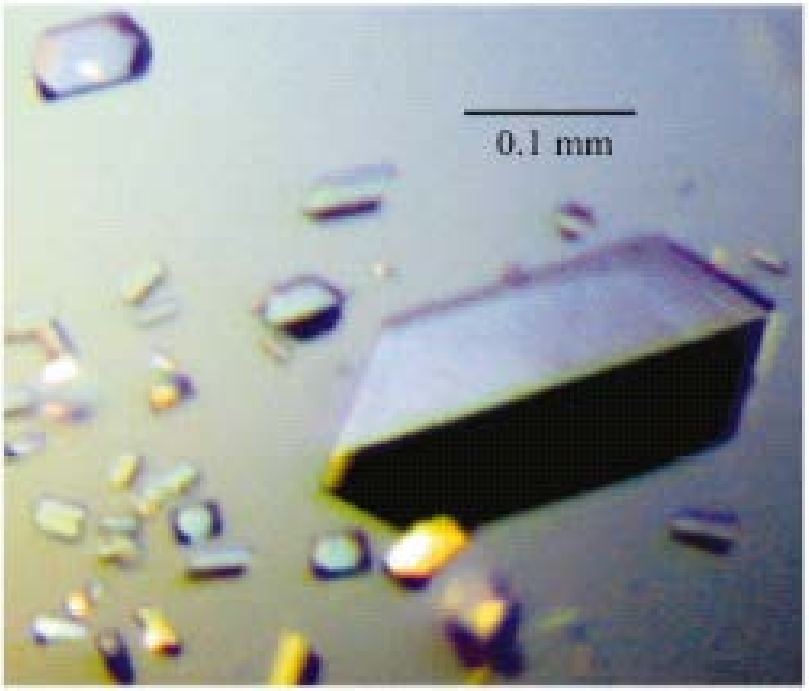
Microseed matrix screening to improve crystals of yeast cytosine deaminase

C.G. Ireton and B. Stoddard ***Acta Cryst.*** **(2004). D60, 601-605**

A crystallization strategy termed `microseed matrix screening' is described where the optimal conditions for nucleation *versus* extended lattice growth are not compatible. This method is an extension of conventional seeding techniques in which microseeds from the nucleation step are systematically seeded into new conditions where all components of the drop are allowed to vary to screen for subsequent growth of well ordered specimens.

Improved crystal morphology?

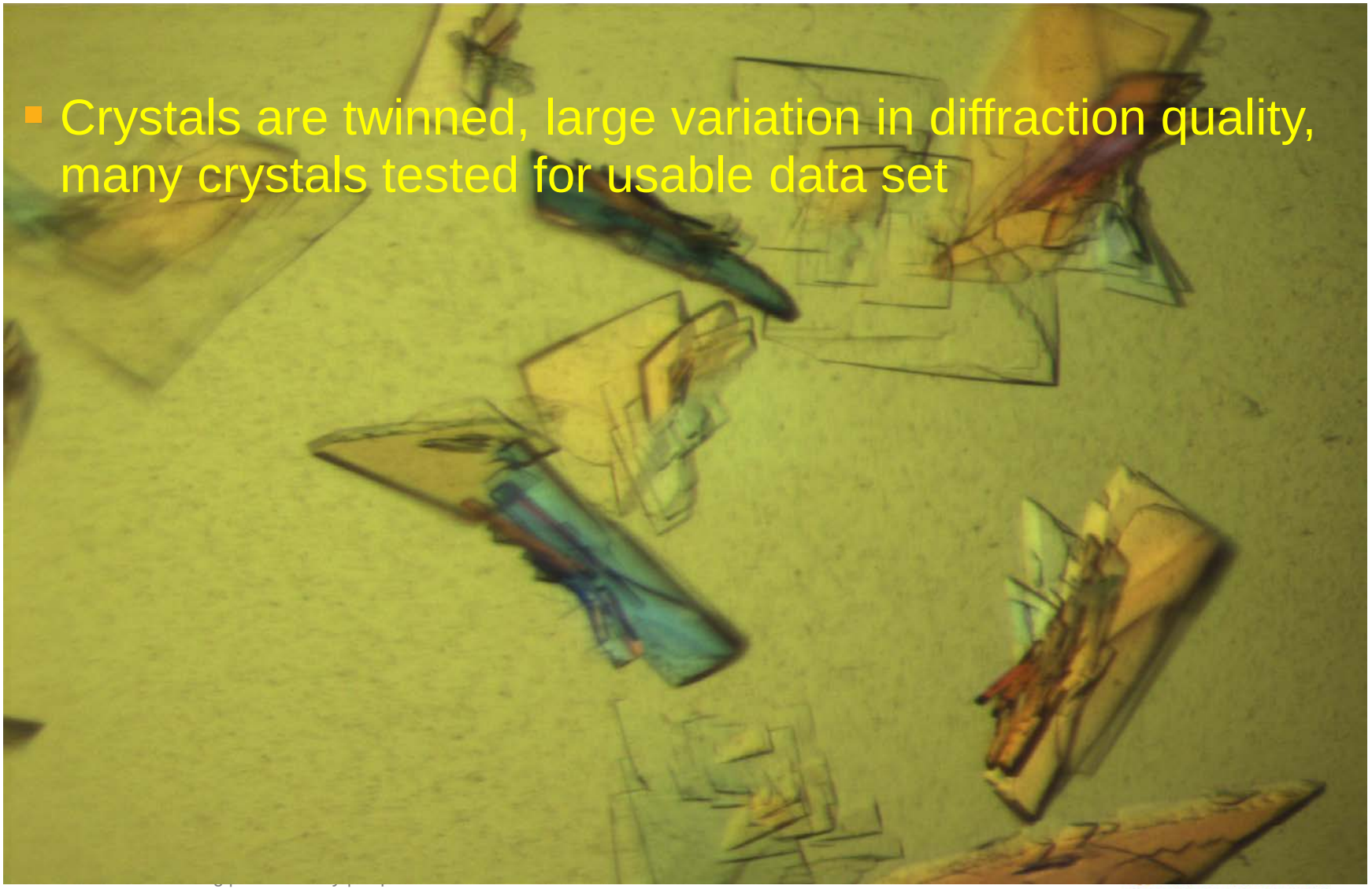




Open questions

- Cross linking
- How far can we go between proteins or species?
- Can we generate new crystals forms?
- Will it work with Membrane proteins

Crystallization: starting point

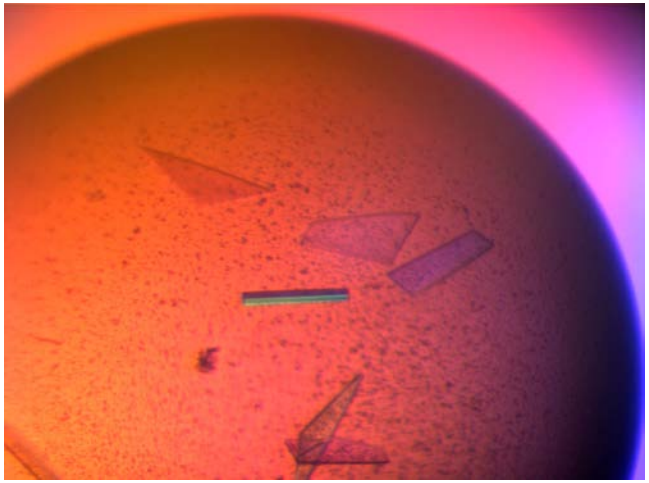


- Crystals are twinned, large variation in diffraction quality, many crystals tested for usable data set

New strategy: matrix seeding!

- Seed stocks of crystals were prepared..
- Index and Nextal PEG screens run: 3 promising hit conditions in Nextal PEG (#37, #38 and #73).

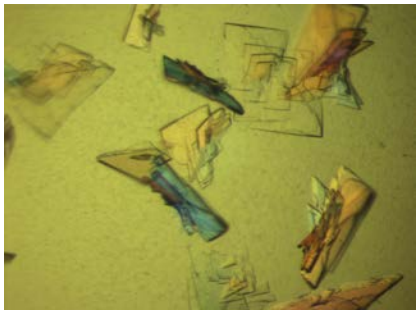
Nextal PEG condition #37 with matrix seeding.

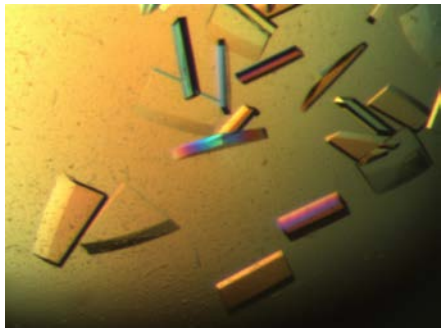


Hit condition with seeding is reproducible .

- Condition 37, 25% PEG 3000, 0.1M HEPES pH7.5, was repeated in 12 drops:

  - Modification of condition 37 using PEG 3350 instead of PEG 3000





  - <u>Both conditions gave well formed, single crystals.</u>

The devil is in the detail!!!

- Identical conditions without seeding => No crystals!
- Optimization around the original conditions: (change of PEG concentration, pH, protein concentration) => No improvement!
- In order to further improve the crystal quality, crystals grown in the absence of salt (Mg)  were used for seeding into the same conditions => No crystals!
- Why are no crystals obtained in 25% PEG 3350, 0.1M HEPES pH7.5 using seeds from crystals grown in the same condition ? ?

What makes the difference?

- In the seeding experiment 0.5ul seeds in the original reservoir are carried over = 22mM $MgCl_2$.
- Test the influence of $MgCl_2$:

  25% peg 3350, 0 mM $MgCl_2$ 100mM hepes 7.5 => no crystals!

  25% peg 3350, 25mM $MgCl_2$ 100mM hepes 7.5 => crystals!

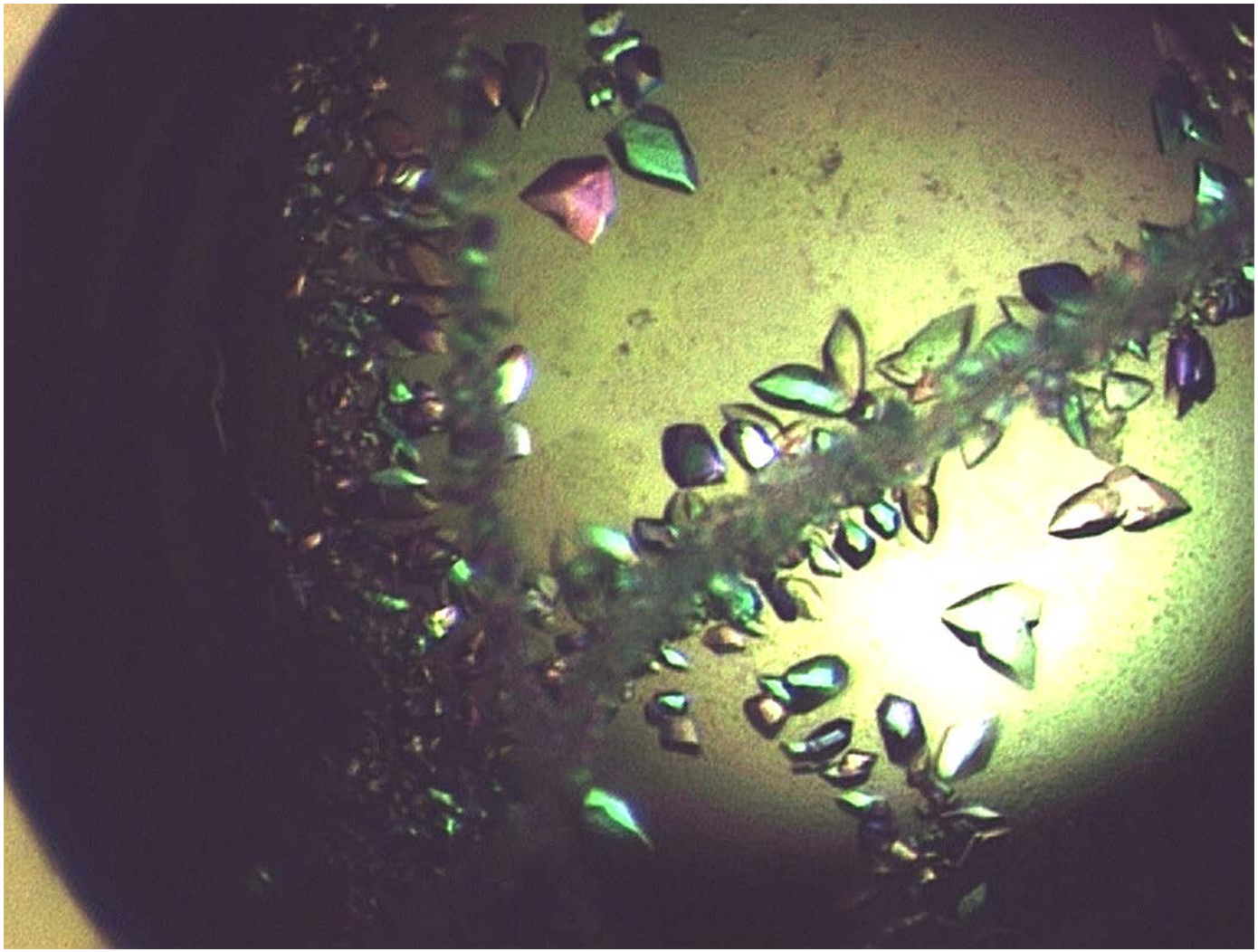
  25% peg 3350, 50mM $MgCl_2$ 100mM hepes 7.5 => crystals!

- $MgCl_2$ is essential for the crystallization

Results from first 5 test proteins tested

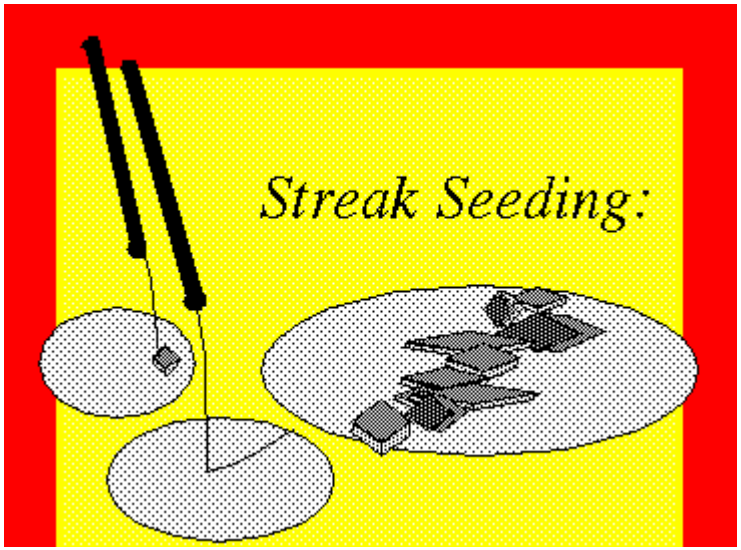
Protein	Number of hits without seeding	Number of hits with seeding
Metallo protease	2	32
Cysteine protease	4	23
Viral protein	1	65
Porcine pancreatic elastase	7	20
Bovine Trypsin	8	22

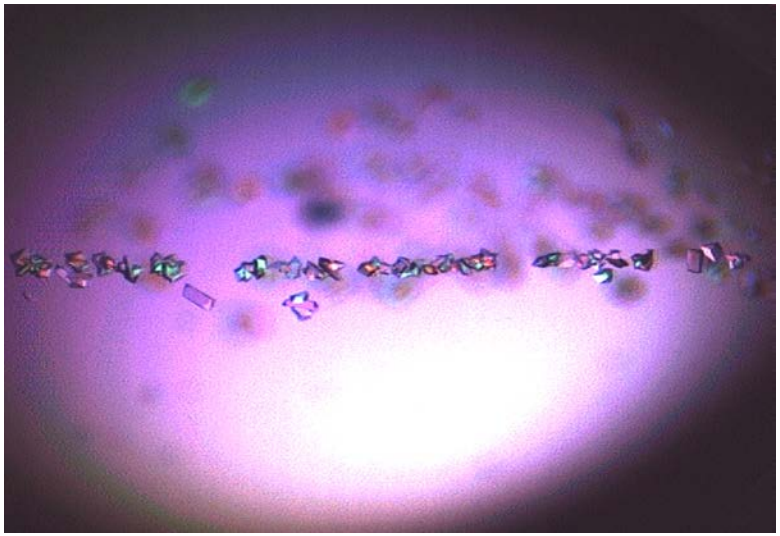
A quick review of seeding methods



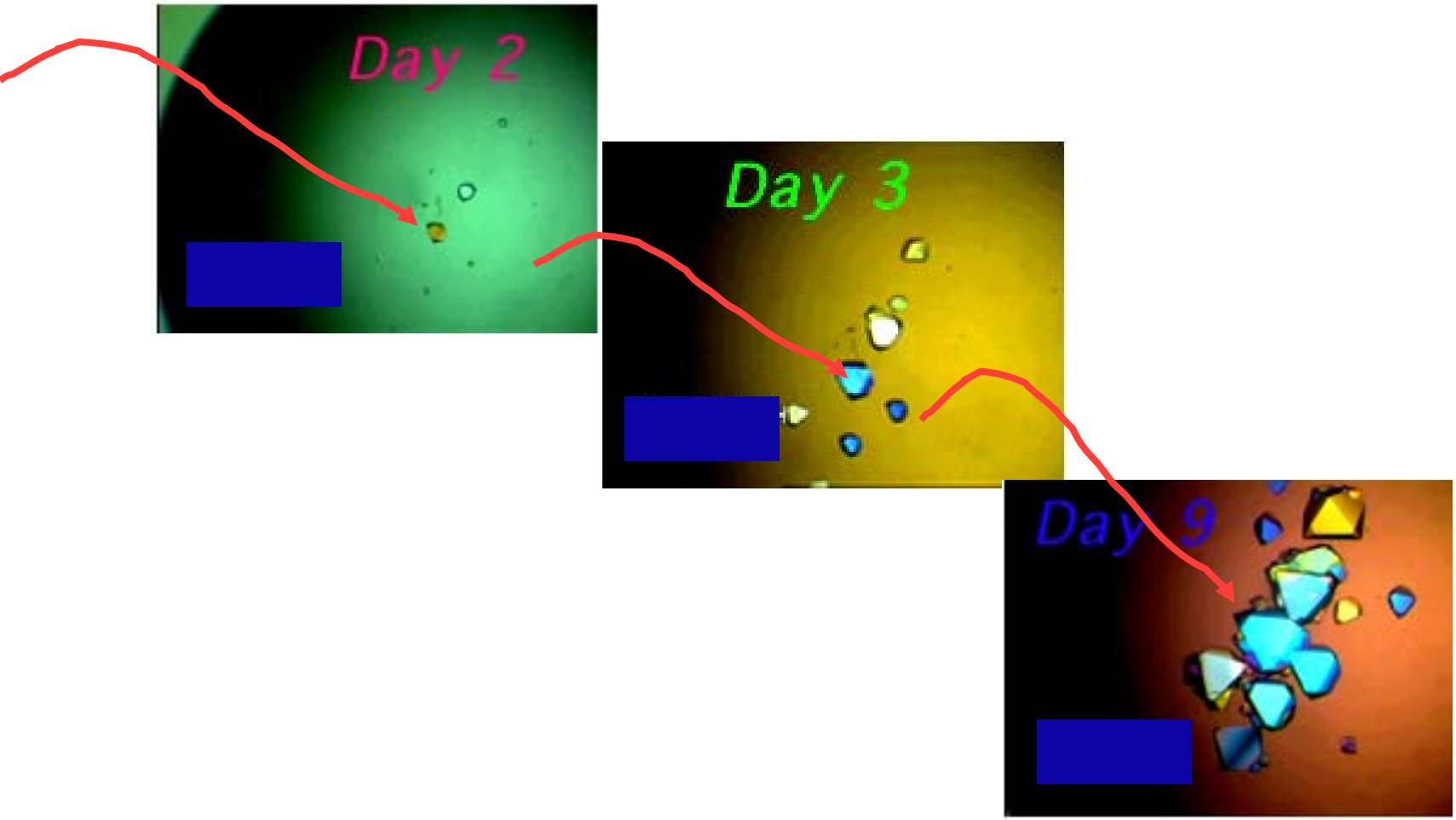
Different seeding methods
1. Streak microseed

Streak seeding with needle or hair





Different seeding methods:
2. Macroseed



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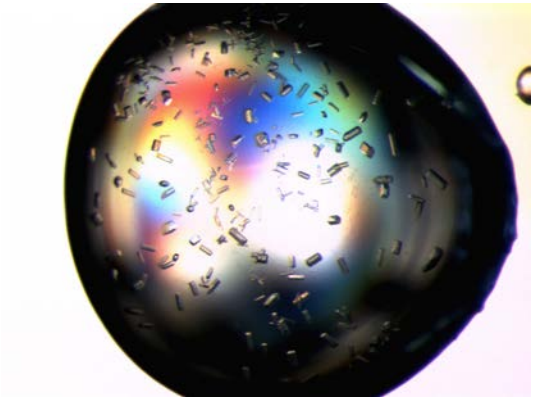
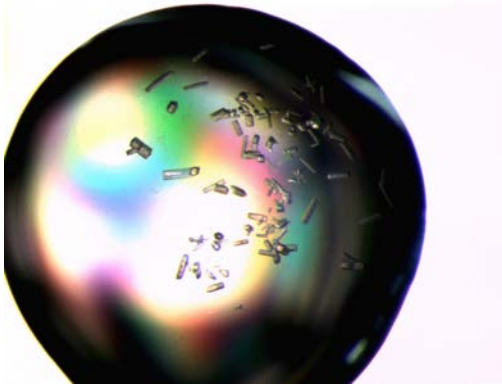
Different seeding methods
3. Microseed dilution

Protein dilution seeded with seed bead

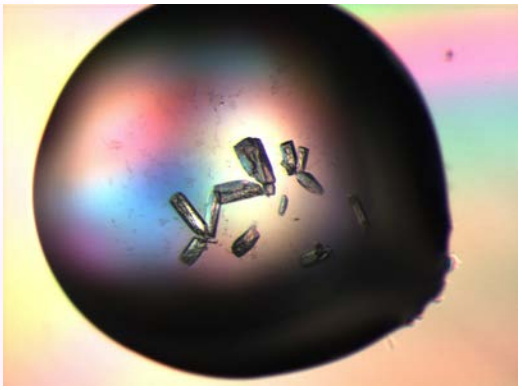
Concentrated seed stock



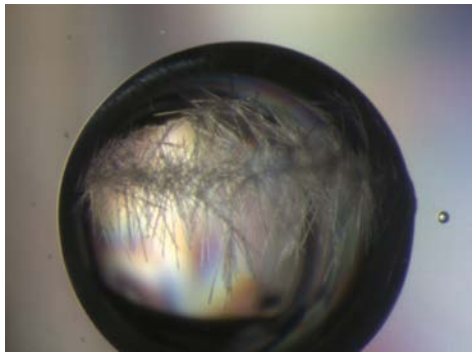
1:5 diluted seed stock

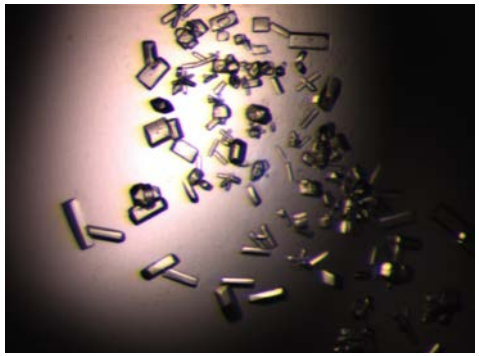


1:25 diluted seed stock

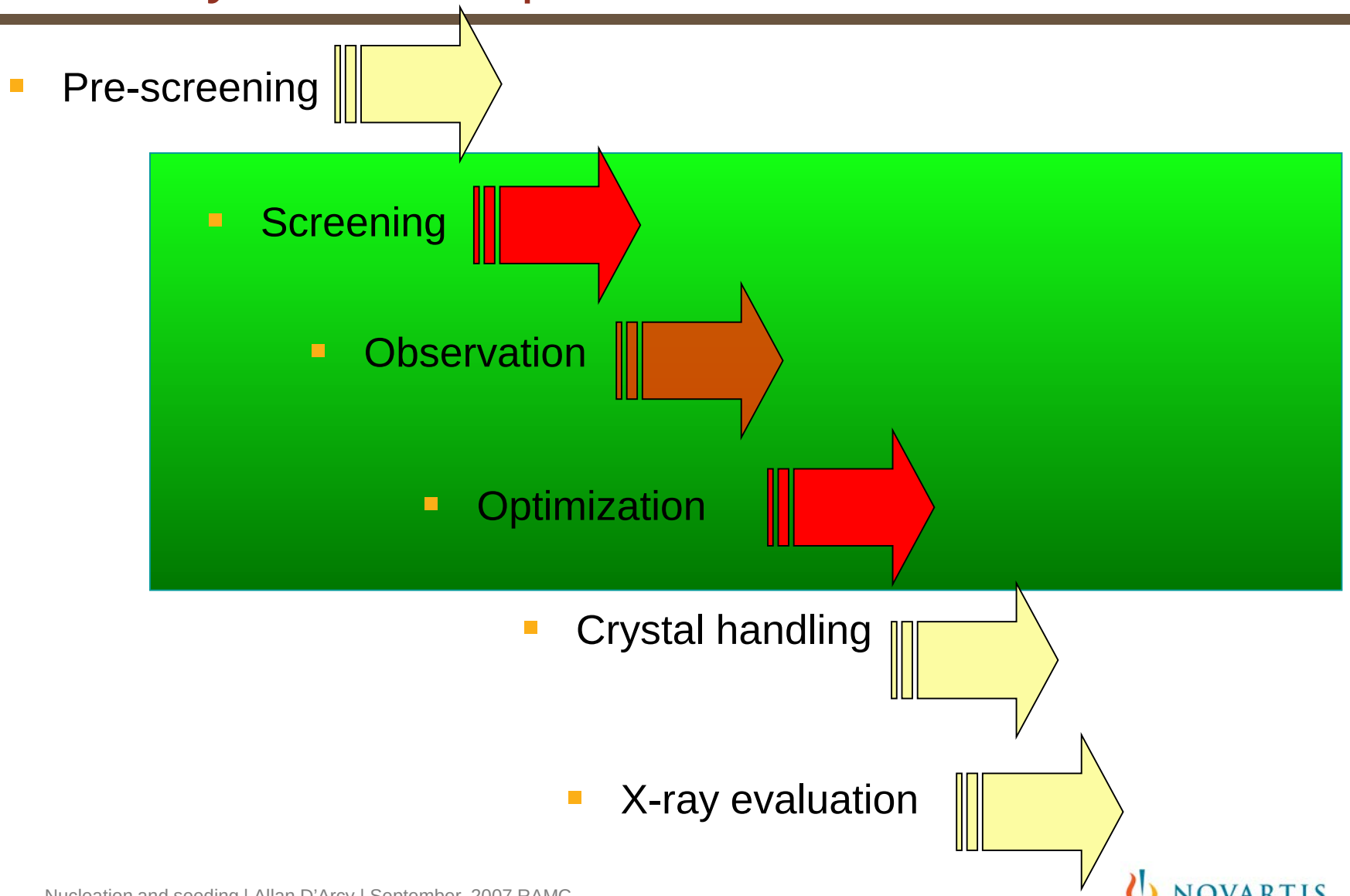


- Same protein
- streak and
- Bead seeded



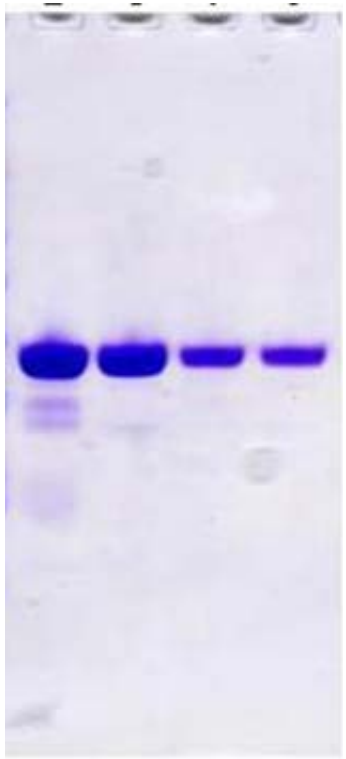


The crystallization process

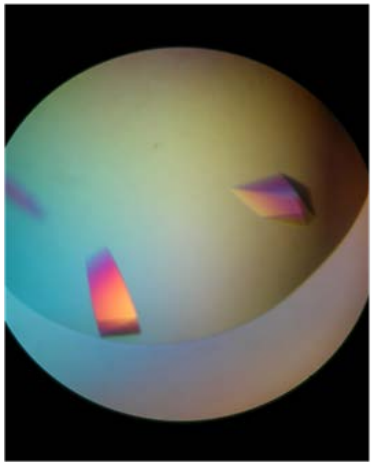


Even perfect proteins can be difficult to crystallize

Example of Dengue virus NS3 protease



- Protein concentration 90mg/ml
- Only highly purified protein gave crystals
- Only 1 hit in initial screen
- Many proteins behave like this
- Could the problem be nucleation?



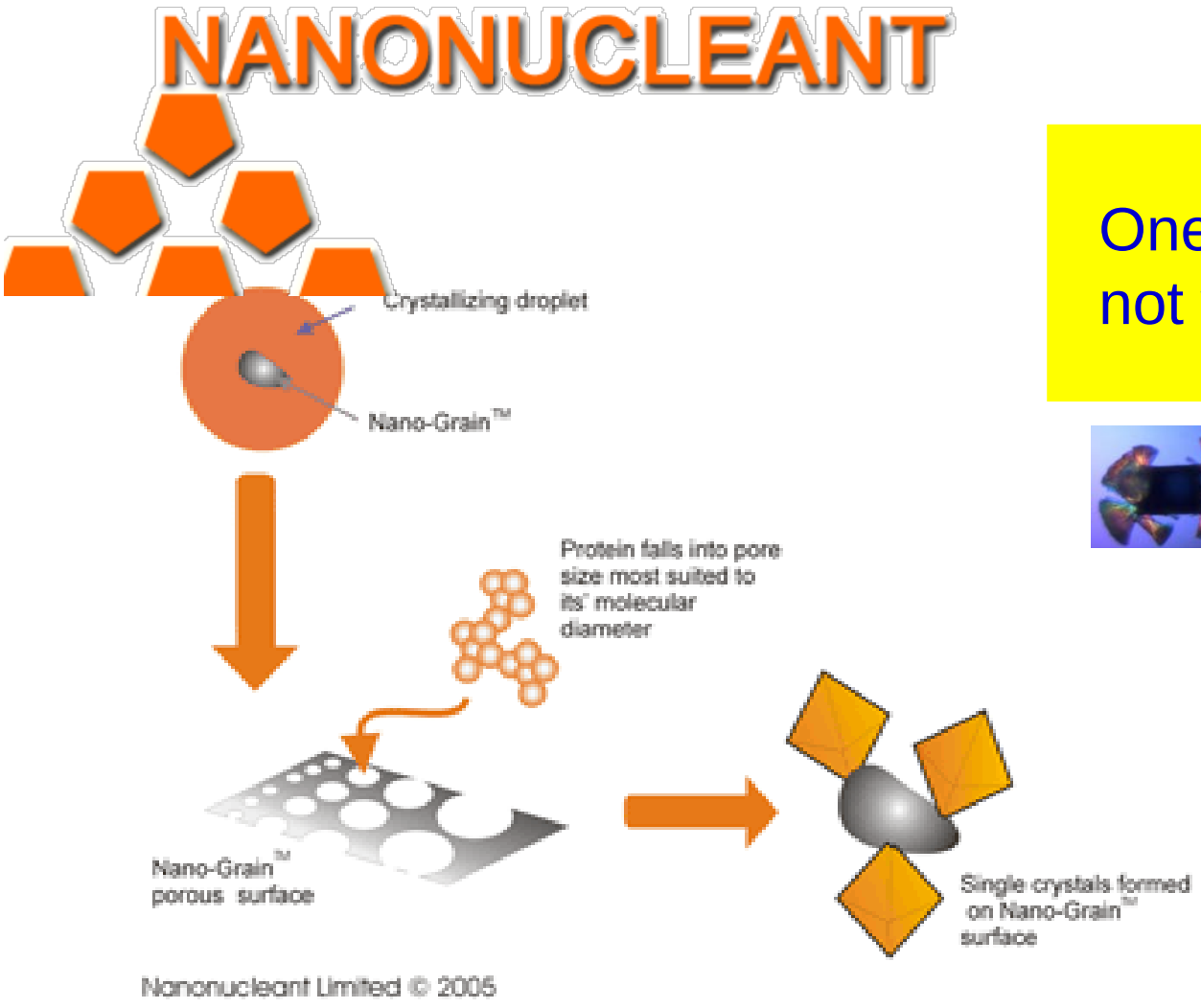
Crystals of chicken triose phosphate ismoerase, the first TIM barrel 1970

- Courtesy of Dave Banner (Roche)

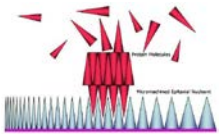
(I. 3.1). A crystal of CTIM (x 100).

(I. 3.2). Crystals of CTIM growing on a hair. (x90)

Can we find a universal nucleant??



One single nucleant will not work for all proteins



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