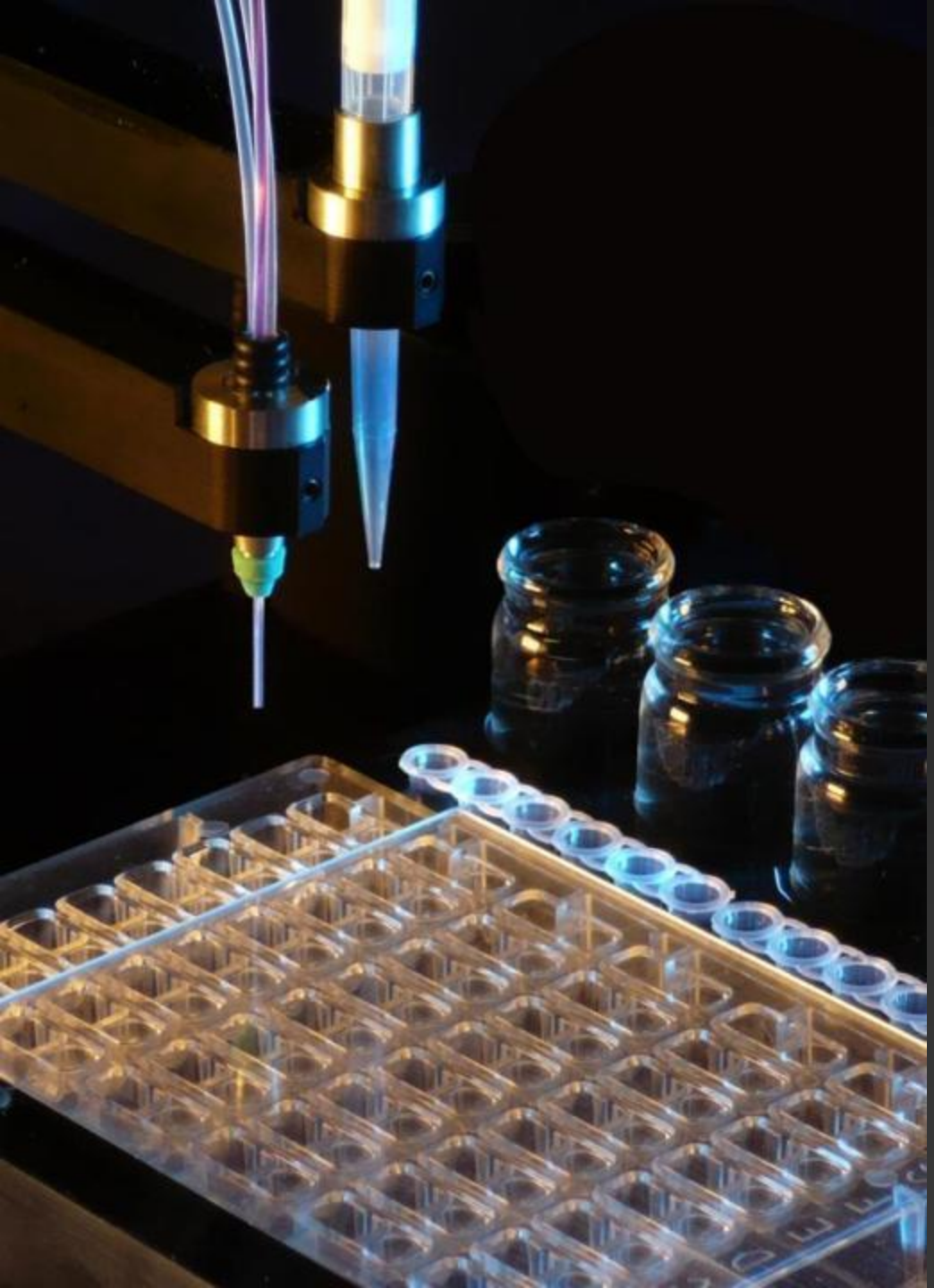


# Microseed it!

## A theoretical and practical exploration of microseeding in protein crystallization

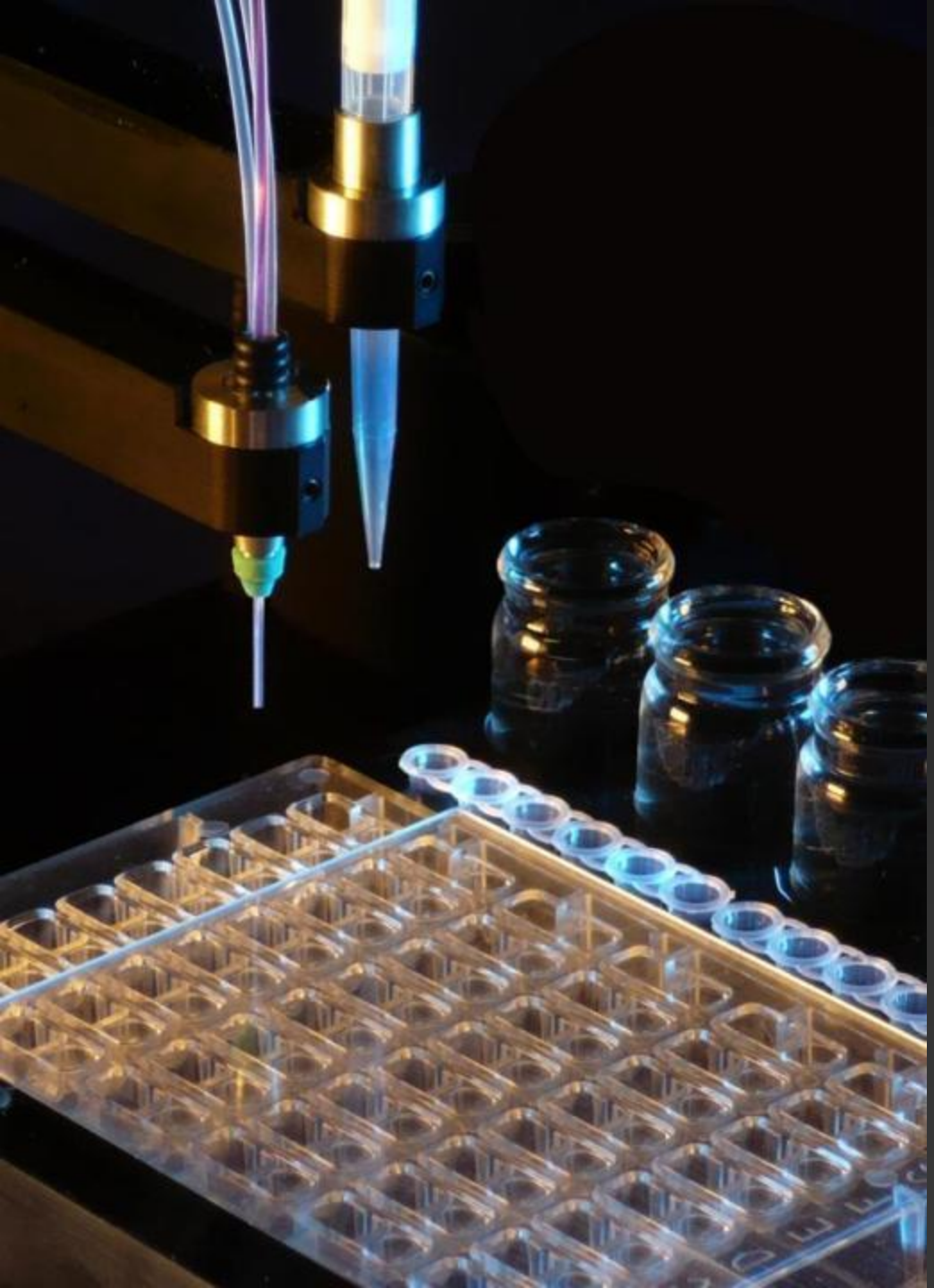

Patrick Shaw Stewart

*Douglas Instruments Limited*
*near Oxford, UK:*

Peter Baldock, Patrick Shaw Stewart, Richard Briggs, Stefan Kolek, Lesley Haire (NIMR).


Douglas Instruments

Random microseeding (random Microseed Matrix Screening, or rMMS) should be part of your normal workflow.



# Microseed it!

1. Introduction to random microseeding
2. Our work
3. New experimental design

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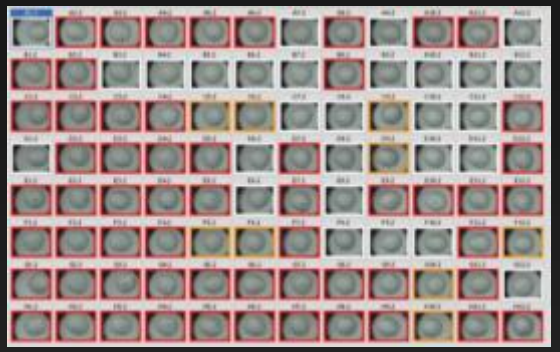
*Advantages for microseeding*

- Contact dispensing allows microseeding
- Almost no protein / seed is wasted
- Optimization
  - 2-d grid
  - 7-d Central Composite etc. (Oryx8)
  - Combinatorial script

Douglas Instruments

# Protein crystallization

Step 1: screening with random solutions that have given crystals before x 96



Douglas Instruments

# Protein crystallization

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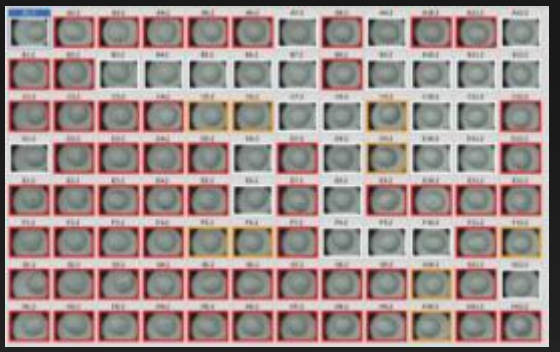
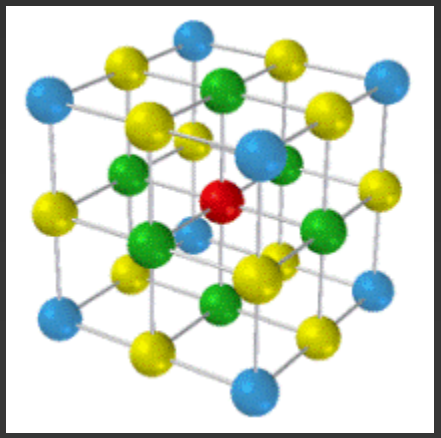


Step 2: optimization by making small changes



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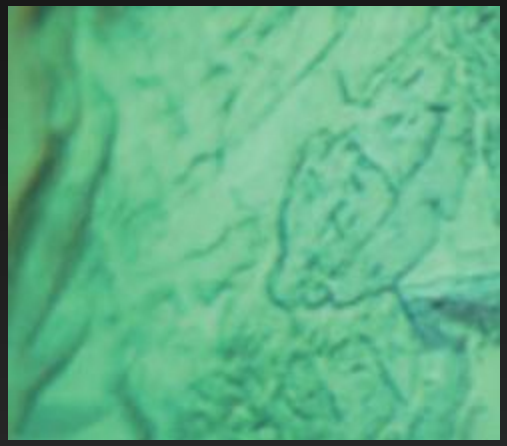
# Protein crystallization



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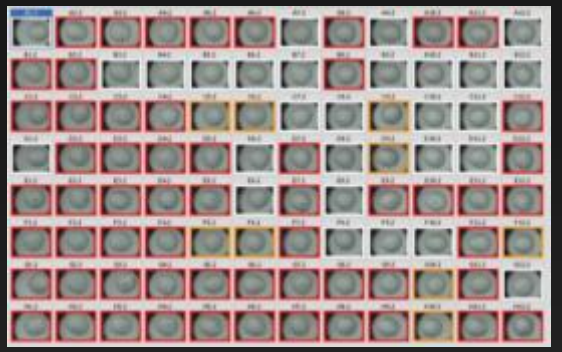

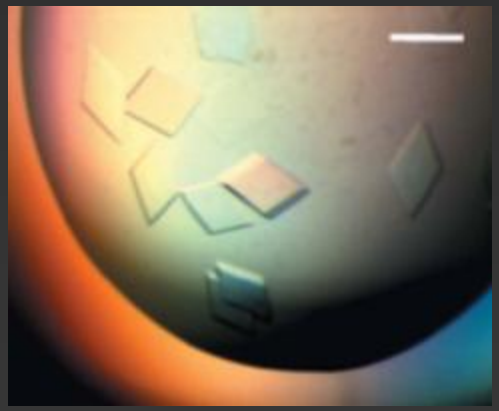

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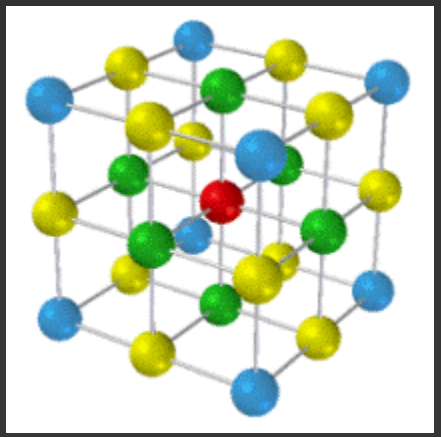

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# Protein crystallization

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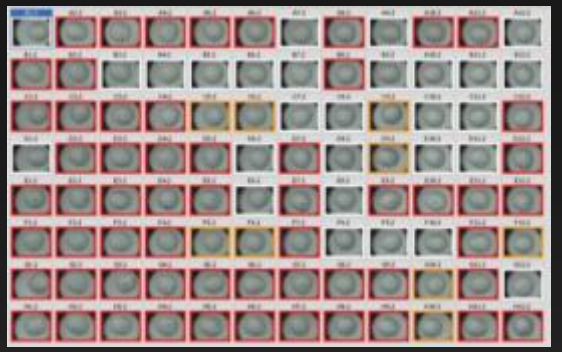

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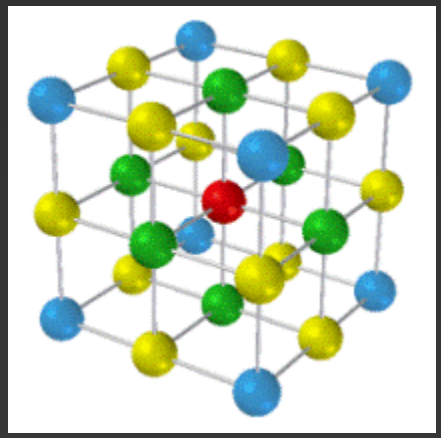

Modify your protein or make a new construct

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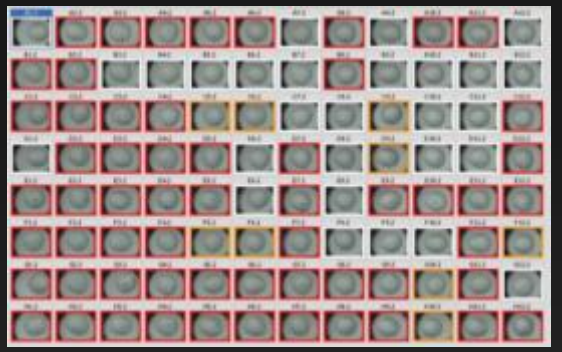
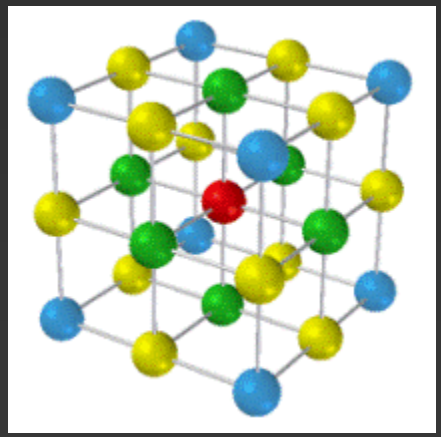
# Protein crystallization

Step 1: screening with random solutions that have given crystals before x 96



Step 1.5: random microseeding

Step 2: optimization by making small changes



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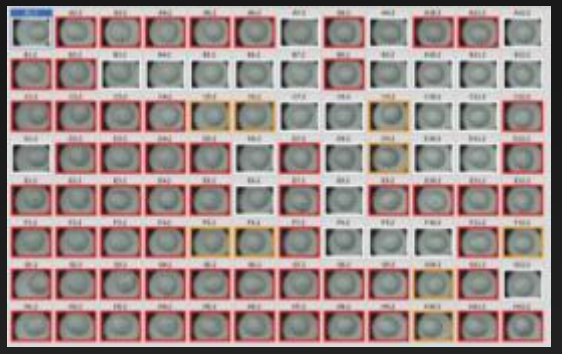
Douglas Instruments

# Protein crystallization

Step 1: screening with random solutions that have given crystals before x 96



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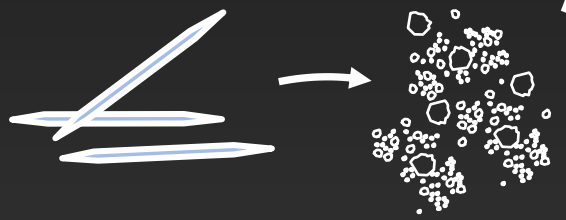

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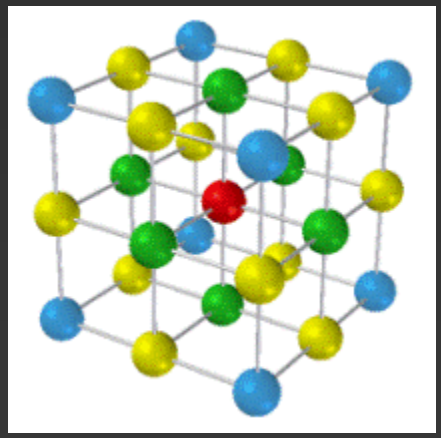

Modify your protein or make a new construct

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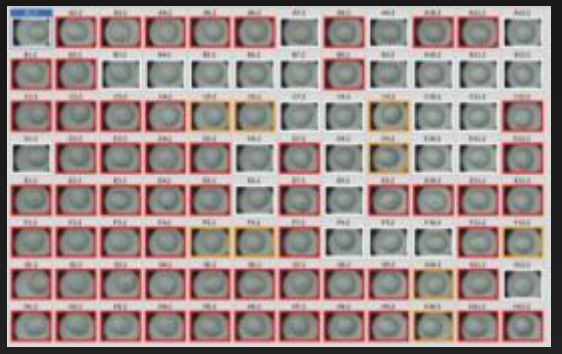
# Protein crystallization

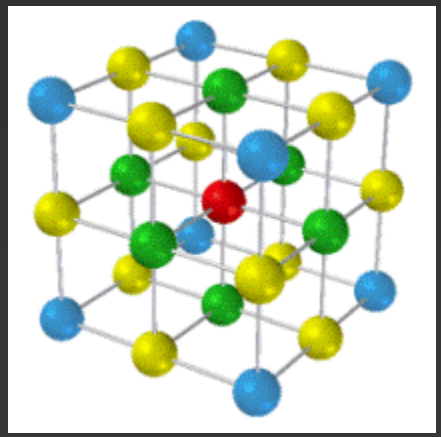
Step 1: screening with random solutions that have given crystals before x 96



Step 1.5: random microseeding

*Easy!*

Step 2: optimization by making small changes



Modify your protein or make a new construct

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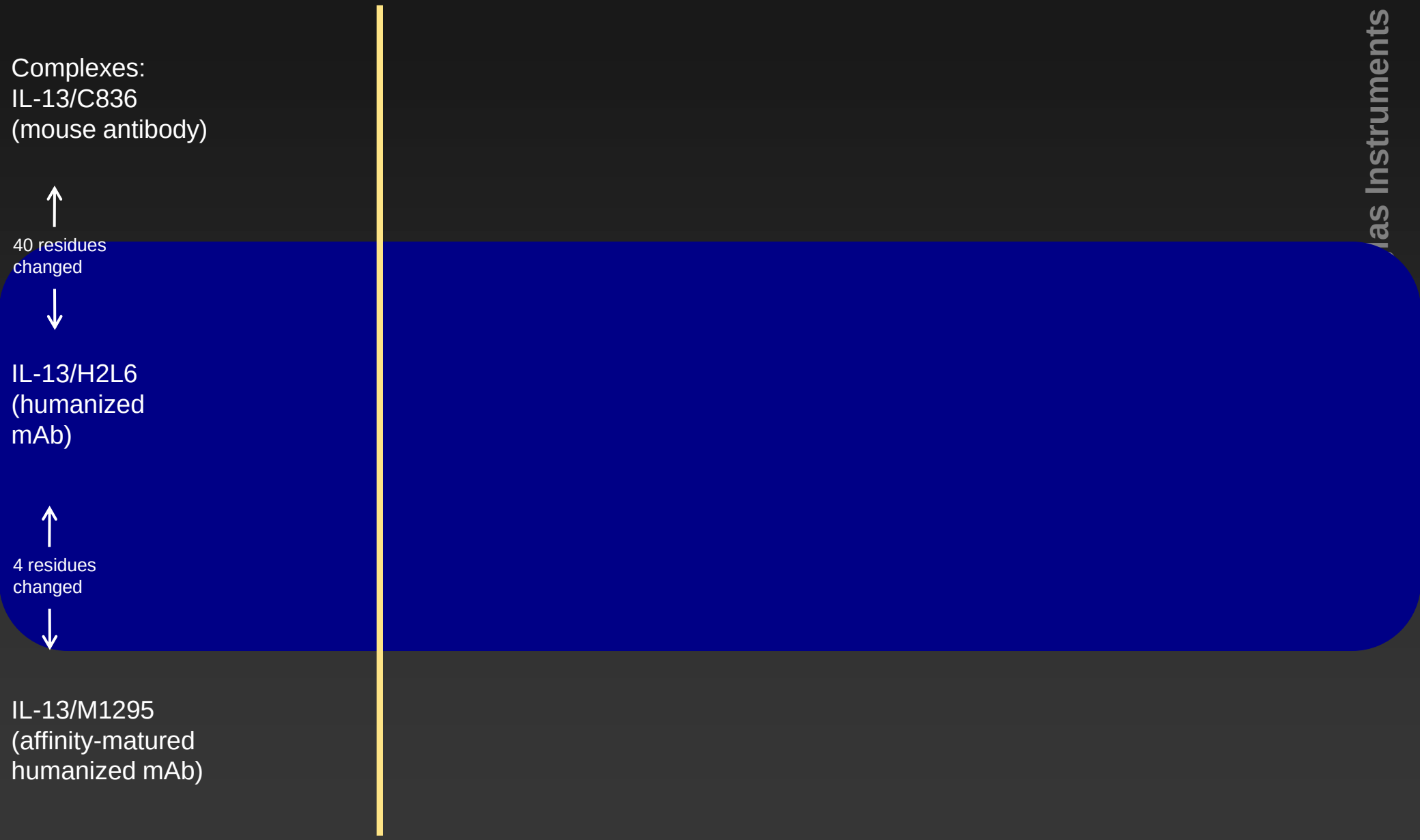
# Case study – Obmolova *et al*, Acta Cryst (2010) D66, 927 - 933

*Conventional methods*



# Case study – Obmolova *et al*, Acta Cryst (2010) D66, 927 - 933

*Conventional methods*

Complexes:
IL-13/C836
(mouse antibody)
*192 conditions*

No hits

40 residues changed

IL-13/H2L6
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*192 conditions*

One hit

4 residues changed

IL-13/M1295
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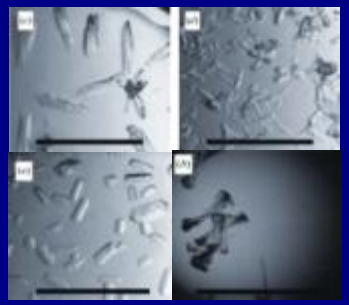
No hits

40 residues changed

Microseeding

IL-13/H2L6
(humanized mAb)

One hit



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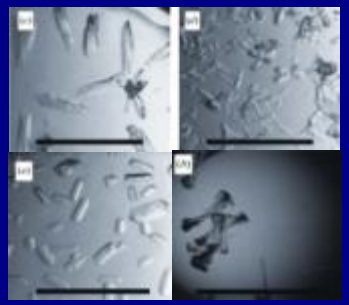
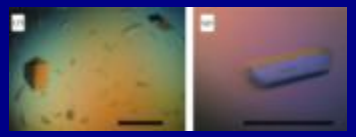


Optimization



Both 1.9 Å resolution
orthorhombic $P2_12_12_1$

4 residues changed

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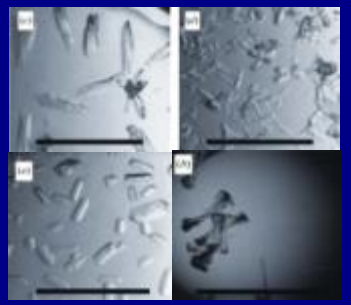

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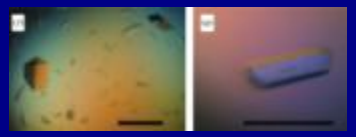

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No hits

Cross-seeding

2.8 Å res.
$P2_12_12_1$

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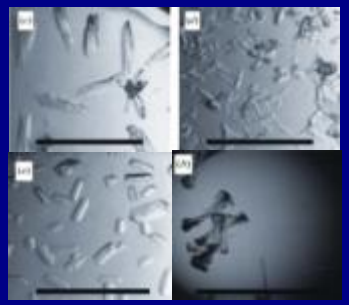

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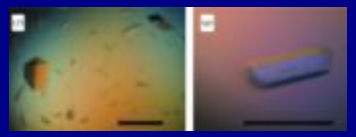

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monoclinic $P2_1$

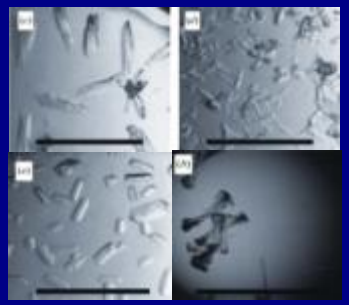
Optimization

40 residues changed

Microseeding

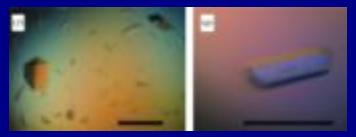
IL-13/H2L6
(humanized
mAb)

One hit



Cross-seeding

Optimization



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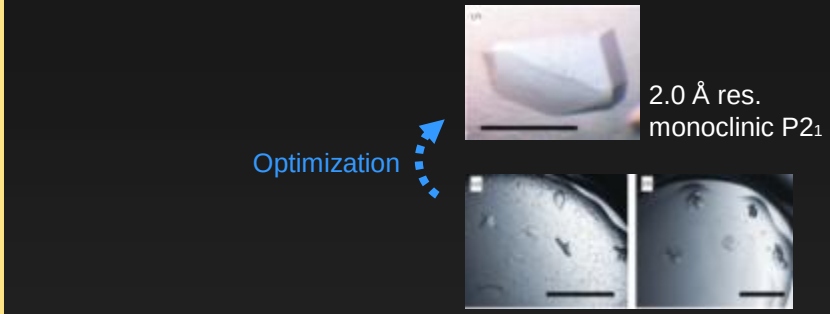

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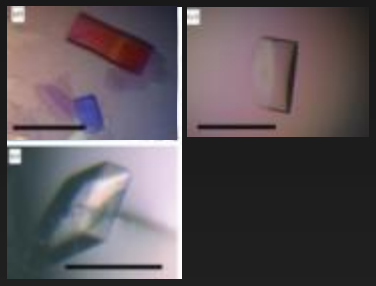

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Microseeding

Microseeding

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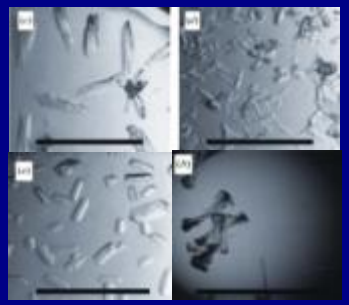
One hit

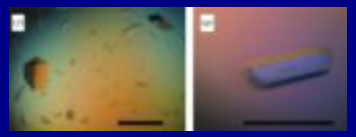


Cross-seeding

4 residues
changed

Optimization

Microseeding



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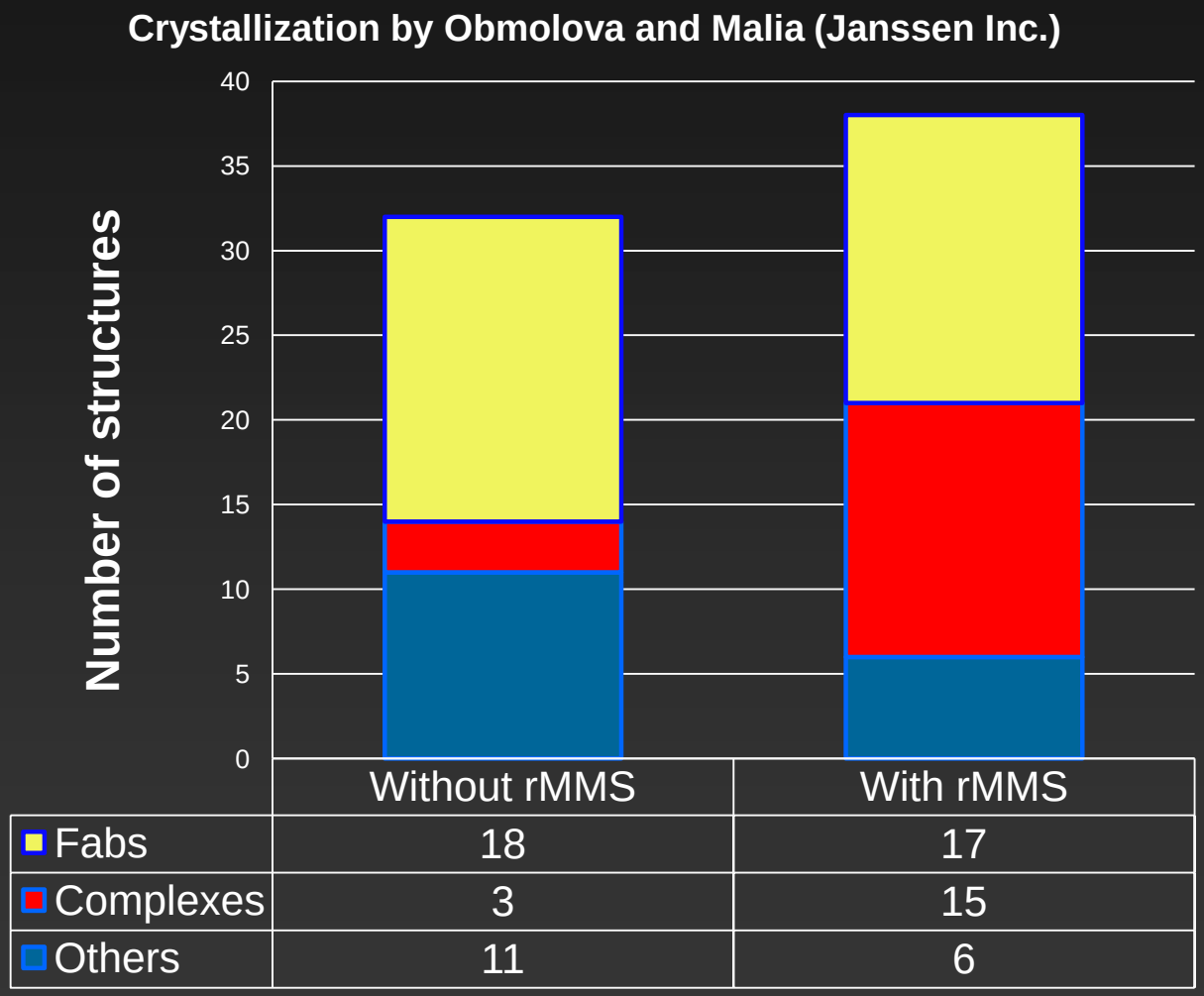
No hits

Cross-seeding

2.8 Å res.
$P2_12_12_1$

# random Microseed Matrix-Screening




| | Without rMMS | With rMMS |
|---|---|---|
| Fabs | 18 | 17 |
| Complexes | 3 | 15 |
| Others | 11 | 6 |

http://hamptonresearch.com/documents/ramc/RAMC2011_T11_Obmolova.pdf

# random Microseed Matrix-Screening

D’Arcy et al. Acta Cryst. (2007). D63. 'An automated microseed matrix-screening method for protein crystallization’

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D’Arcy et al. Acta Cryst. (2007). D63. 'An automated microseed matrix-screening method for protein crystallization’

1. Add seed crystals to a random screen

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D’Arcy et al. Acta Cryst. (2007). D63. 'An automated microseed matrix-screening method for protein crystallization’

1. Add seed crystals to a random screen

2. Suspend crushed crystals in the reservoir solution that gave the hits used (“hit solution”)

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*To get:*

*(1) more hits*

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*To get:*

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*(2) better crystals*

Douglas Instruments

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1. Add seed crystals to a random screen
2. Suspend crushed crystals in the reservoir solution that gave the hits used ("hit solution")
3. Automate!

*To get:*

*(1) more hits*

*(2) better crystals*

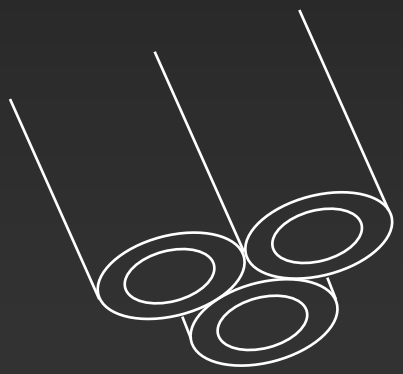
*(3) the right number of crystals (e.g. for soaking)*

# Microseeding in *screening* experiments

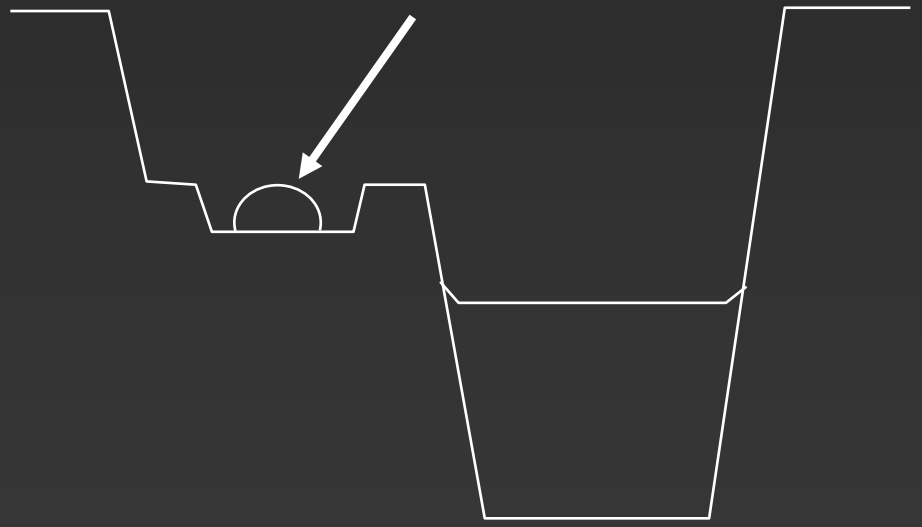
Allan D'Arcy
Novartis, Basle
2006 'Matrix-seeding script'

Douglas Instruments

3-bore tip



1. protein
2. reservoir solution



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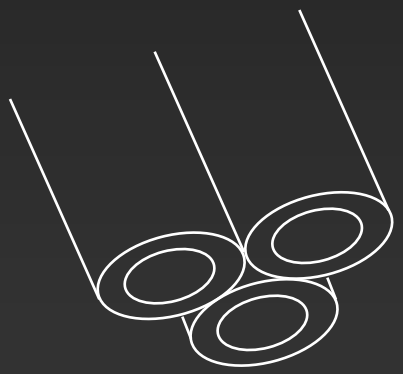
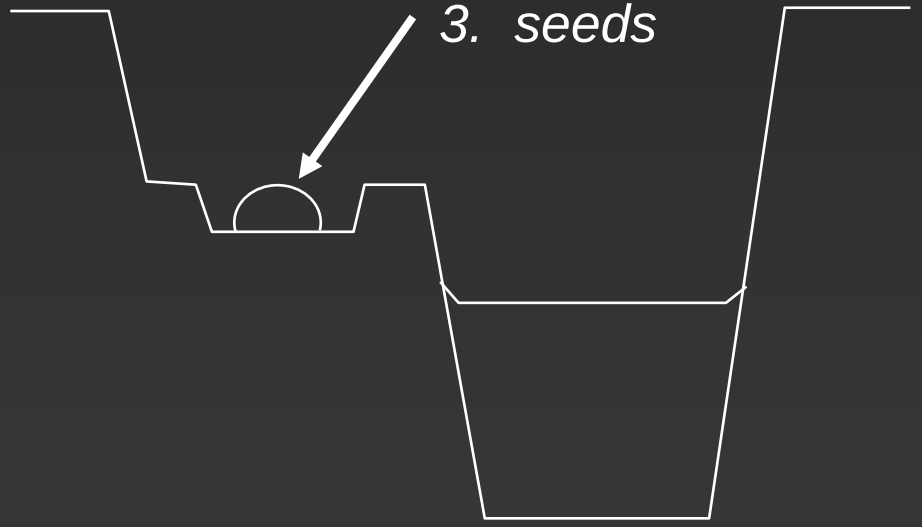
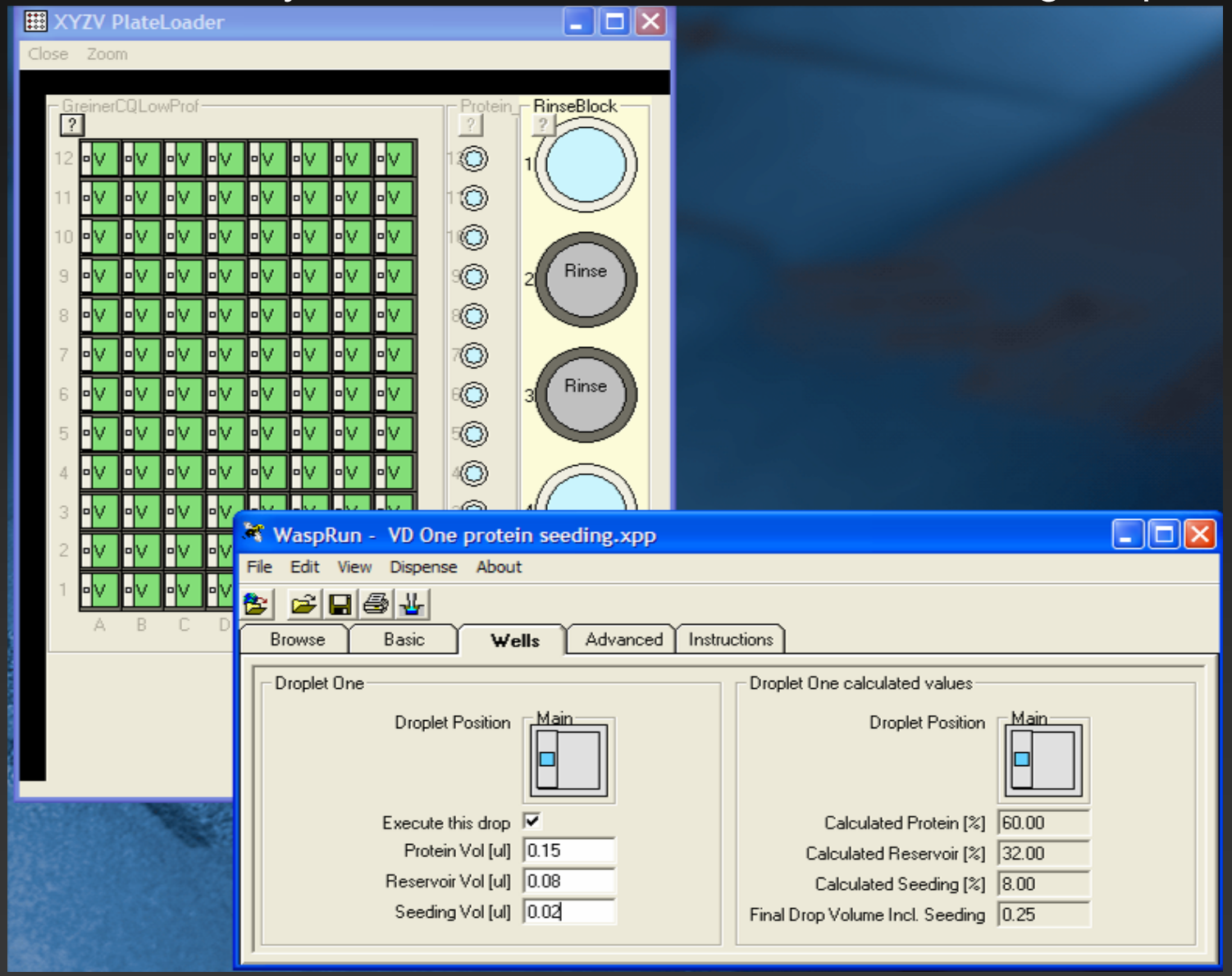


1. protein
2. reservoir solution
3. *seeds*



# Microseeding in *screening* experiments

Allan D'Arcy, Novartis, Basle. 2006 'Matrix-seeding script'

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Matrix seeding volumes:

0.3 µl protein

+ 0.2 µl reservoir solution

+ 0.1 µl seed stock

# Microseeding in *screening* experiments

**Regular screen** **Screen with seeds**

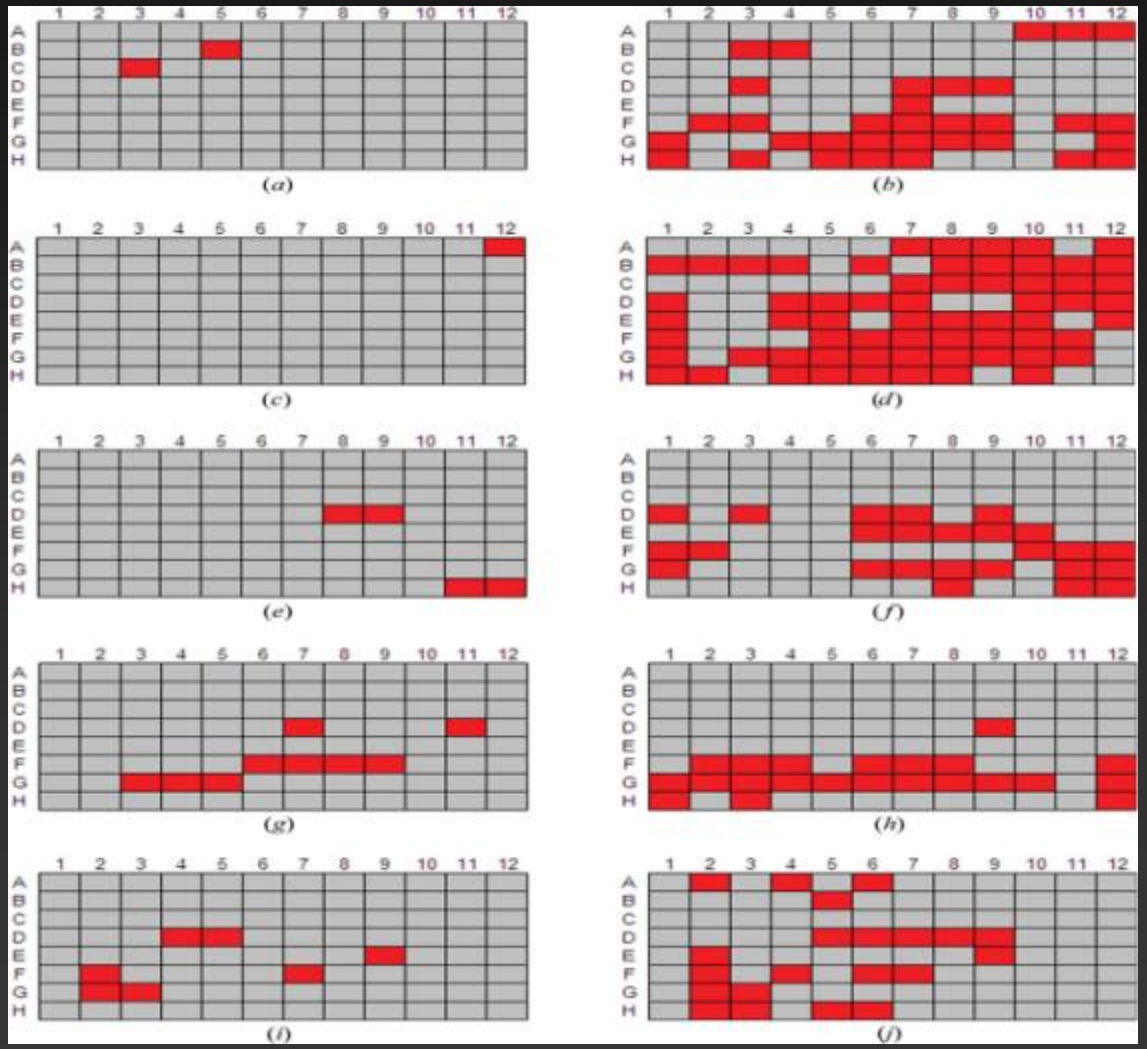
MMP12

BVP

USP7

Trypsin

PPE

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Douglas Instruments

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**Regular screen** **Screen with seeds**

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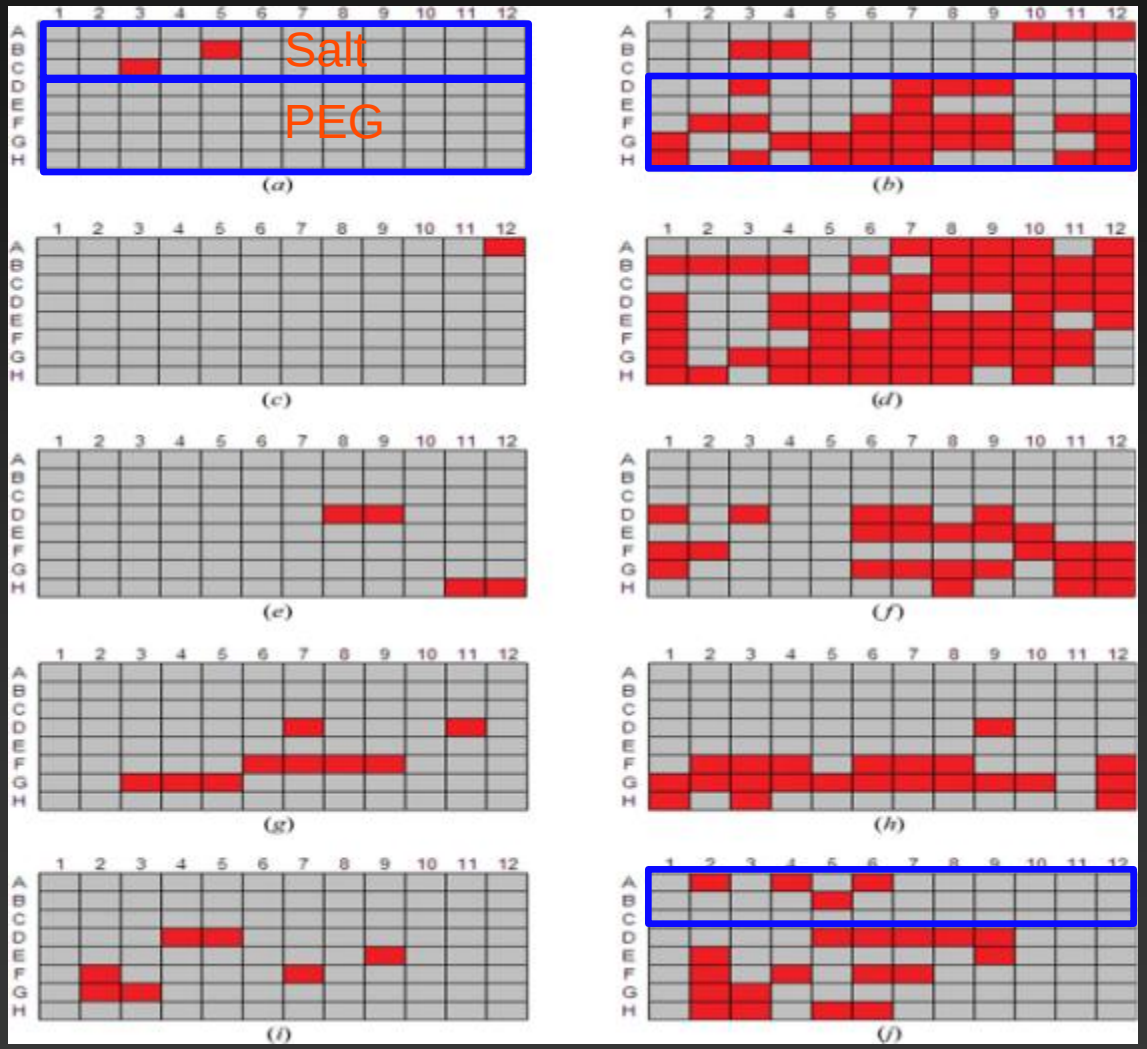
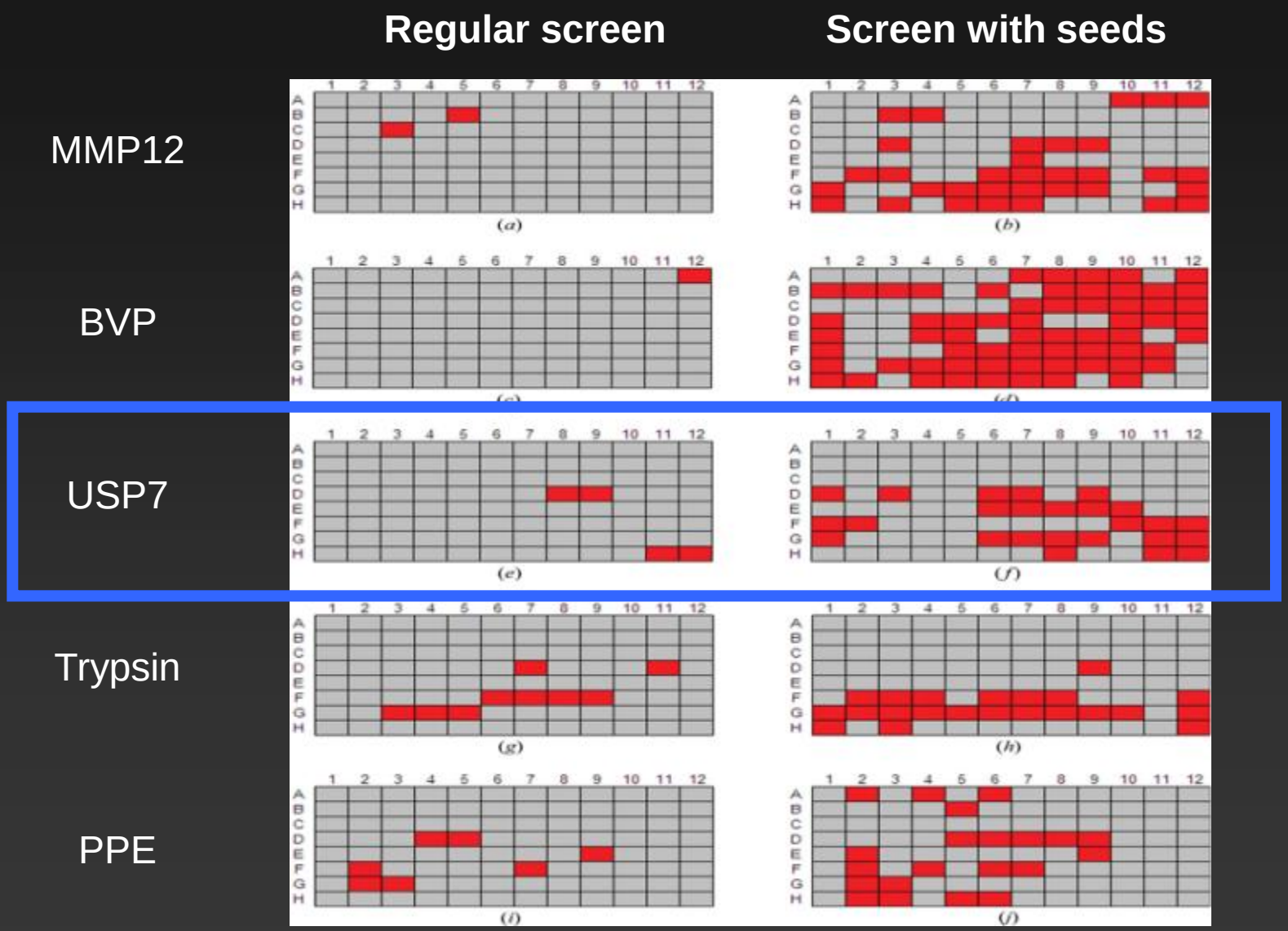


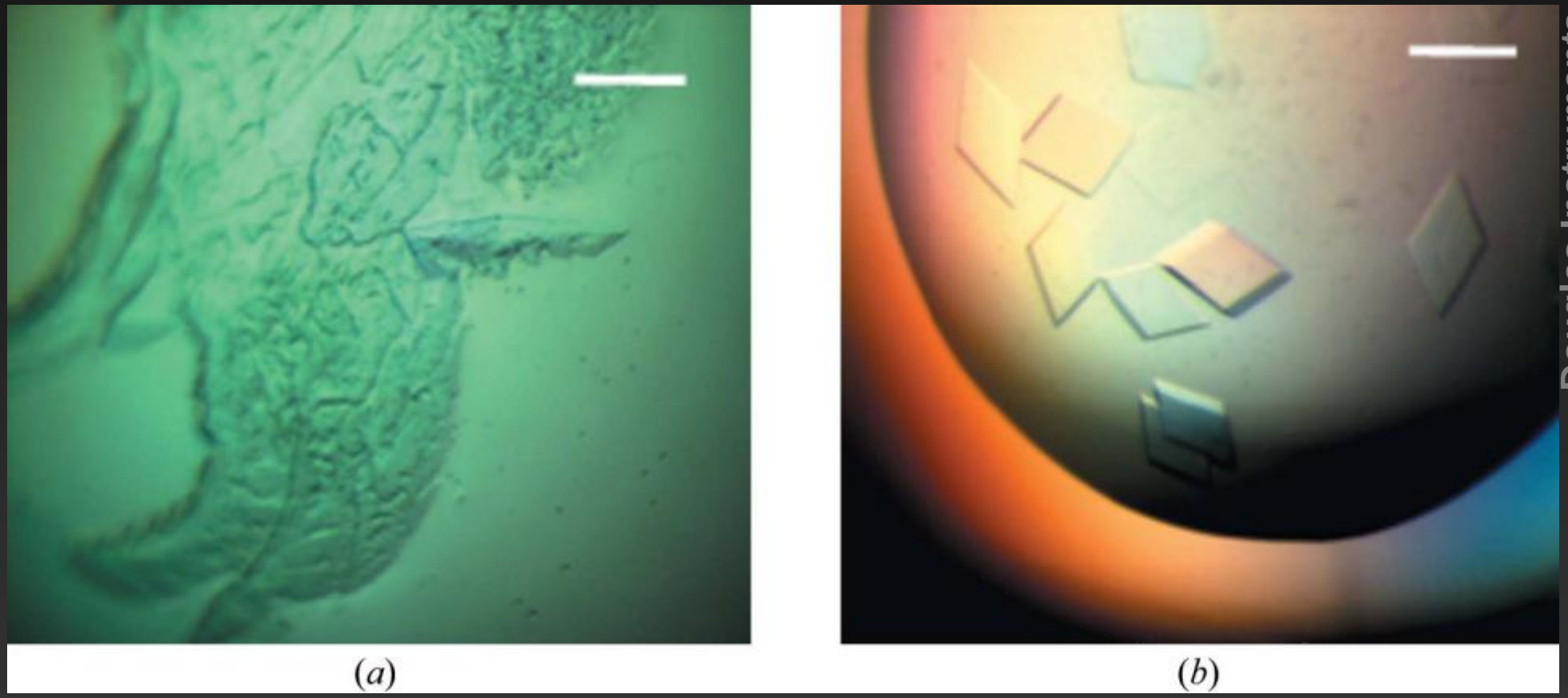

D'Arcy et al. Acta Cryst. (2007). D63

# Microseeding in *screening* experiments




D'Arcy et al. Acta Cryst. (2007). D63

# Microseeding in *screening* experiments




USP7 crystals used for seeds grown in 30% PEG 3350, 100 mM HEPES pH 7.0

USP7 crystals after seeding in 20% PEG 3350, 200 mM magnesium hexahydrate

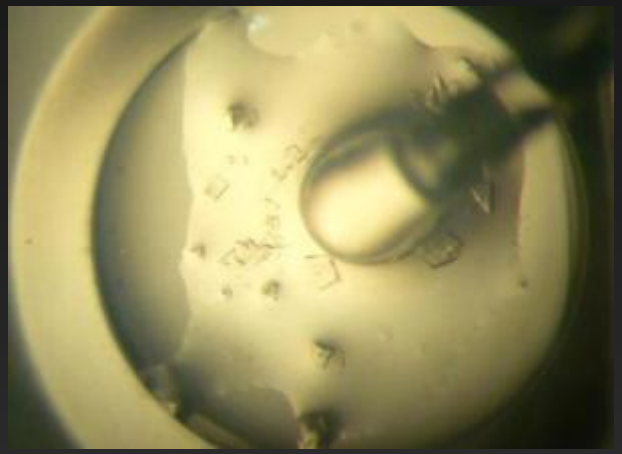
D'Arcy et al. Acta Cryst. (2007). D63

# How to make the seed stock

*See www.douglas.co.uk/mms.htm or sheet*

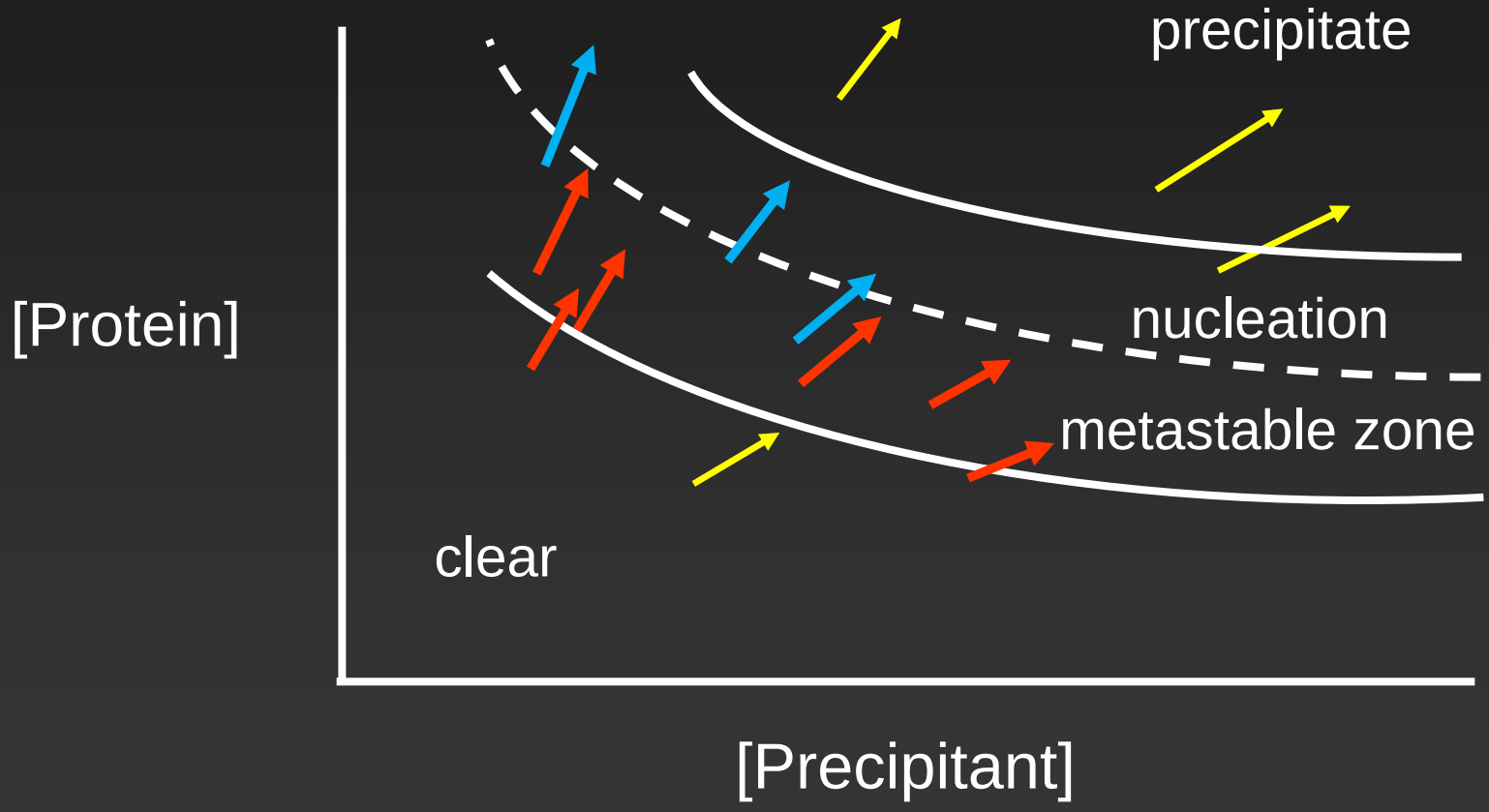
1. Make a seed stock as soon as your crystals stop growing
2. Break crystals with a probe
3. Take 50 µl of reservoir solution and add everything from a drop
4. Vortex with a Hampton “Seed Bead”
5. Make a dilution series immediately
6. Freeze

Look after your seeds!



Douglas Instruments

# Phase diagram of a protein




Douglas Instruments

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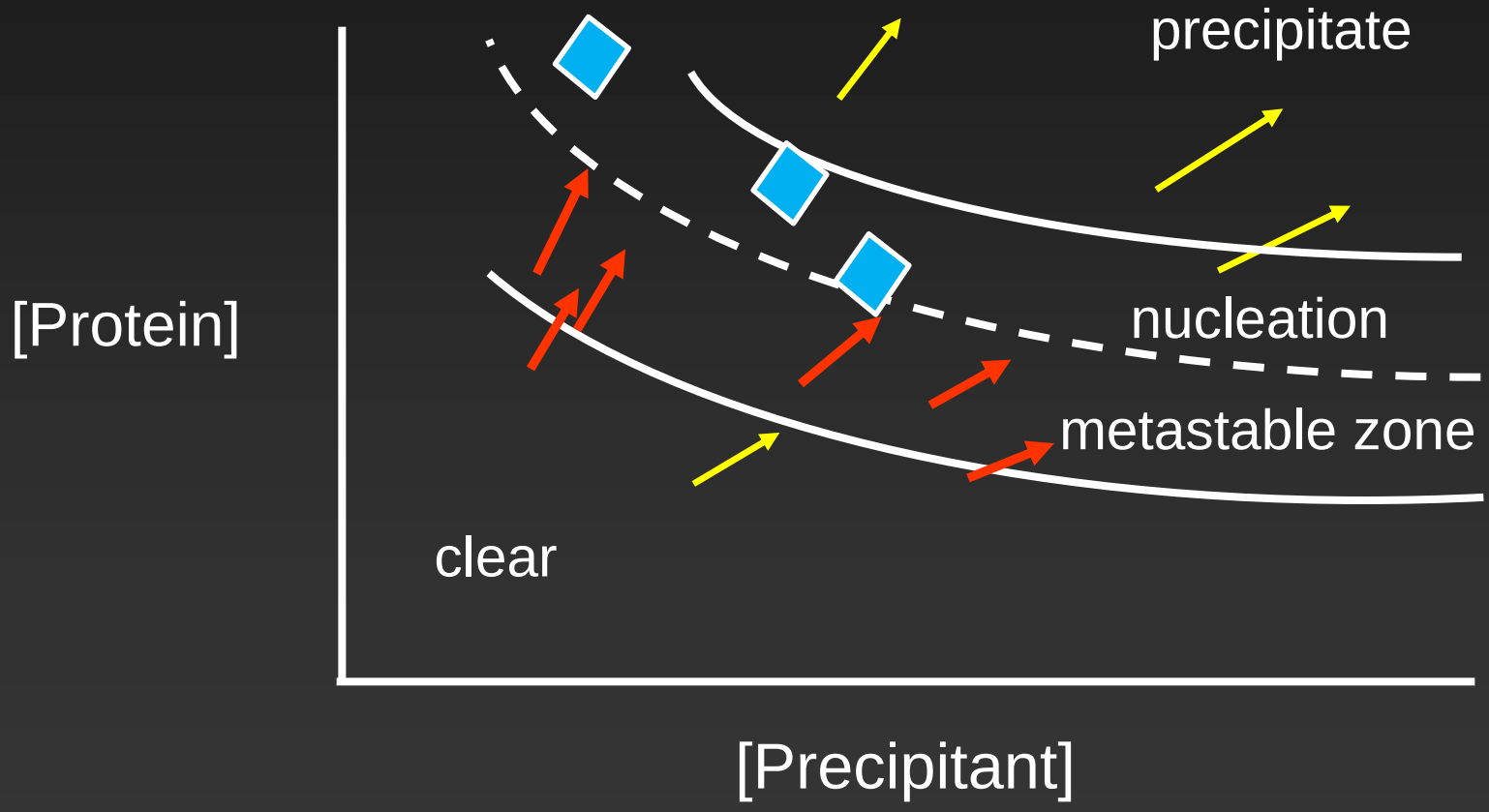
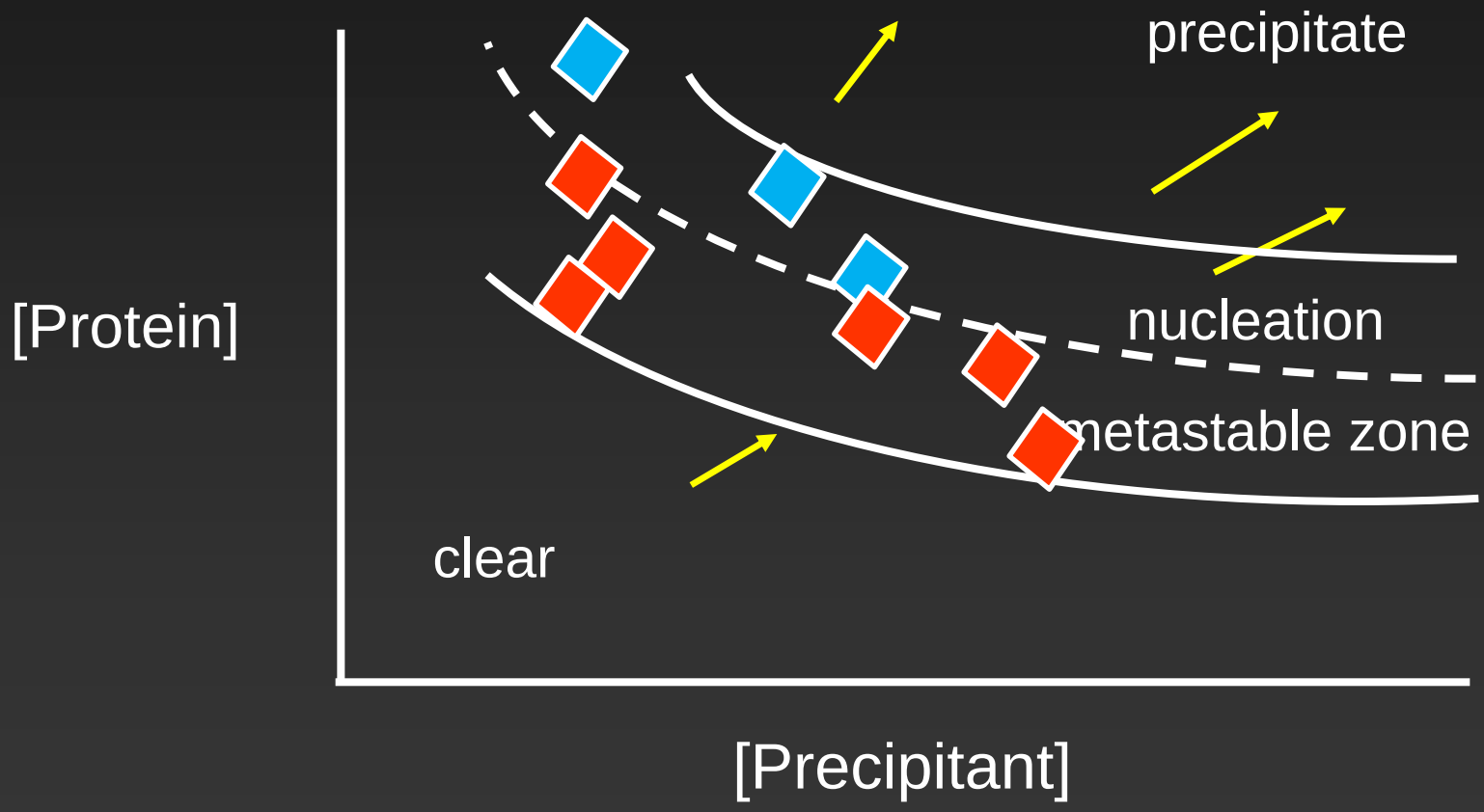



Douglas Instruments

# Phase diagram of a protein




Douglas Instruments

# random Microseed Matrix-Screening

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Most labs don’t use this method routinely

You need a robot that uses contact dispensing

We don’t know why it’s not more popular!



# rMMS with membrane proteins

Crystals of membrane proteins are often unstable

Remember that the reservoir normally has no detergent!

Harvest several large drops *without dilution*

*1.5 µl is enough - if you have the right kind of robot!*

See http://www.douglas.co.uk/MMS_proc.htm

Matrix seeding volumes:

0.3 µl protein

+ 0.2 µl reservoir solution

+ 0.1 µl seed stock

Matrix seeding volumes:

0.3 µl protein

+ 0.2 µl reservoir solution

+ 0.1 µl seed stock

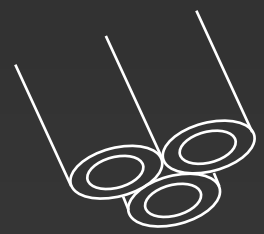
E.g. for membrane proteins:

0.3 µl protein

+ 0.29 µl reservoir solution

+ 0.01 µl seed stock

*3-bore tip*



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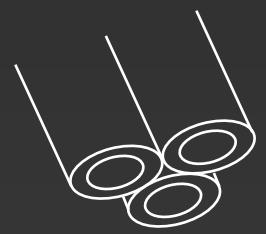


or: for membrane proteins:

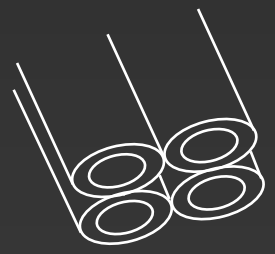
0.3 µl protein

+ 0.2 µl reservoir solution

+ 0.09 ul “hit solution” (additive)

+ 0.01 µl seed stock

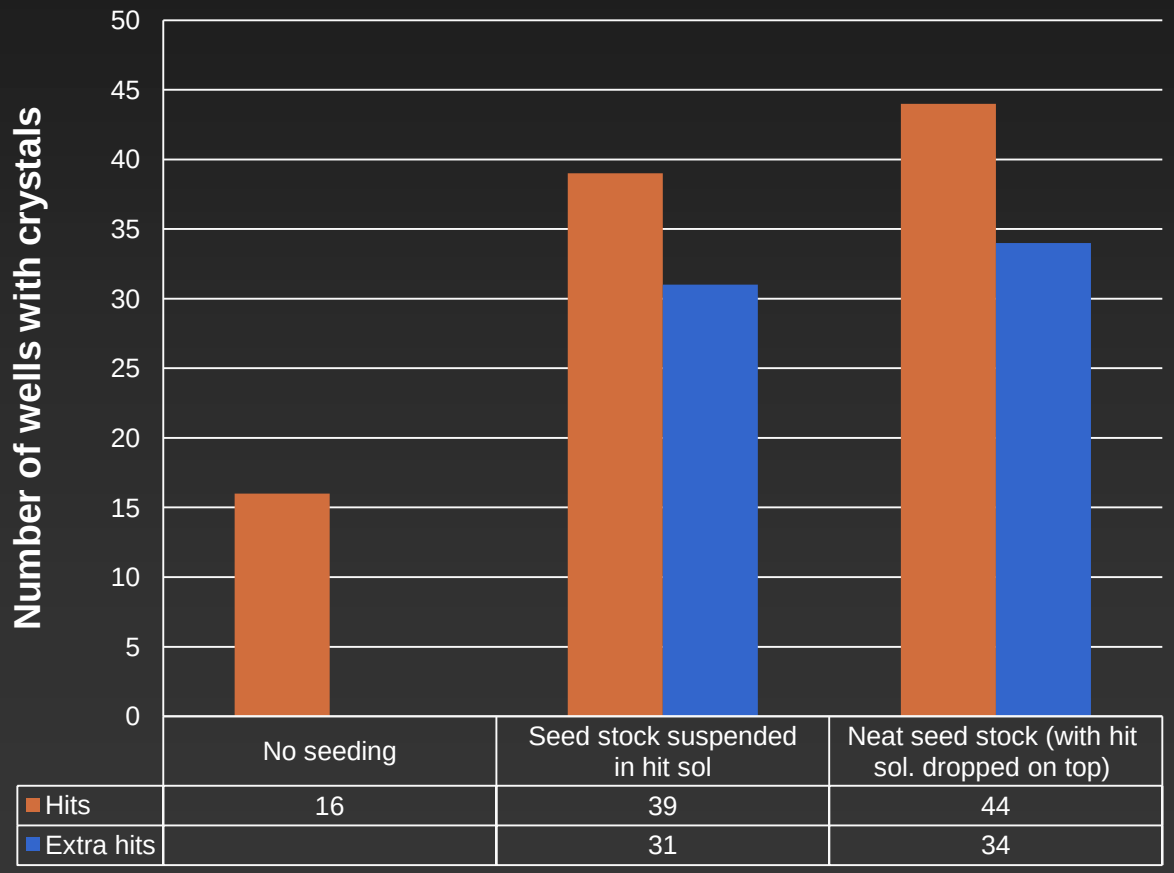
*4-bore tip*



Douglas Instruments

# rMMS with membrane proteins

See http://www.douglas.co.uk/MMS_proc.htm




A well-known bacterial membrane protein, crystallized by Christine Oswald (Goethe University, Frankfurt)

Douglas Instruments

# Microseeding toolkit

Douglas Instruments

If you want to know more:

“Random Microseeding: A Theoretical and Practical Exploration of Seed Stability and Seeding Techniques for Successful Protein Crystallization”

Patrick D. Shaw Stewart, Stefan A. Kolek, Richard A. Briggs, Naomi E. Chayen and Peter F.M. Baldock.

*Crystal Growth and Design*, 2011, 11 (8), p3432.

On-line at http://pubs.acs.org/doi/abs/10.1021/cg2001442

# Microseeding

*Opticryst* – a consortium of European institutions and companies aiming to improve crystal optimization.  2007 – 2010.

# Microseeding

Douglas Instruments

*Opticryst* – a consortium of European institutions and companies aiming to improve crystal optimization.  2007 – 2010.

We decided to look into microseeding, especially the stability of seeds.

# Microseeding



Douglas Instruments

*Opticryst* – a consortium of European institutions and companies aiming to improve crystal optimization.  2007 – 2010.

# Microseeding



*Opticryst* – a consortium of European institutions and companies aiming to improve crystal optimization.  2007 – 2010.

Stefan set up 30,000 drops and estimated the number of crystals In 15,000 drops!

# random Microseed Matrix-Screening

| *Our questions:* | *Take-home practical suggestions:* |
|---|---|
| ***(1) How can we get as many hits as possible?*** | |
| ***(2) How stable are the seed stocks?*** | |
| *(3) Is "preseeding" the protein stock helpful?* | |
| *(4) How can we avoid salt crystals?* | |
| *(5) How can we get more diverse crystals?* | |
| ***(6) How can we stabilize protein complexes?*** | |
| *(7) Can we harvest seed crystals from microfluidic devices?* | |
| *(8) What can you do if you have no crystals?* | |

Douglas Instruments

| Protein | Source | Concentration |
|---|---|---|
| Glucose Isomerase | Hampton Research | 33 mg/ml |
| Hemoglobin | Sigma Aldrich | 60 mg/ml |
| Thaumatin | Sigma Aldrich | 30 mg/ml |
| Thermolysin | Sigma Aldrich | 15 mg/ml |
| Trypsin | Sigma Aldrich | 30 mg/ml |
| Xylanase | Macro Crystal | 36 mg/ml |

*“Receptive” conditions*

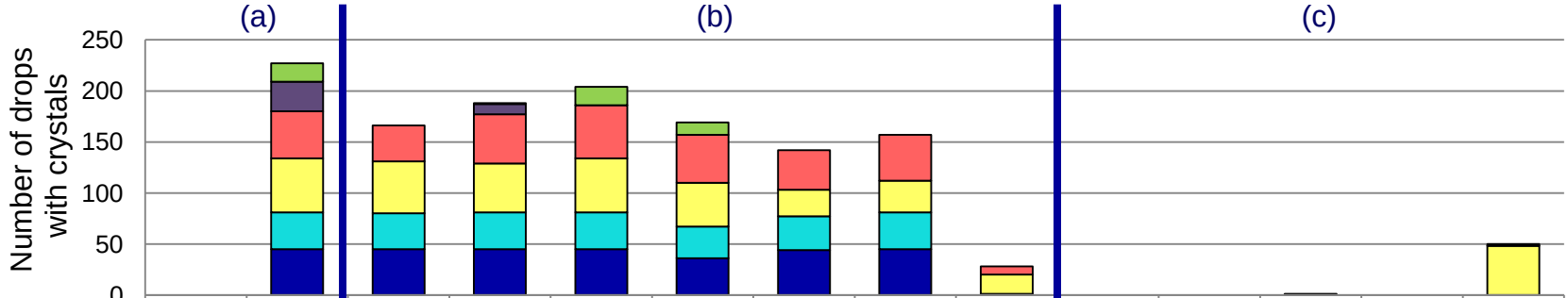
*Conditions where:*

*(1) crystals don’t grow without seeds in four drops, but*

*(2) crystals grow in at least three out of four drops with seeds.*

*Do any other precipitants work better than the Hit Solution for suspending seed crystals?*

# *Focusing on “receptive” conditions*




| | Regular screens (x2) | Seeds in Hit Sol. 10nl | Seeds in isopro 10nl | Seeds in PEG 600 10nl | Seeds in am.sul 10nl | Seeds in 50:50 am.su, Hit 10nl | Seeds in NaCl 10nl | Seeds in 50:50 NaCl, Hit 10nl | Seeds in prot. 10nl | Screen with Hit Sol. 10nl | Screen with PEG 600 10nl | Screen with am.sul. 10nl | Screen with NaCl 10nl | Screen with Hit Sol. 100nl |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gluc. Isom. | 0 | 18 | 0 | 1 | 18 | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| Hemoglob. | 0 | 29 | 0 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Thaumatin | 0 | 46 | 35 | 48 | 52 | 47 | 39 | 45 | 8 | 0 | 0 | 0 | 0 | 0 |
| Thermolys. | 0 | 53 | 51 | 48 | 53 | 43 | 26 | 31 | 19 | 0 | 0 | 1 | 0 | 48 |
| Trypsin | 0 | 36 | 35 | 36 | 36 | 31 | 33 | 36 | 1 | 0 | 0 | 0 | 0 | 0 |
| Xylanase | 0 | 45 | 45 | 45 | 45 | 36 | 44 | 45 | 0 | 0 | 0 | 0 | 0 | 0 |

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# random Microseed Matrix-Screening

| *Our questions:* | *Take-home practical suggestions:* |
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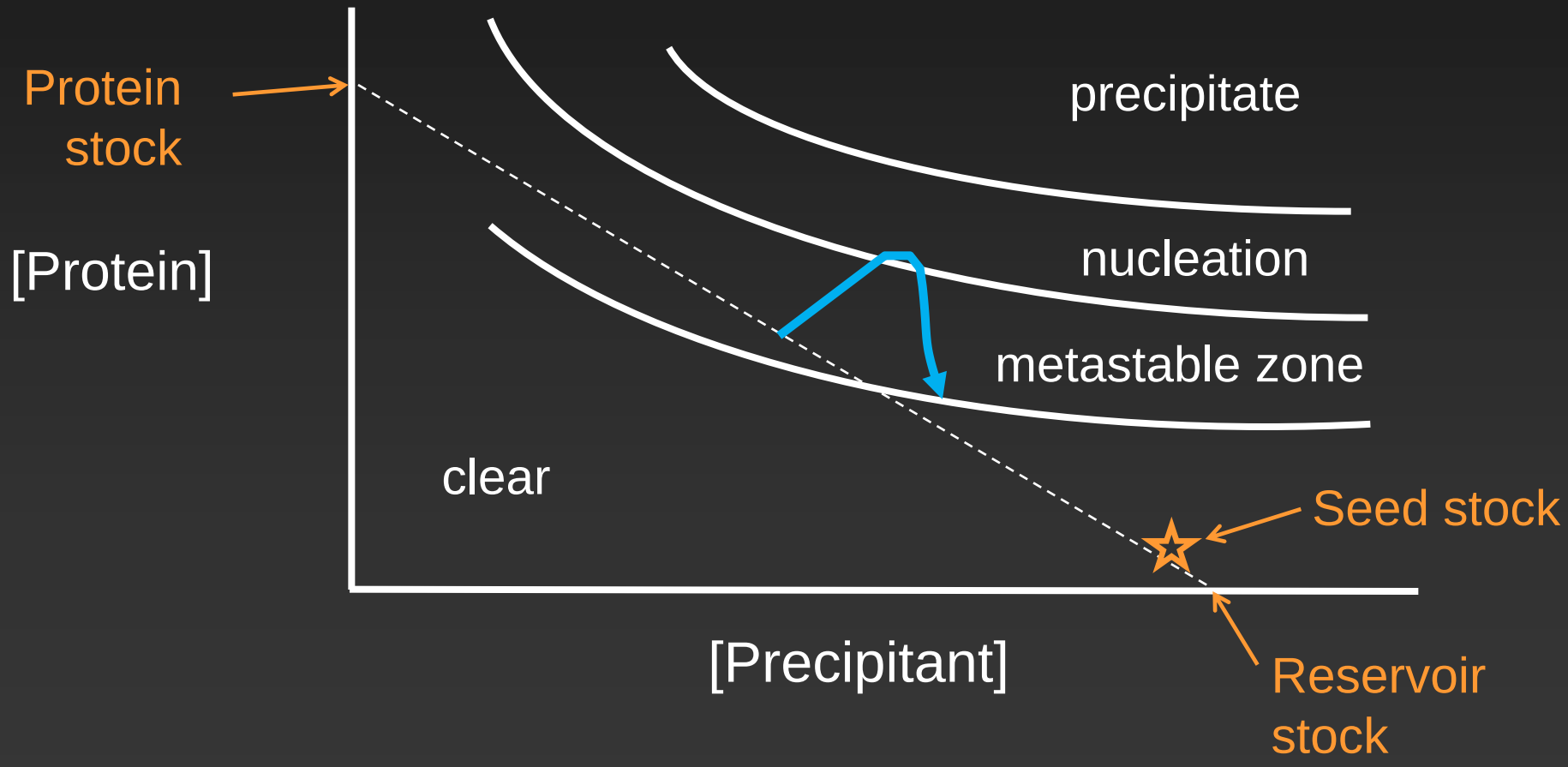
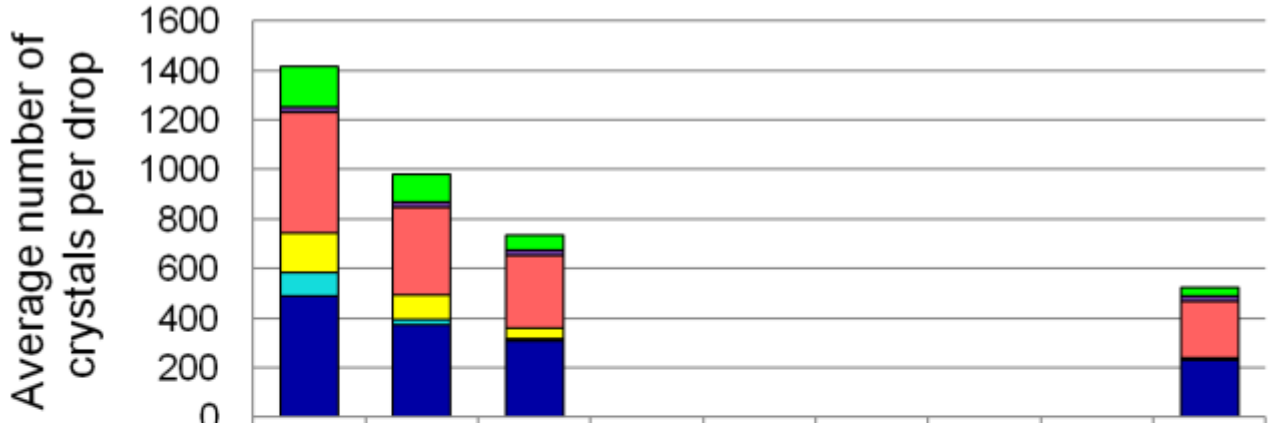
# Phase diagram of a protein




Douglas Instruments




| | 0 hrs | 3 hrs | 6 hrs | 9 hrs | 12 hrs | 15 hrs | 18 hrs | 21 hrs | 24 hrs |
|---|---|---|---|---|---|---|---|---|---|
| Gluc. isom. | 166 | 113 | 63 | | | | | | 36 |
| Hemoglob. | 19 | 18 | 19 | | | | | | 20 |
| Thaumatin | 489 | 357 | 293 | | | | | | 228 |
| Thermolysin | 158 | 96 | 46 | | | | | | 11 |
| Trypsin | 95 | 23 | 6.0 | | | | | | 0.1 |
| Xylanase | 490 | 372 | 309 | | | | | | 229 |

# random Microseed Matrix-Screening

| *Our questions:* | *Take-home practical suggestions:* |
|---|---|
| *(1) How can we get as many hits as possible?* | *Stick to the 'hit solution' for suspending seed crystals for routine rMMS* |
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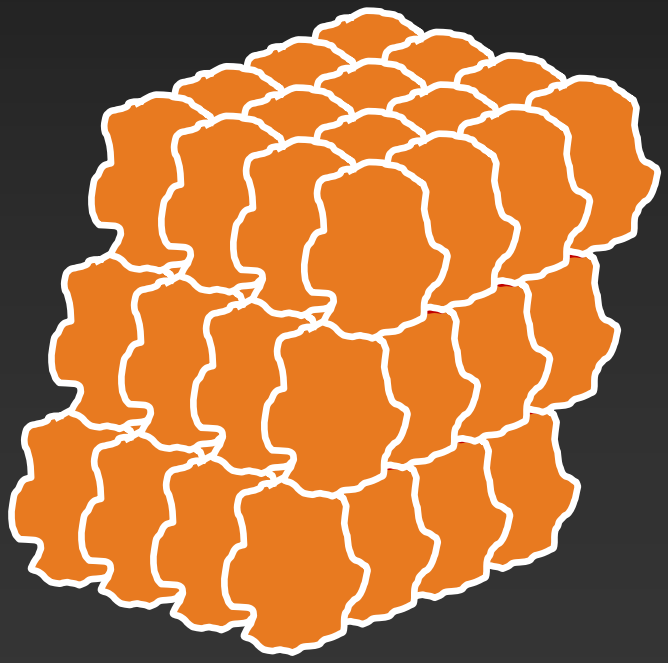
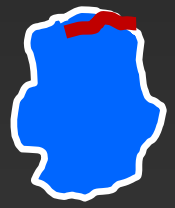
*Suggested by Lesley Haire, National Institute for Medical Research*

# Cross-seeding

A natural approach, especially when you are adding something small e.g. a peptide or nucleic acid



Uncomplexed protein crystals



Complex

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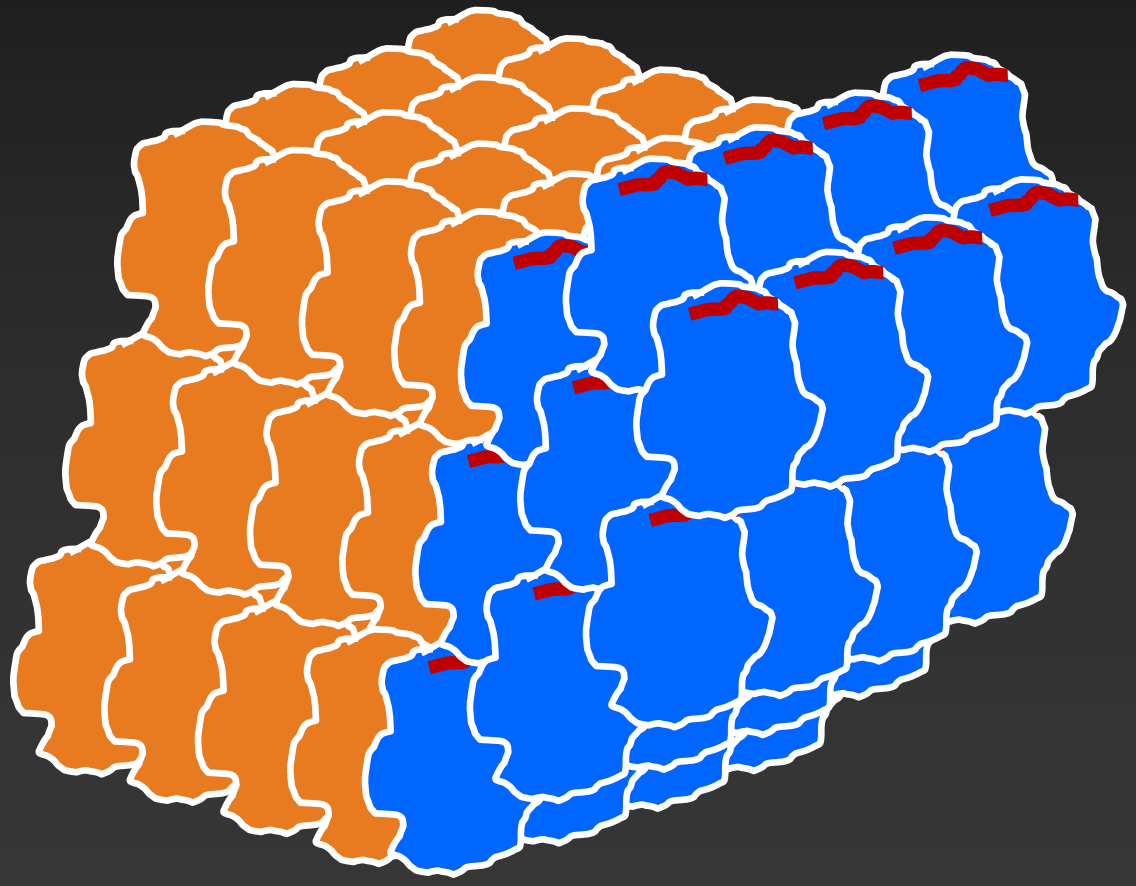
# Cross-seeding

You don’t have to match the unit cell, only one of the structural planes of the crystals



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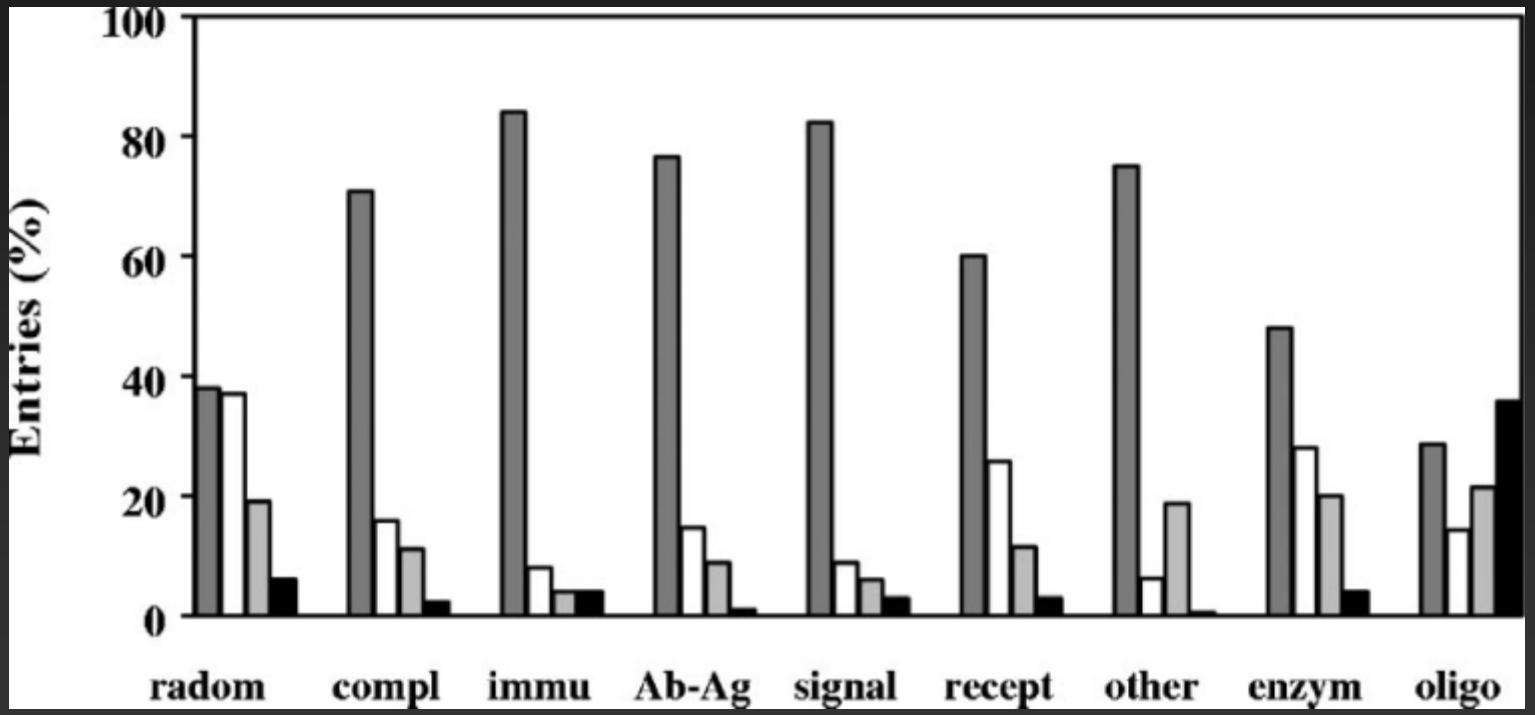
# Crystallizing complexes

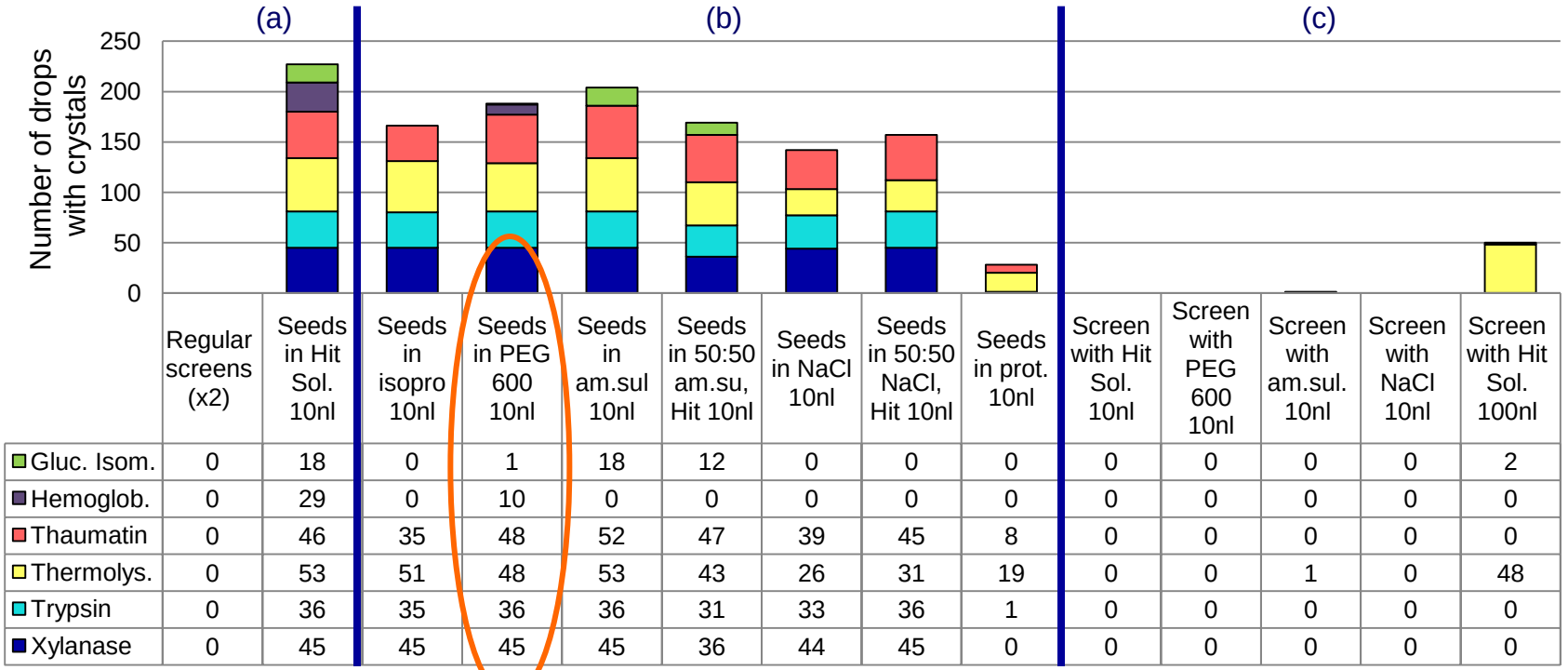
Radaev and Sun. Crystallization of protein-protein complexes.
J. Appl. Cryst. (2002). 35, 674-676




- PEG / (NH4)2SO4 / other salts / organic solvents (including 2-propanol, MPD, ethanol)

*Random uncomplexed samples*, all *protein-protein complexes* included in this survey, immune complexes, antibody-antigen complexes, signal transduction complexes, receptor and ligand complexes, miscellaneous protein-protein complexes, enzyme related complexes, *oligomeric protein complexes*

# *What can we replace the Hit Solution with?*

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| Hemoglob. | 0 | 29 | 0 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Thaumatin | 0 | 46 | 35 | 48 | 52 | 47 | 39 | 45 | 8 | 0 | 0 | 0 | 0 | 0 |
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Douglas Instruments

# random Microseed Matrix-Screening

| *Our questions:* | *Take-home practical suggestions:* |
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| *(4) How can we avoid salt crystals?* | *Please read the paper!* |
| *(5) How can we get more diverse crystals?* | *Please read the paper!* |
| *(6) How can we stabilize protein complexes?* | *Avoid high salt in your seed stock;* |
| *(7) Can we harvest seed crystals from microfluidic devices?* | *Please read the paper!* |
| *(8) What can you do if you have no crystals?* | *Please read the paper!* |

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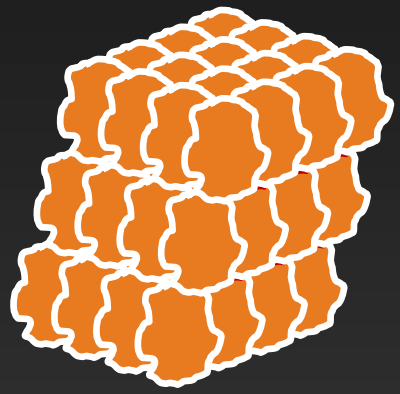
*Can we predict which solutions the seed crystals will be stable in?*

*Appearance of uncrushed crystals after incubation for one day*

| Protein | Crystals in Hit Sol. | Crystals in Isopropanol | Crystals in PEG 600 | Crystals in Amm.sul. | Crystals in NaCl | Crystals in protein stock |
|---|---|---|---|---|---|---|
| Gluc. Isom. | **OK** | Cracked | Shattered | **Cracked** | Dissolved | Dissolved |
| Hemoglobin | **OK** | Cracked | **OK** | Dissolved | Dissolved | Dissolved |
| Thaumatin | **OK** | **Cracked** | **OK** | **OK** | **OK** | **Grew** |
| Thermolysin | **OK** | **OK** | **Shattered** | **OK** | **Dissolved** | **Grew** |
| Trypsin | **OK** | **OK** | **Dissolved** | **OK** | **OK** | Dissolved |
| Xylanase | **OK** | **OK** | **Cracked** | **OK** | **OK** | Dissolved |

# Try to find a solution that both the seed crystals *and* the complex are stable in



*Investigate stability of complex with isothermal calorimetry, fluorescence anisotropy, thermal shift assay etc.*

*Test stability of seed crystals by incubation of uncrushed crystals in the suggested solution for 1 day*

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| *(5) How can we get more diverse crystals?* | *Please read the paper!* |
| *(6) How can we stabilize protein complexes?* | *Avoid high salt in your seed stock; remove ingredients .... test by incubation for 1 day* |
| *(7) Can we harvest seed crystals from microfluidic devices?* | *Please read the paper!* |
| *(8) What can you do if you have no crystals?* | *Please read the paper!* |

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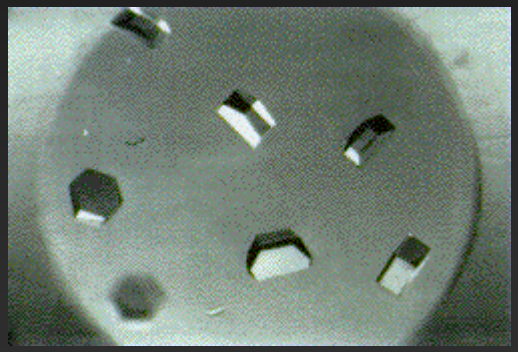
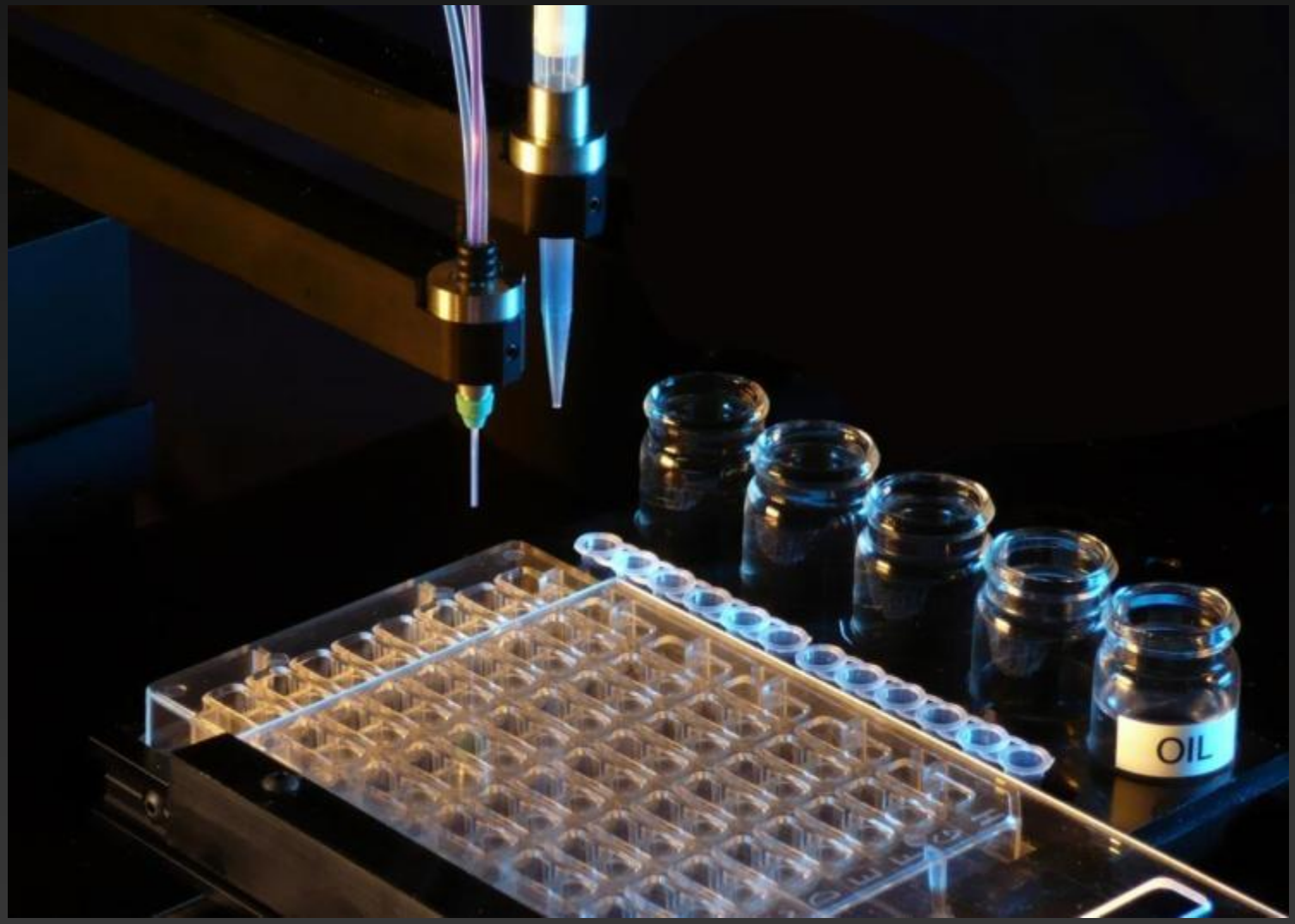
# *Soaking experiments*

*You need a good supply of wells with about 5 crystals per drop*



*According a user in Basle, seeding with diluted seed stock is “the only reliable way” to achieve this*

# New “combinatorial” experimental design

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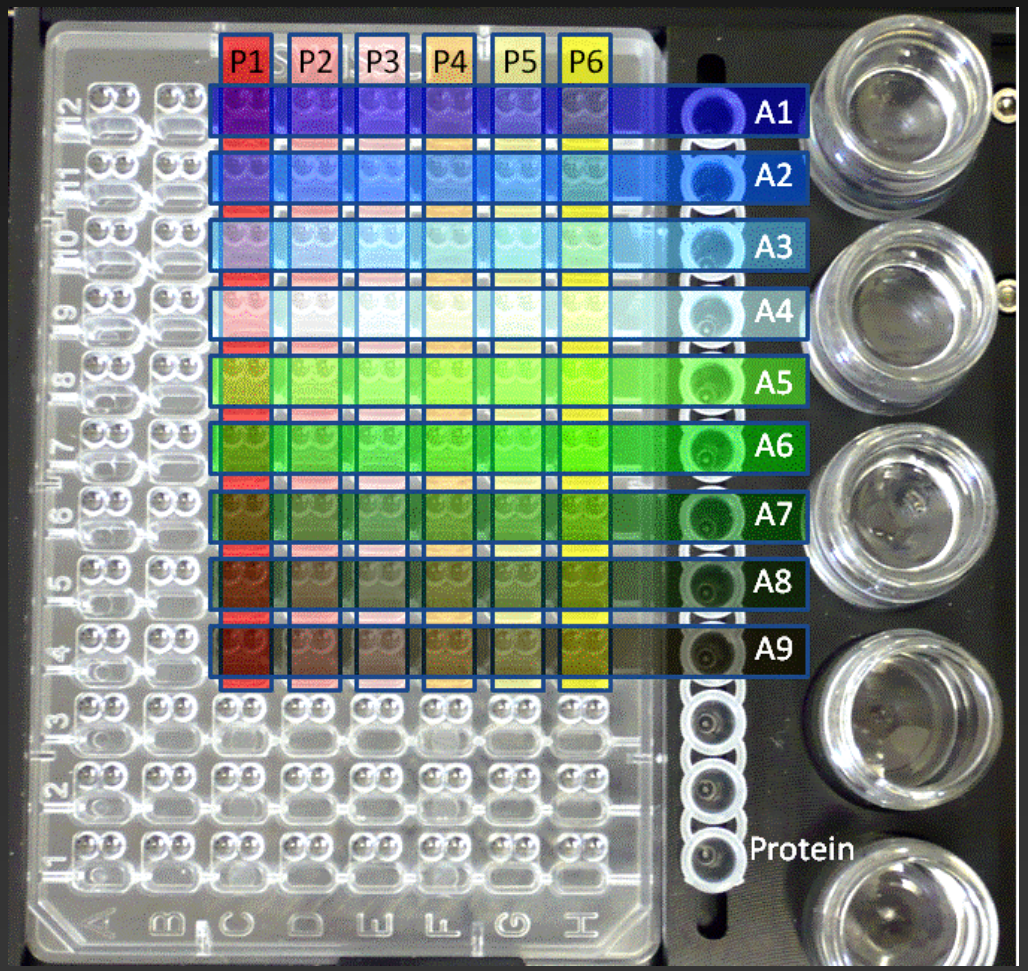
# New “combinatorial” experimental design

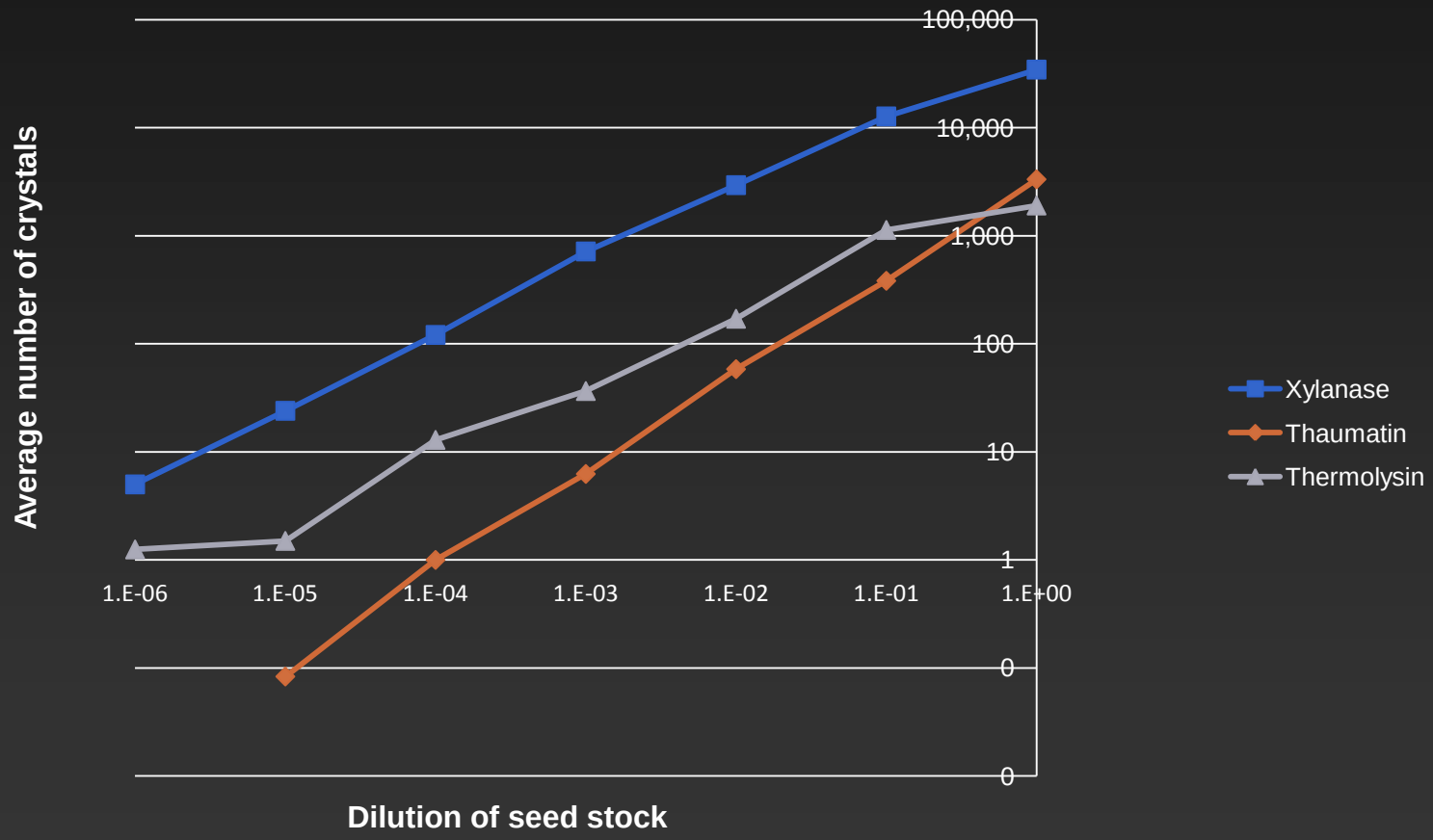



Microseeding:

A1: 100% seed stock
A2: 25% seed stock
A3: 6.3% seed stock
A4: 1.6% seed stock
A5: 0.4% seed stock
A6: 0.1% seed stock
A7: 0.02% seed stock
A8: 0.006% seed stock
A9: 0.002% seed stock

Douglas Instruments

# New “combinatorial” experimental design

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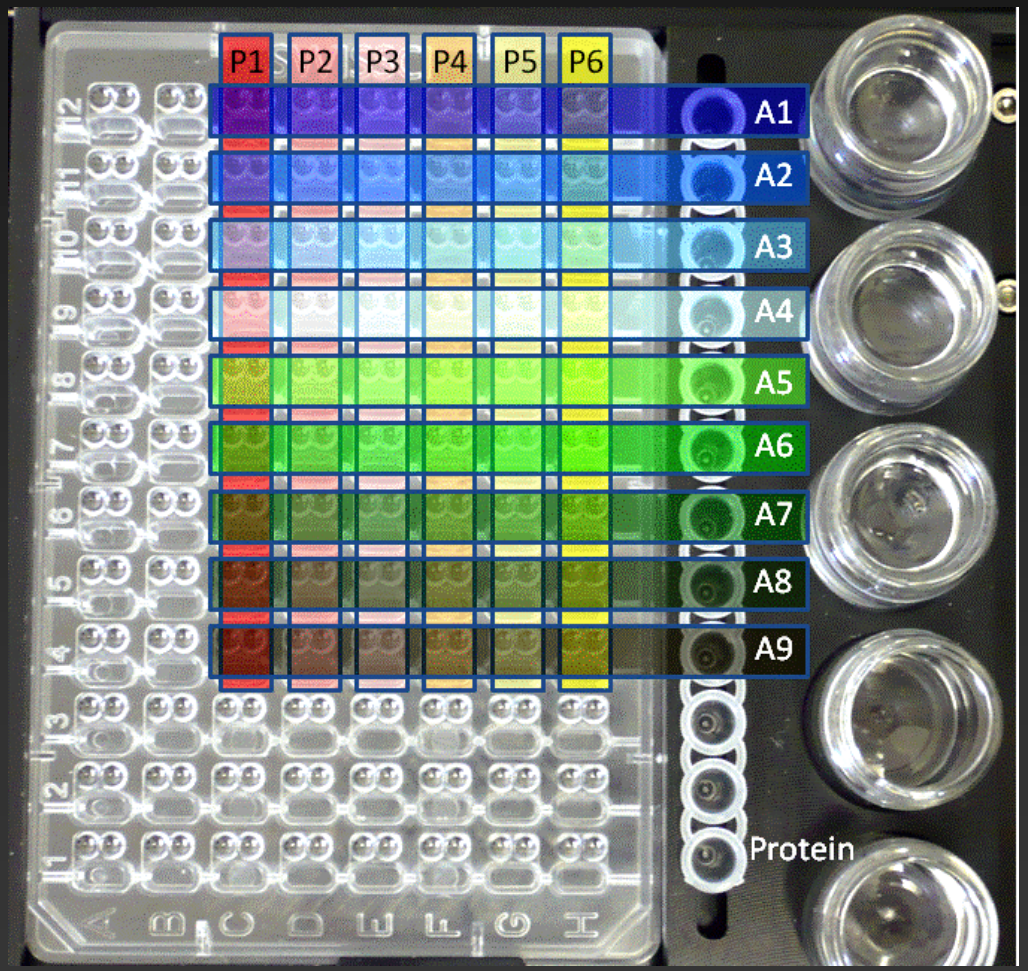
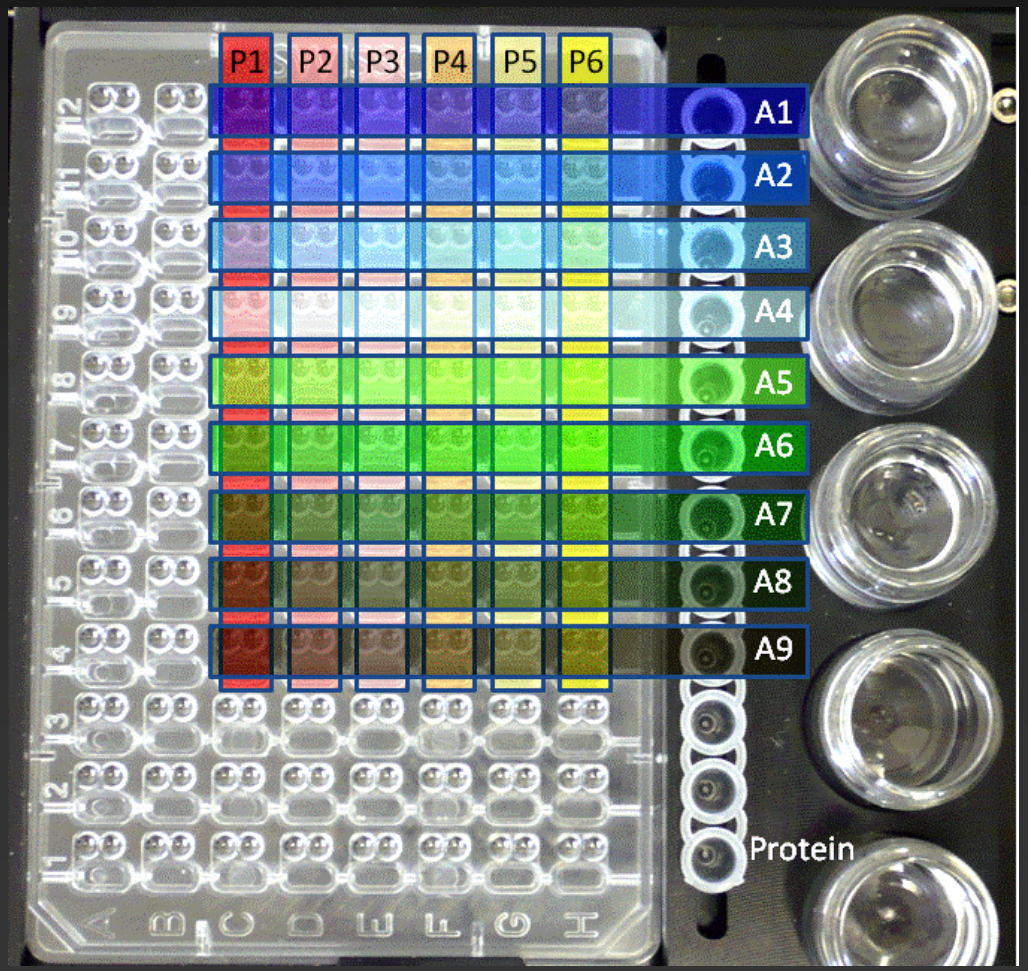



Or test up to 12
inhibitors or ligands

Douglas Instruments

# New “combinatorial” experimental design




Or test up to 12 inhibitors or ligands

Or 12 proteins

Douglas Instruments

# New “combinatorial” experimental design

A fourth use – re-shuffling

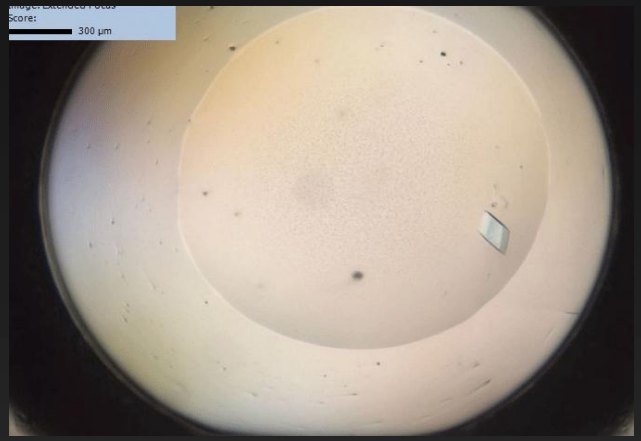

Douglas Instruments

# Presentation by Lesley Haire, NIMR at BCA 2012

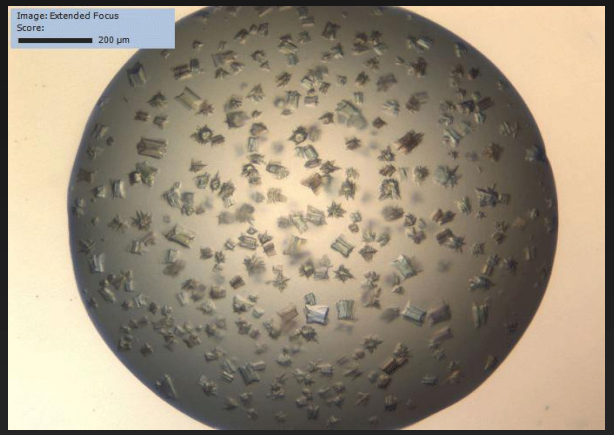
**Original Hits – note color coding:**

1. **40% MPD, KI**
2. **30% peg 550MME, HEPES pH 7.5, MgCl2**
3. **30% pentaerythritol propoxylate, Hepes pH 7.5, KCl**
4. **30% pentaerythritol ethoxylate, AmS04, bistris pH 6.**
5. **30% Jeffamine ED 2001, Hepes pH 7.0**

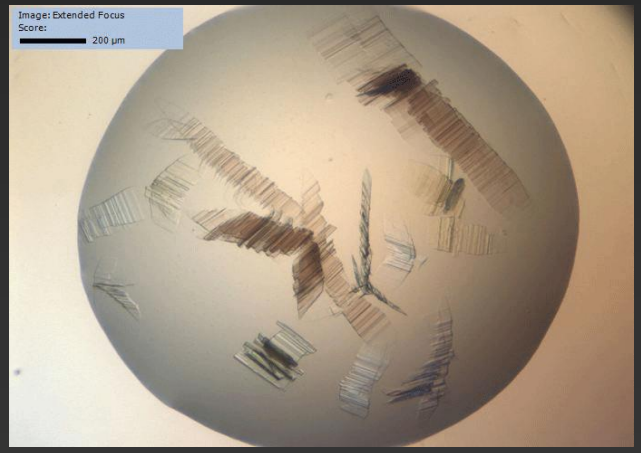
# Original Hits



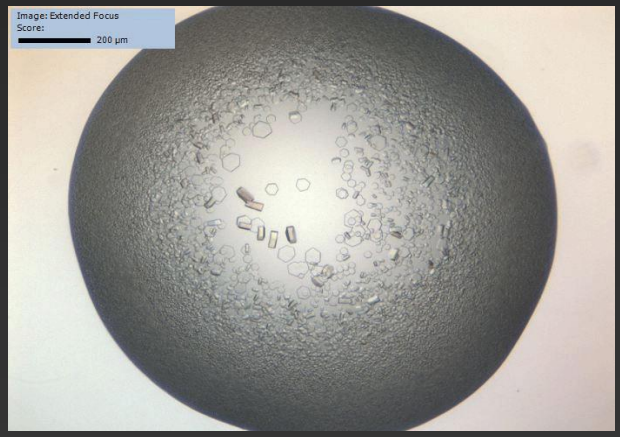

MPD, KI




550MME , MgCl2 pH 7.5




Pentaerythritol propoxylate ,KCl, pH7.5




Ethoxylate, AmS04, pH 6.5

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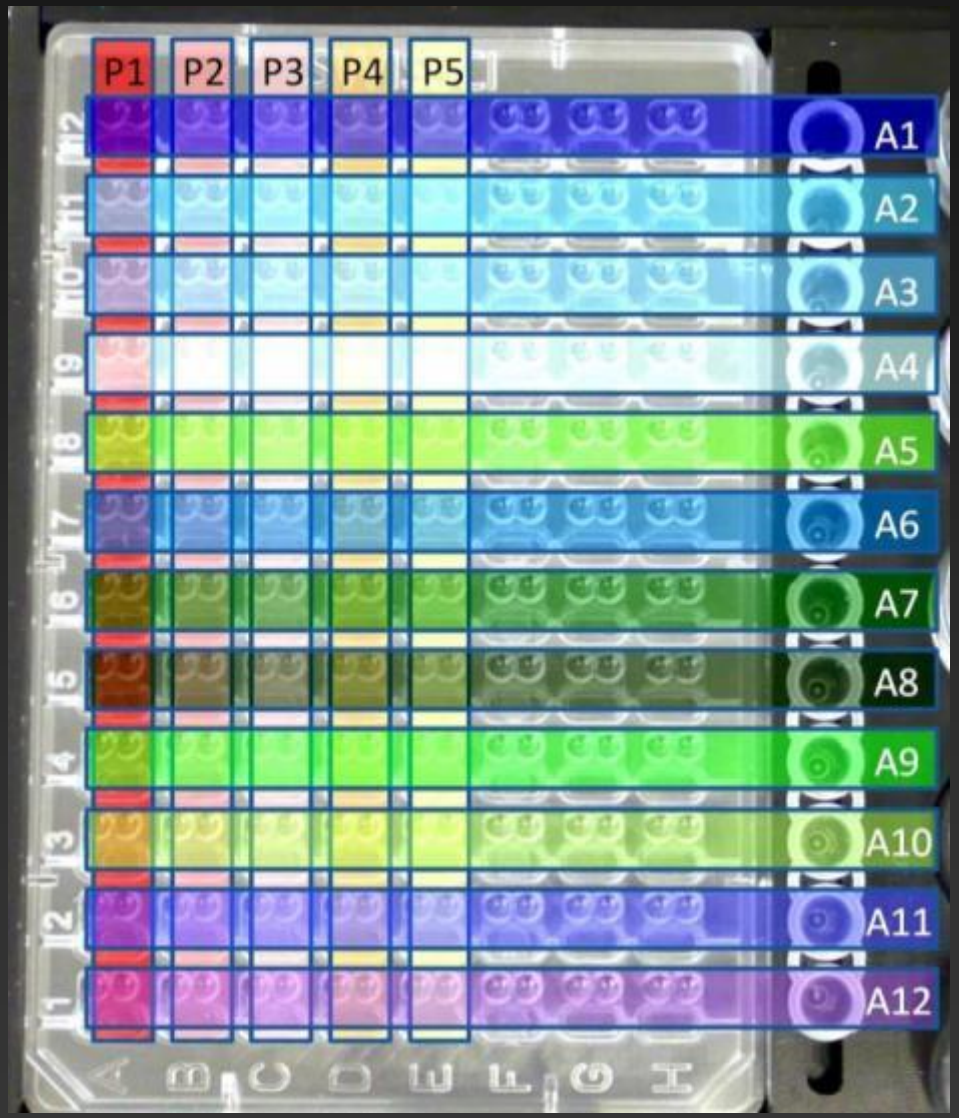


**Precipitants**

P1: **40% MPD**

P2: **30% peg 550MME**

P3: **30% pentaerythritol propoxylate**

P4: **30% pentaerythritol ethoxylate**

P5: 30% Jeffamine ED 2001

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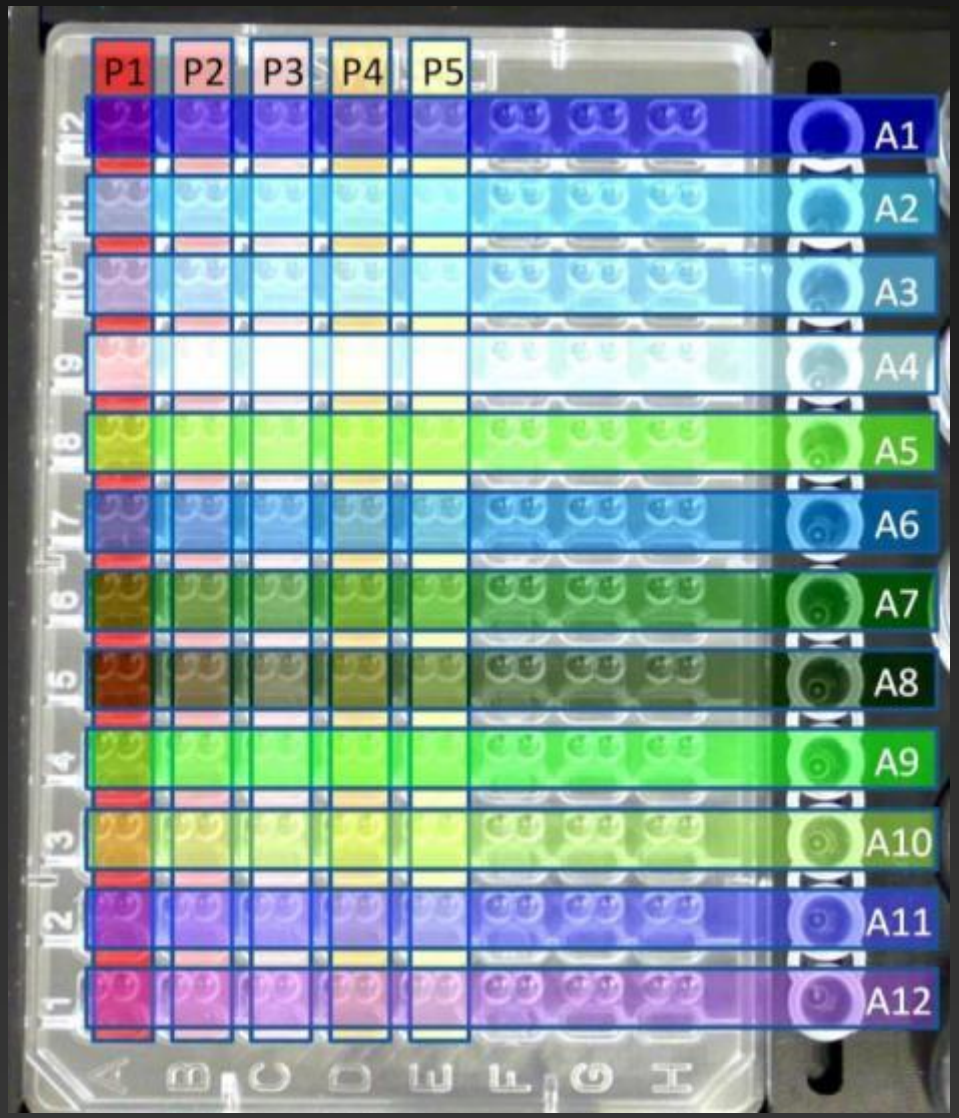
## Additives: final conc. in drop




A1: 0.05M MgCl2, 0.1M bistris pH 6.5

A2: 0.05M MgCl2, 0.1M Hepes pH 7.0

A3: 0.05M MgCl2, 0.1M Hepes pH 7.5

A4: 0.05M AmS04, 0.1M bistris pH 6.5

A5: 0.05M AmS04, 0.1M Hepes pH 7.0

A6: 0.05M AmS04, 0.1M Hepes pH 7.5

A7: 0.2M KCl, 0.1M bistris pH 6.5

A8: 0.2M KCl, 0.1M Hepes pH 7.0

A9: 0.2M KCl, 0.1M Hepes pH 7.5

A10: 0.1M KI, 0.1M bistris pH 6.5

A11: 0.1M KI, 0.1M Hepes pH 7.0

A12: 0.1M KI, 0.1M Hepes pH 7.5

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No crystals with P1 - P3

| | Peg 550MME | Jeffamine 200 |
|---|---|---|
| 1. MgCl2 pH 6.5 | precipitate | tiny crystals |
| 2. MgCl2 pH 7.0 | precipitate | tiny crystals |
| 3. MgCl2 pH 7.5 | crystals | tiny crystals |
| 4. AmS04 pH 6.5 | crystals | precipitate |
| 5. AmS04 pH 7.0 | tiny crystals | precipitate |
| 6. AmS04 pH 7.5 | crystals | precipitate |
| 7. KCl pH 6.5 | crystals | tiny crystals |
| 8. KCl pH 7.0 | crystals | tiny crystals |
| 9. KCl pH 7.5 | clear drop | precipitate |
| 10. KI pH 6.5 | spheroids | crystals |
| 11. KI pH 7.0 | spheroids | precipitate |
| 12. KI pH 7.5 | crystals | light precipitate |

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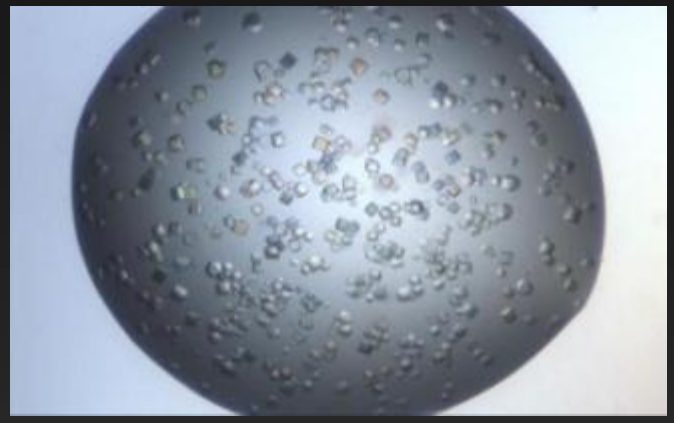
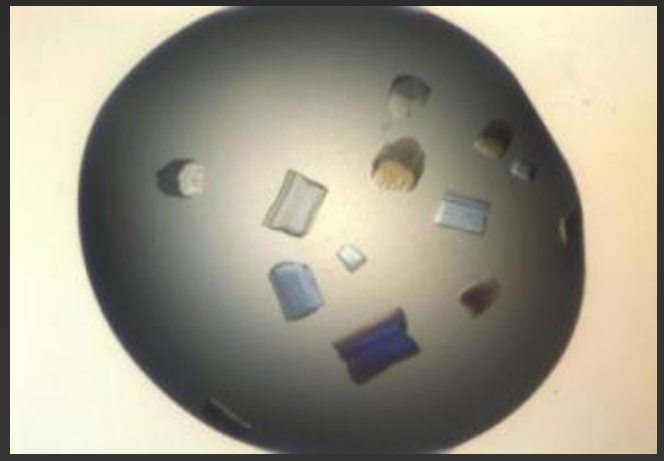
## Reshuffling - crystals from 30% peg 550MME

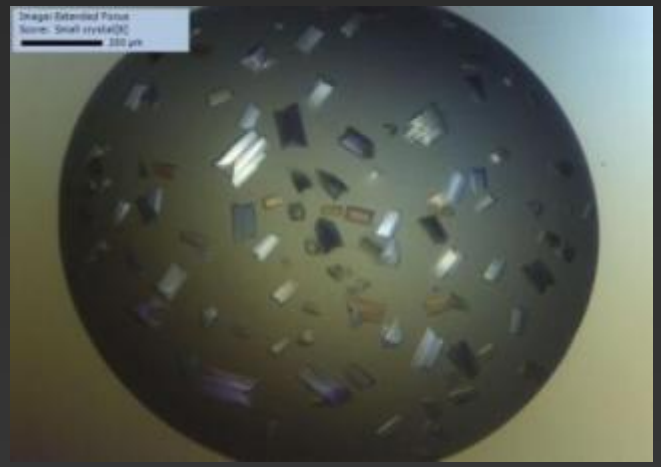
MgCl2, pH 7.5



KI, pH 6.5



KCl, pH 6.5



KI, pH 7.5

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# Analysis of the trends

**Peg 550MME** + AmS04 gave crystals with similar morphology to the original hit with pentaerythritol ethoxylate + AmS04

KI and AmS04 gave crystals with “nice” morphology

From this experiment, **30% peg 550MME** gives the most hits

pH 6.5 and **7.5** gave most crystals

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# rMMS: comments by Allan D'Arcy

1. Freeze your seed stock – then you can always reproduce your crystals (even years later)

2. rMMS greatly reduces the need for crystal optimization

3. Make it part of your routine workflow !

# Thank you for listening

Microseeding paper:

Shaw Stewart *et al.,* Cryst. Growth Des., 2011, 11 (8), p3432.

Talk and papers are on my memory sticks

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