The Abdus Salam
International Centre for Theoretical Physics

United Nations
Educational, Scientific and
Cultural Organization

IAEA
International Atomic Energy Agency

2336-12

Advanced School: From Genes to Atomic Structures: an Introduction to Synchrotron-Based Structural Biology

23 - 27 April 2012

Random Microseeding

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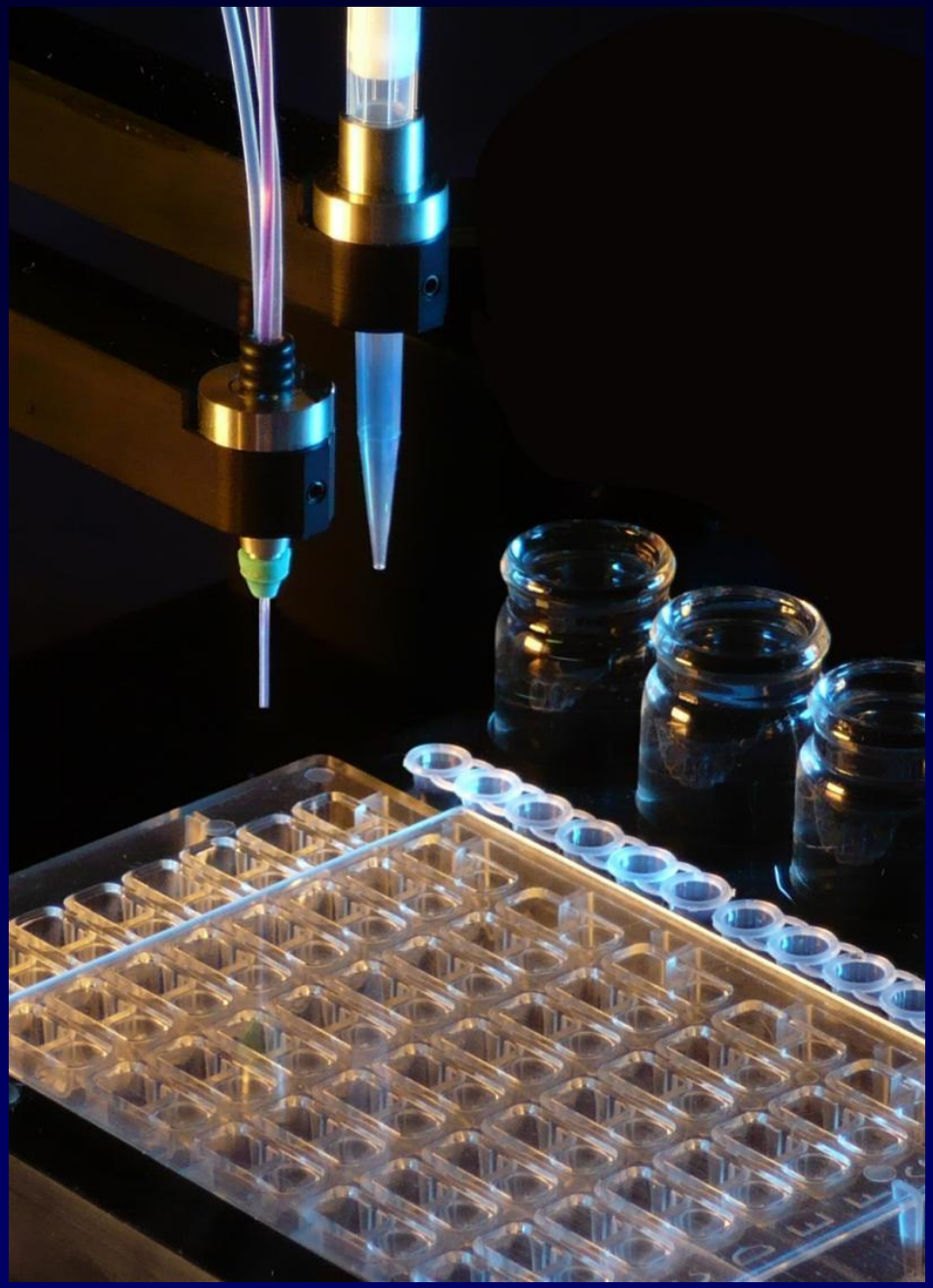
U.K.

Strada Costiera 11, 34151 Trieste, Italy - Tel.+39 040 2240 111; Fax +39 040 224 163 - sci_info@ictp.it



Microseed it!

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

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

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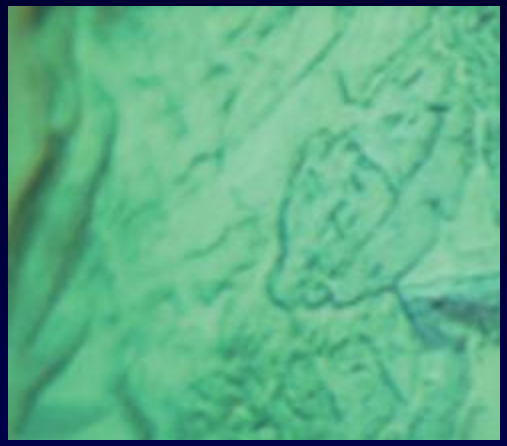
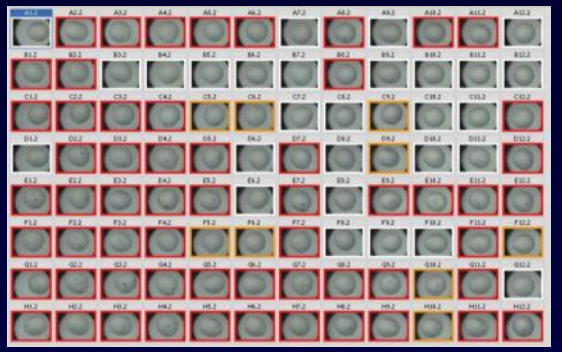
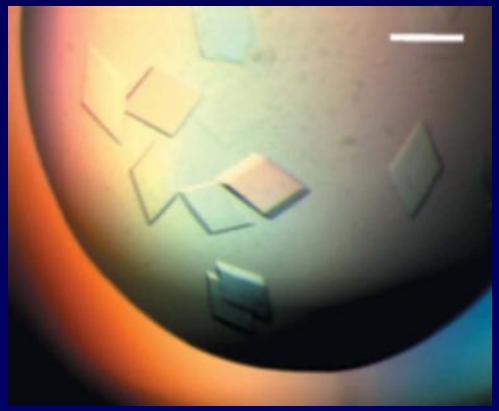
Microseeding slide 2

Protein crystallization

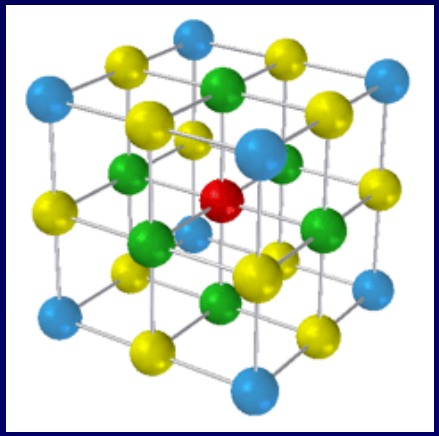


Step 1: screening with random solutions that have given crystals before x 96





Step 2: optimization by making small changes



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Protein crystallization

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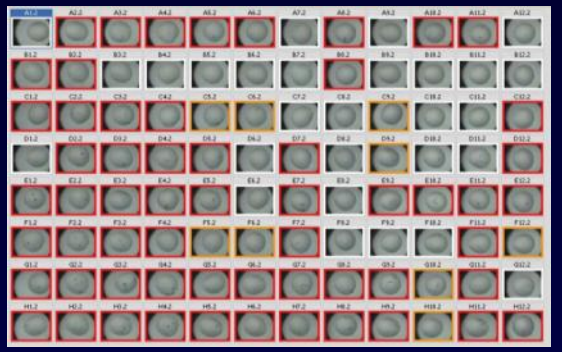

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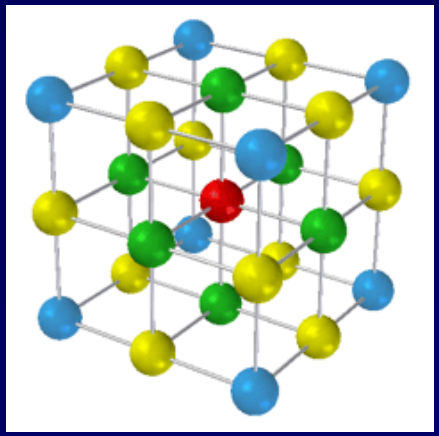

Modify your protein or make a new construct

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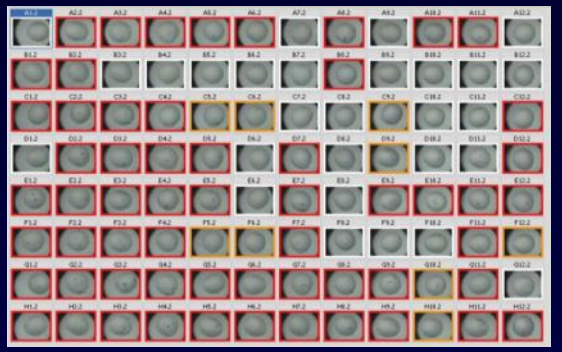
Protein crystallization

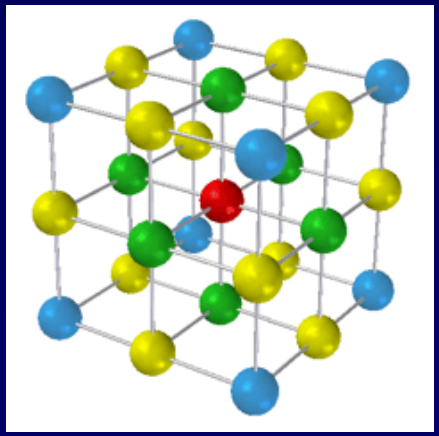
Step 1: screening with random solutions that have given crystals before x 96



Step 1.5: random microseeding

Step 2: optimization by making small changes

Modify your protein or make a new construct



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Protein crystallization

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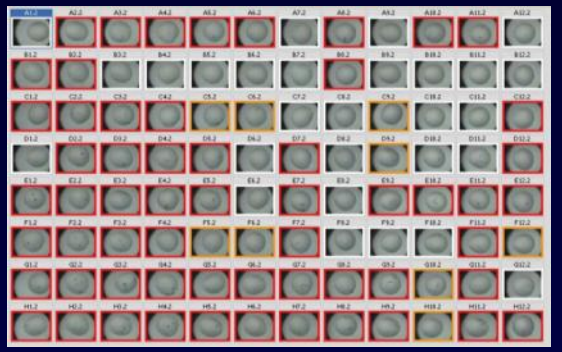
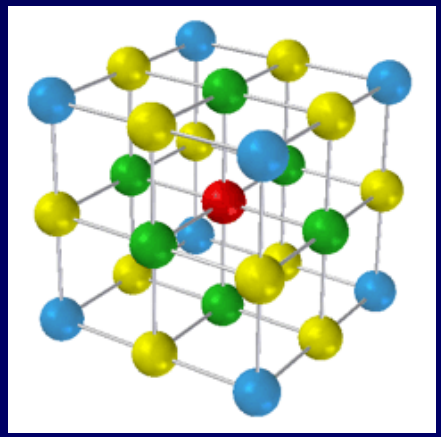


Step 1.5: random microseeding

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

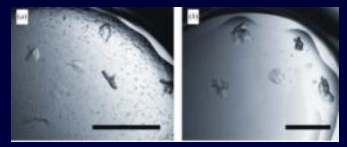
Conventional methods

Random microseeding (rMMS)

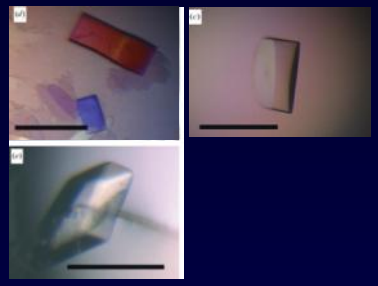
Complexes:
IL-13/C836
(mouse antibody)

No hits

Optimization



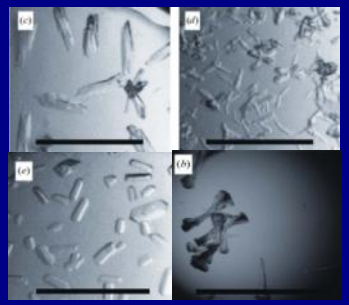
2.0 Å res.
monoclinic $P2_1$



2.0 Å res.
monoclinic $P2_1$

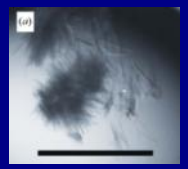
40 residues
changed

Microseeding



Microseeding

One hit

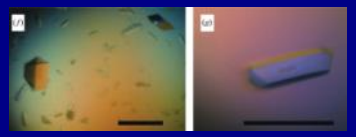


IL-13/H2L6
(humanized
mAb)

Cross-seeding

4 residues
changed

Optimization



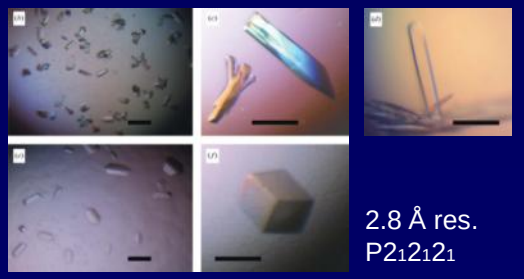
Microseeding

Both 1.9 Å resolution
orthorhombic $P2_12_12_1$

IL-13/M1295
(affinity-matured
humanized mAb)

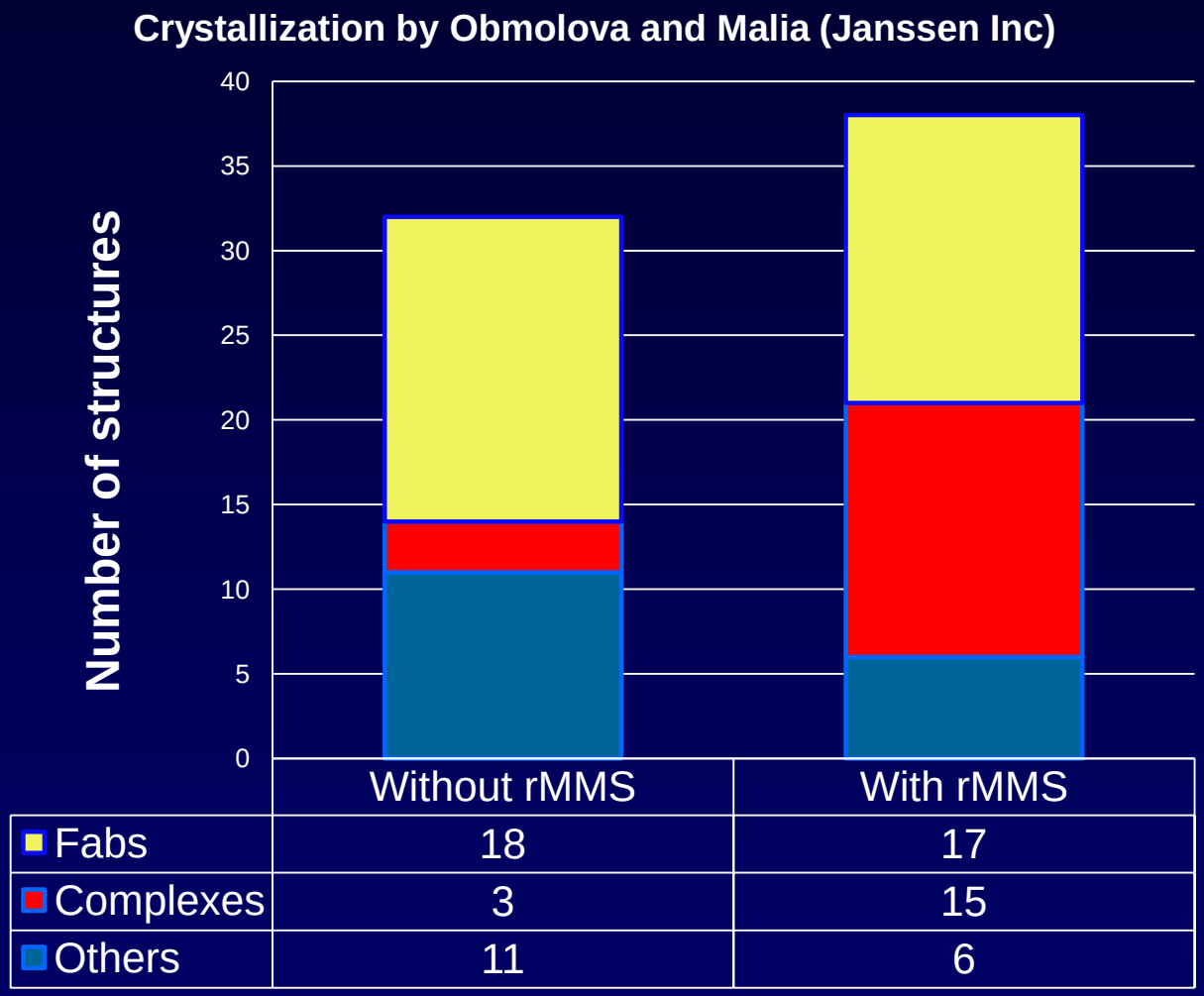
No hits

Cross-seeding



2.8 Å res.
$P2_12_12_1$

random Microseed Matrix-Screening



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'

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)

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Microseeding in *screening* experiments

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

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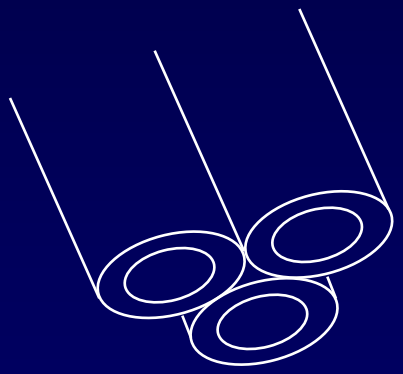
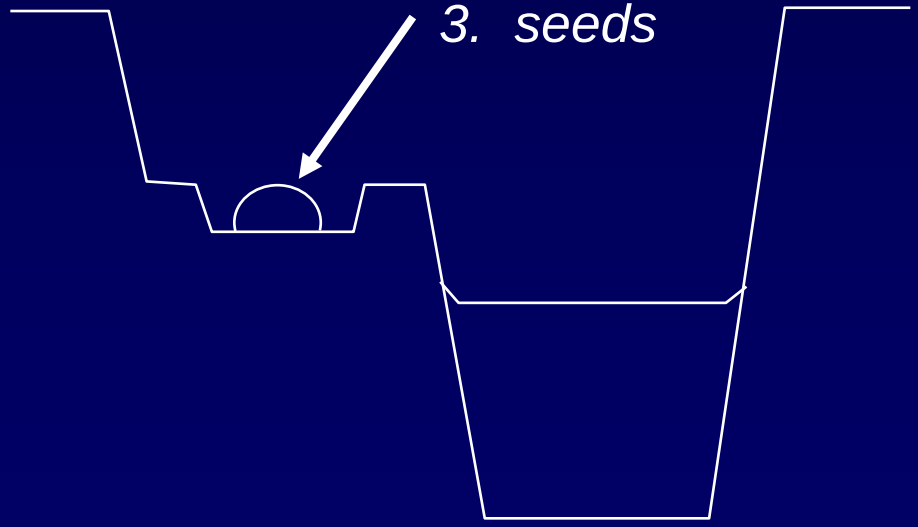
3-bore tip

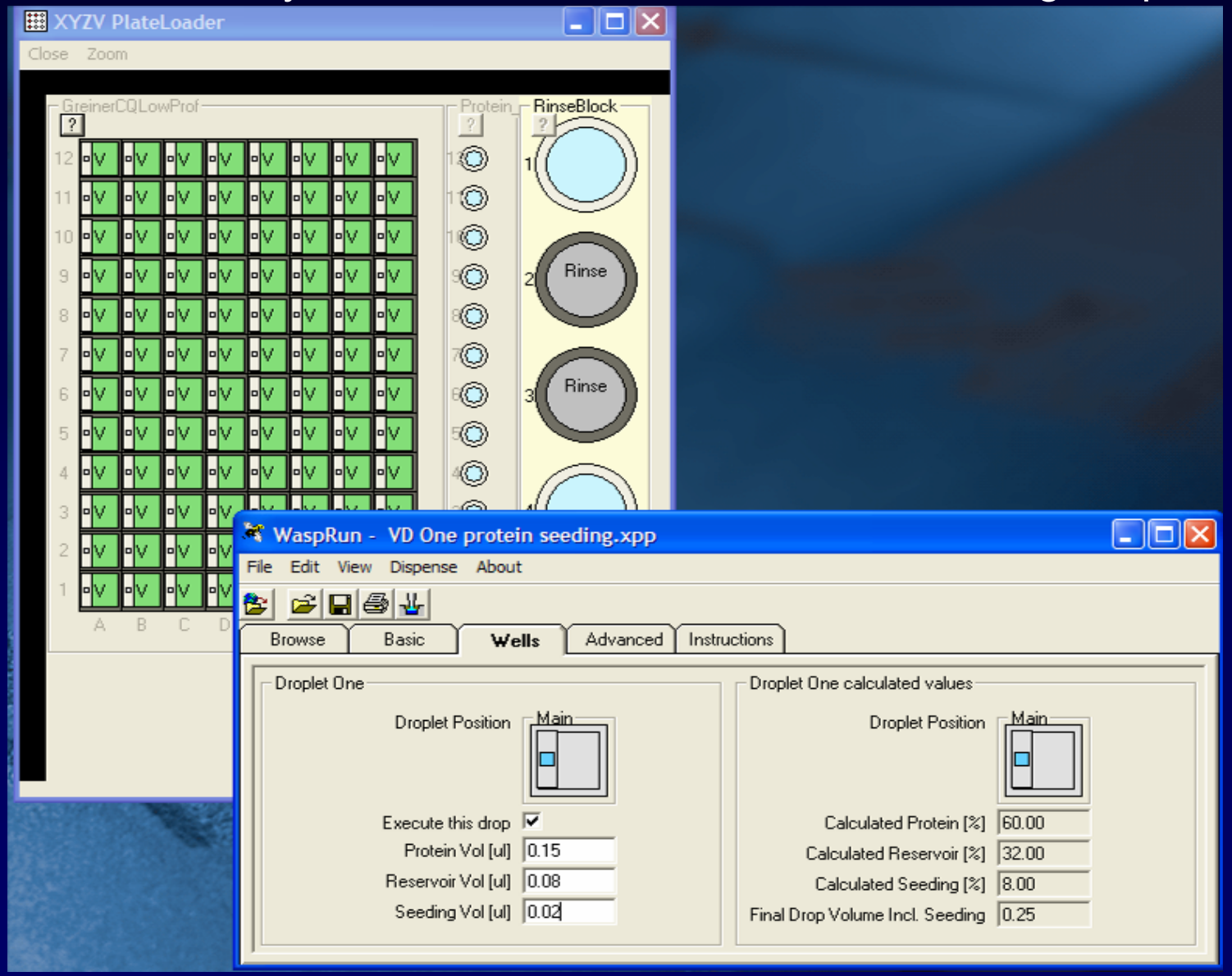


1. protein
2. reservoir solution
3. *seeds*



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**

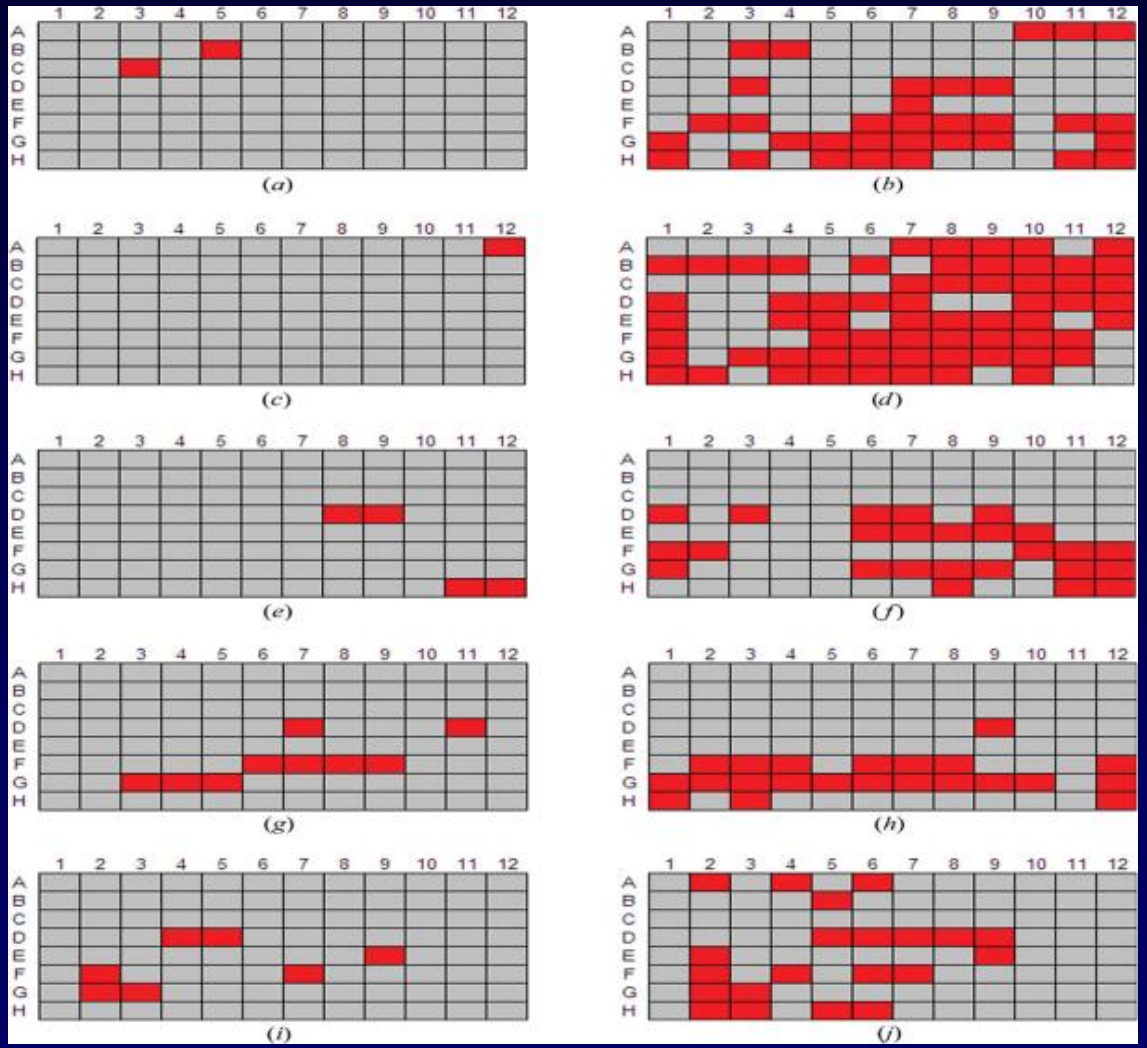
MMP12

BVP

USP7

Trypsin

PPE



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Microseeding in *screening* experiments

Regular screen **Screen with seeds**

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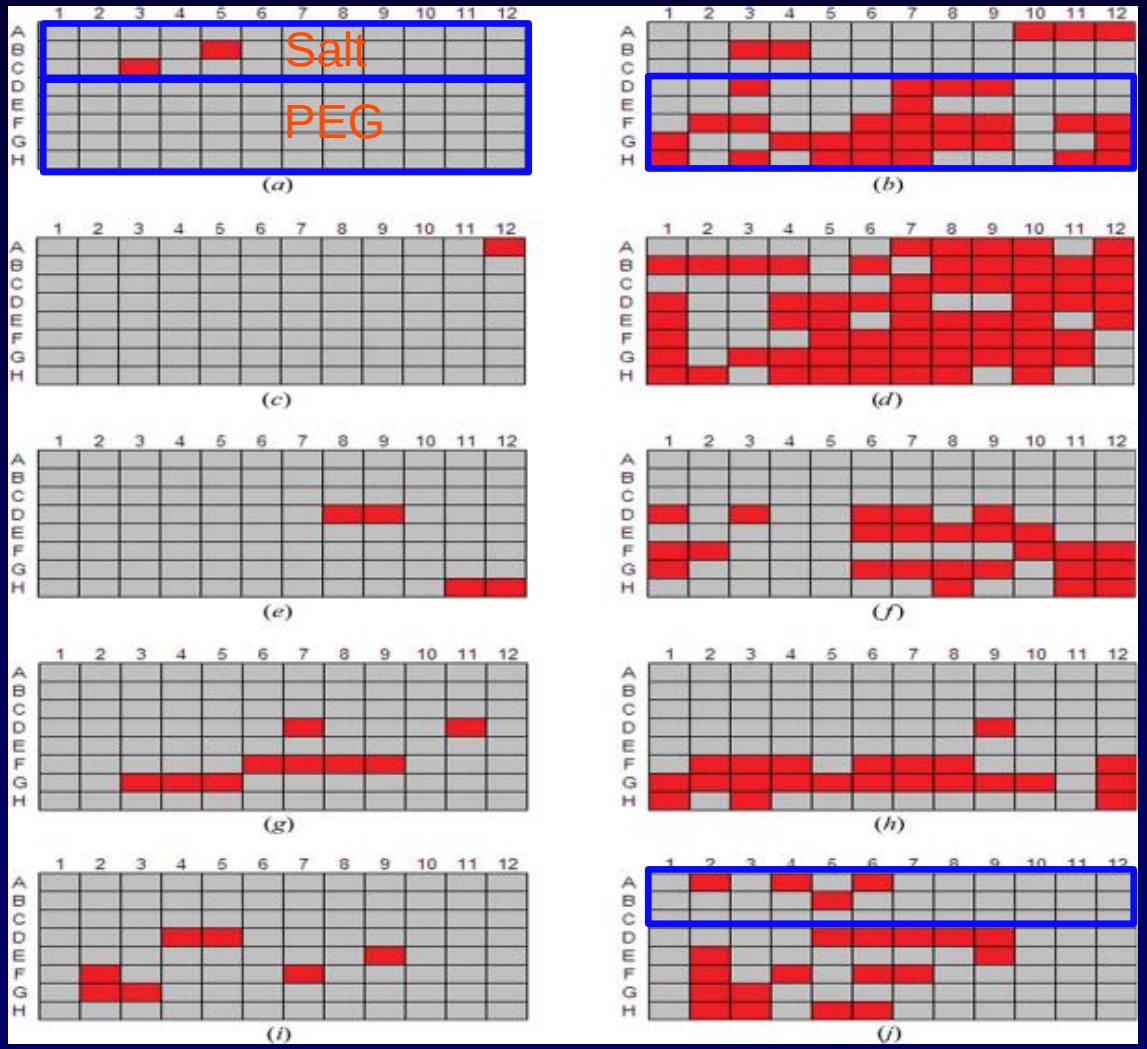

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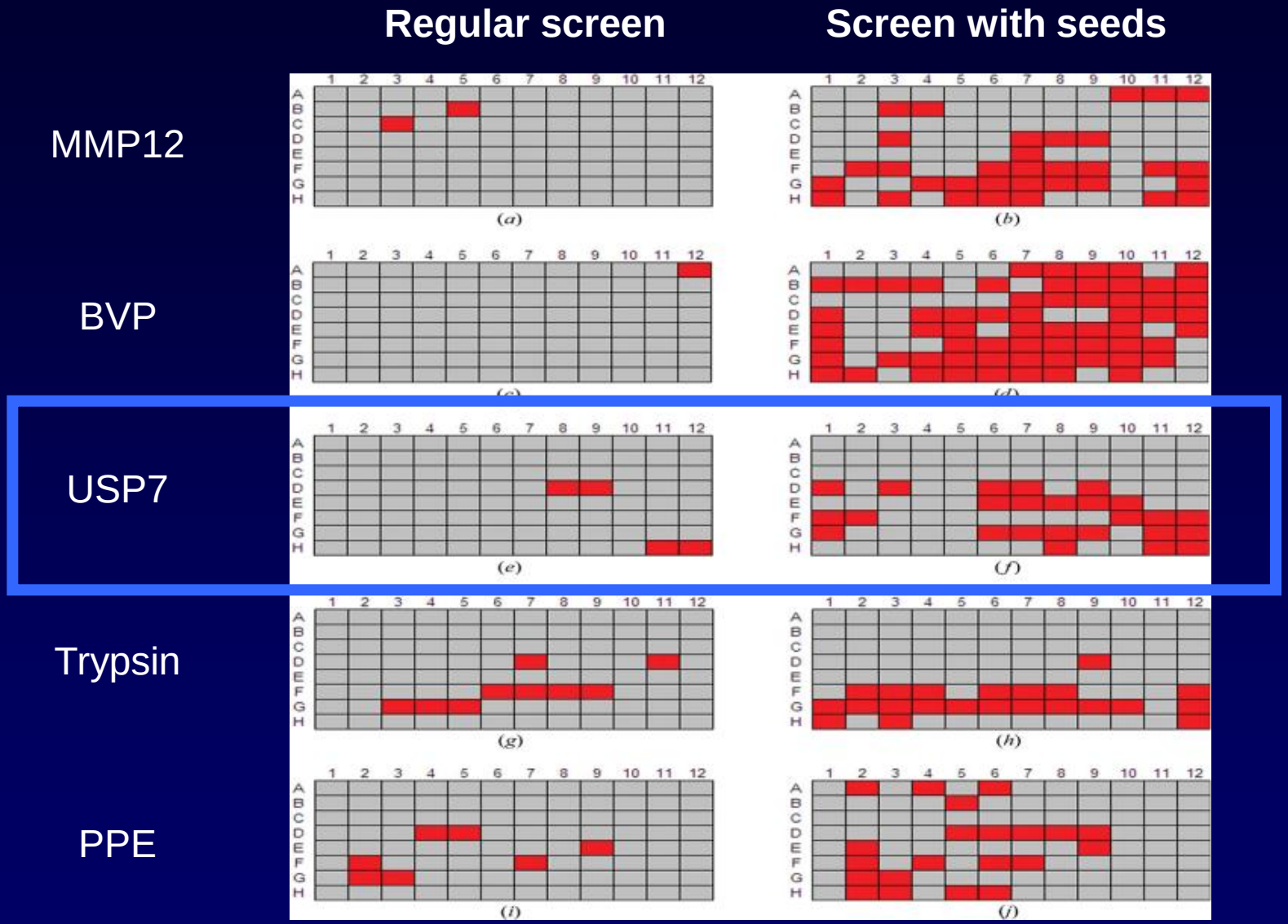
Microseeding in *screening* experiments



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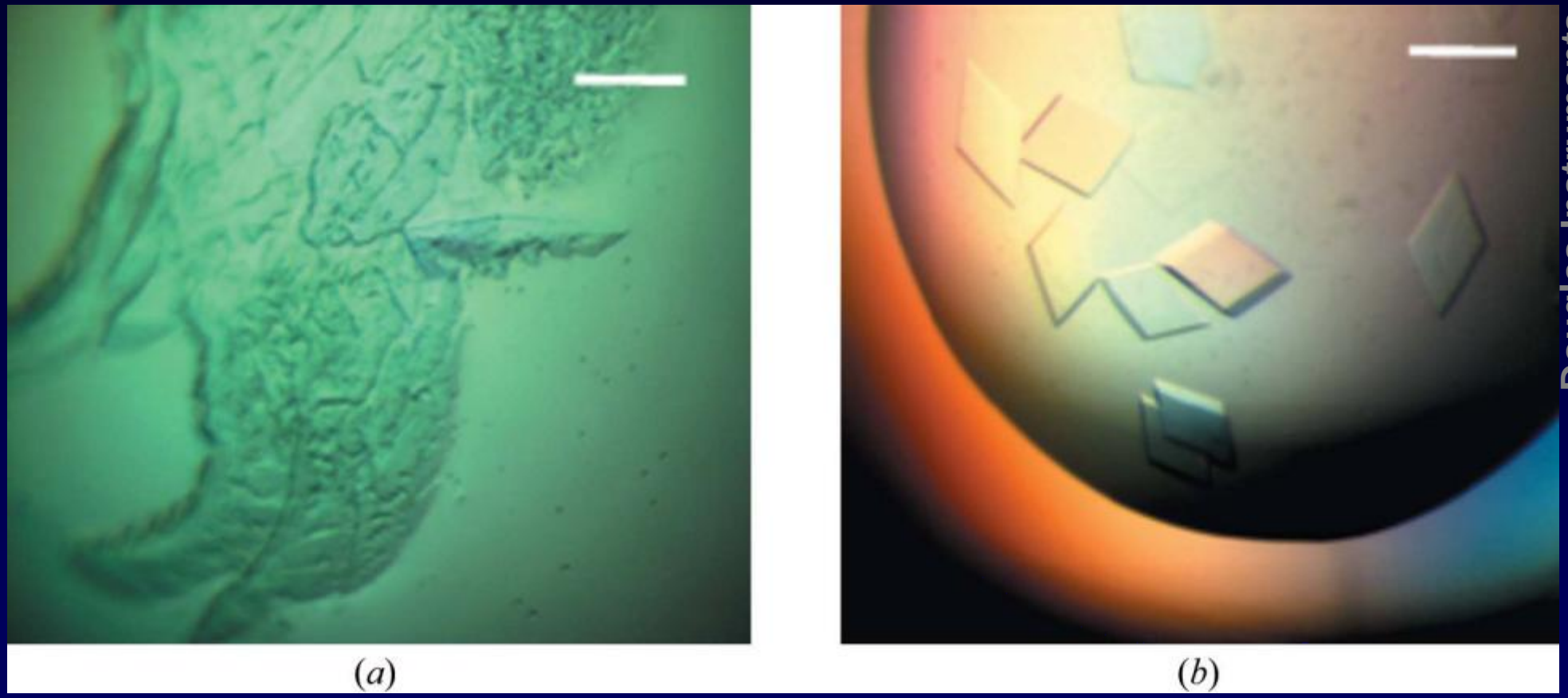
Microseeding in *screening* experiments



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

random Microseed Matrix-Screening (rMMS)

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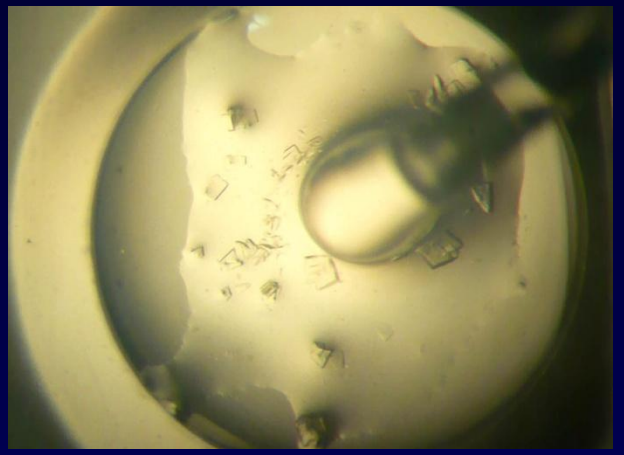
“rMMS”
D’Arcy et al. Acta Cryst. (2007). D63. 'An automated microseed matrix-screening method for protein crystallization’

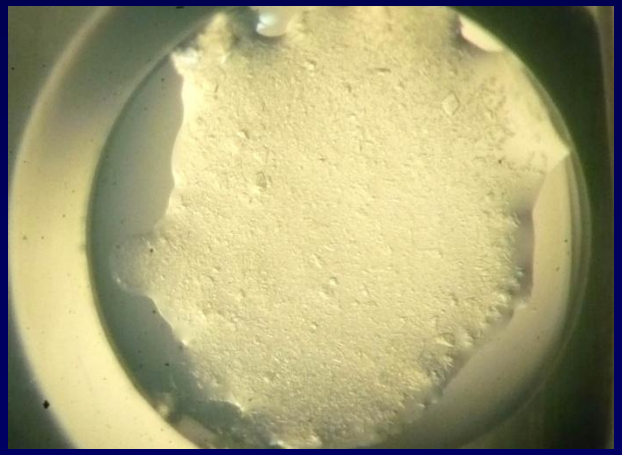
How to make the seed stock

See www.douglas.co.uk/mms.com or sheet

1. Break crystals with a probe
2. Place contents of well in 50 µl of reservoir solution
3. Vortex with Hampton “Seed Bead”
4. Make a dilution series immediately
5. Freeze

Look after your seeds!





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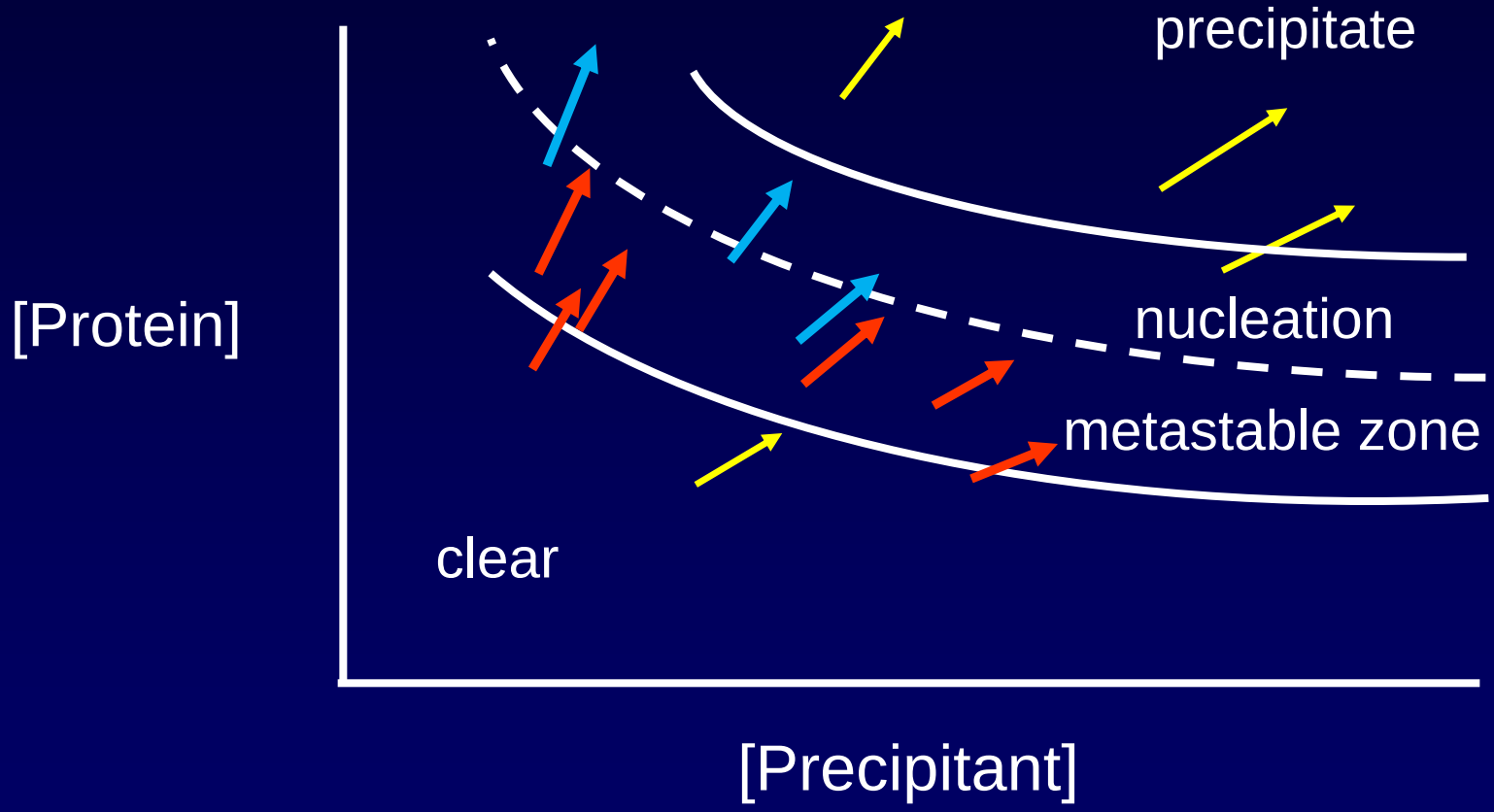
Phase diagram of a protein



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Microseeding toolkit

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If you want to know more:

Patrick D. Shaw Stewart, Stefan A. Kolek, Richard A. Briggs, Naomi E. Chayen and Peter F.M. Baldock. “Random Microseeding: A Theoretical and Practical Exploration of Seed Stability and Seeding Techniques for Successful Protein Crystallization”

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

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

Microseeding

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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

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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, including heavy atom, small molecule and peptide derivatives ?	
(7) Can we harvest seed crystals from microfluidic devices?	
(8) What can you do if you have no crystals?	

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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

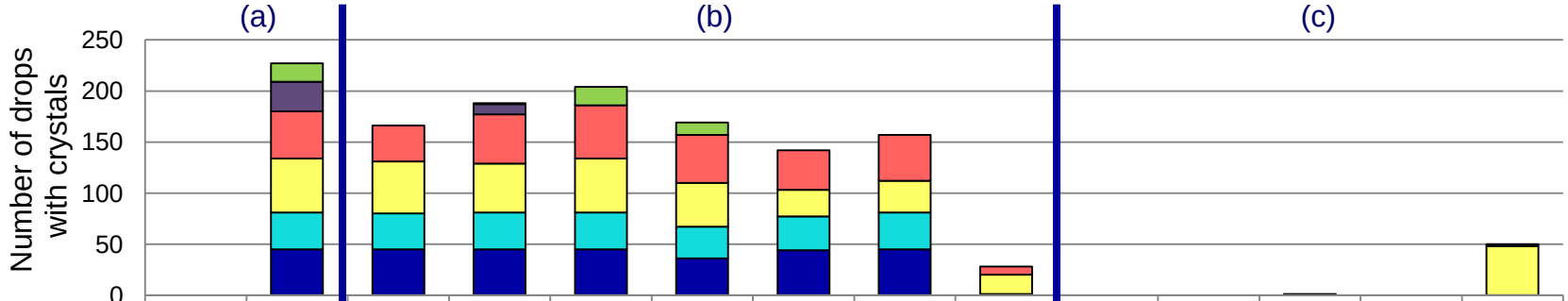
Conditions where:

(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 "pregnant" 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:*
(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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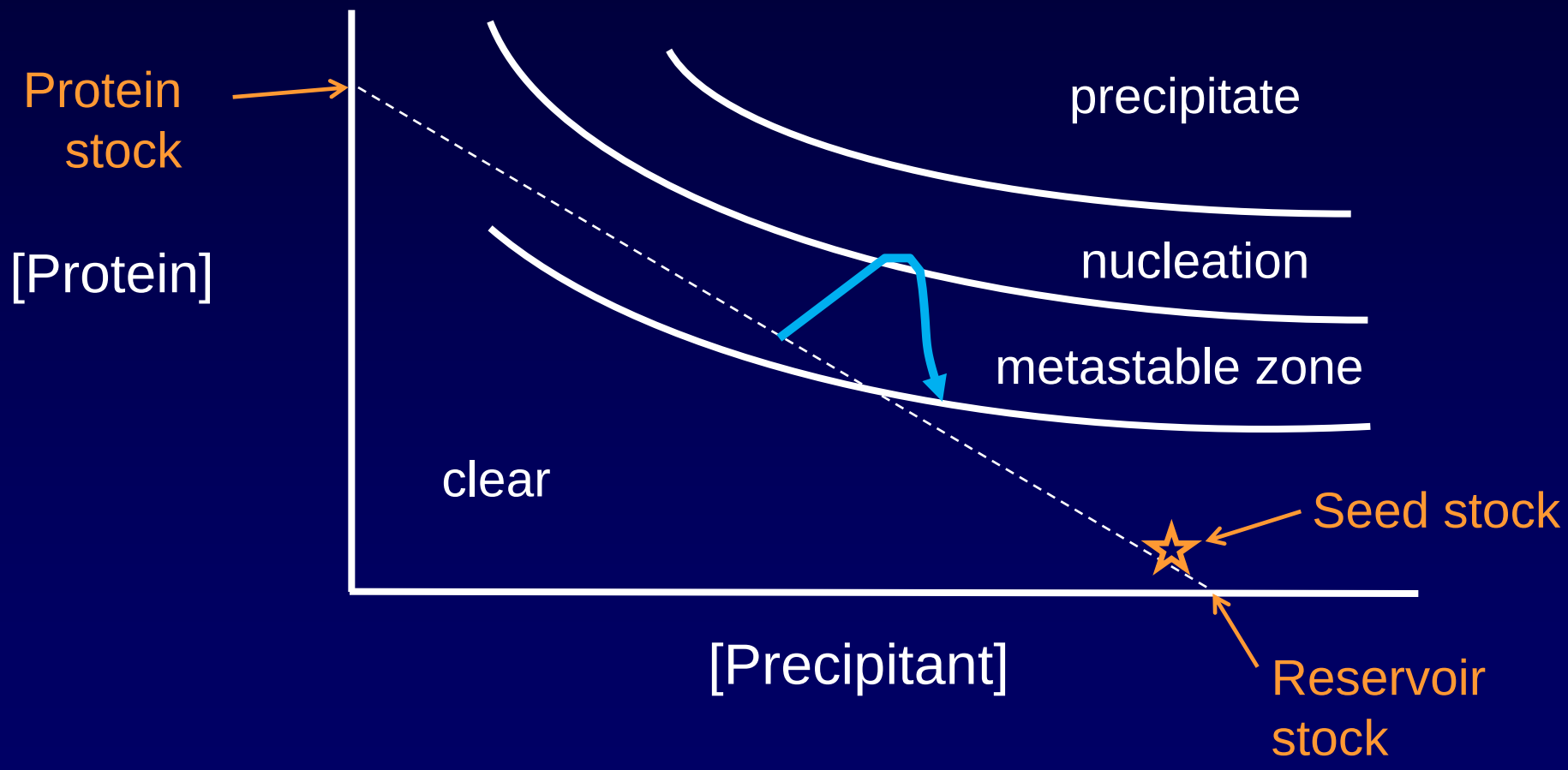
Phase diagram of a protein



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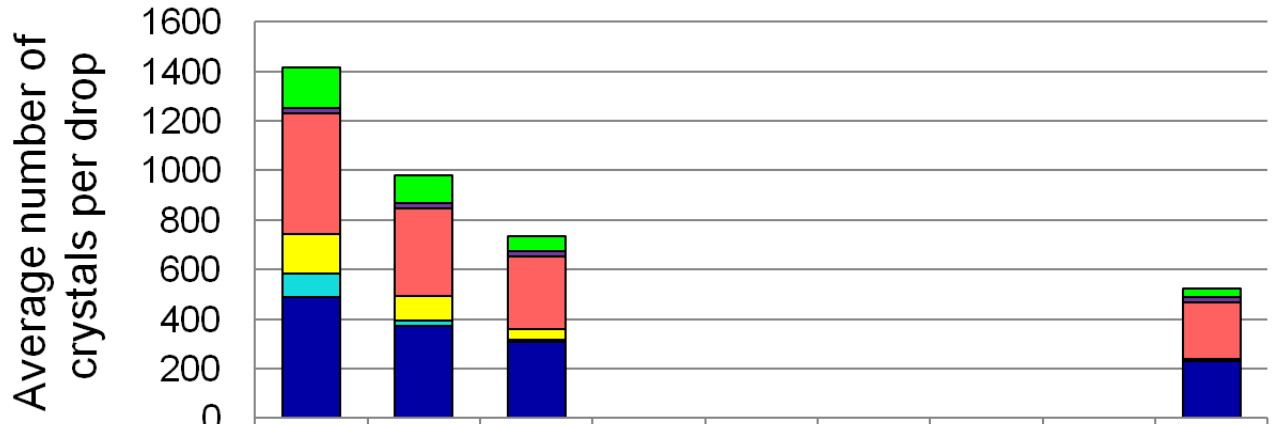

	0 hrs	3 hrs	6 hrs	9 hrs	12 hrs	15 hrs	18 hrs	21 hrs	24 hrs
Gluc. isom.	166	113	63						36
Hemoglob.	19	18	19						20
Thaumatin	489	357	293						228
Thermolysin	158	96	46						11
Trypsin	95	23	6.0						0.1
Xylanase	490	372	309						229

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*
(2) How stable are the seed stocks?	*Not completely stable so use your seed stock quickly, then freeze. Or cross-link.*
(3) Is "preseeding" the protein stock helpful?	
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(2) How stable are the seed stocks?	*Not completely stable so use your seed stock quickly, then freeze. Or cross-link.*
(3) Is "preseeding" the protein stock helpful?	*Please read the paper!*
(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, including heavy atom, small molecule and peptide derivatives ?	
(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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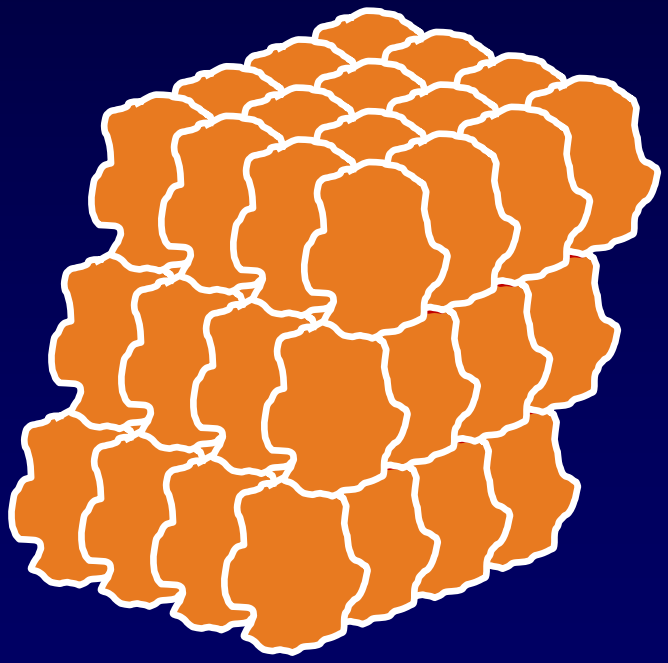
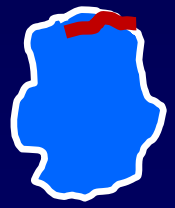
Suggested by Lesley Haire, National Institute for Medical Research

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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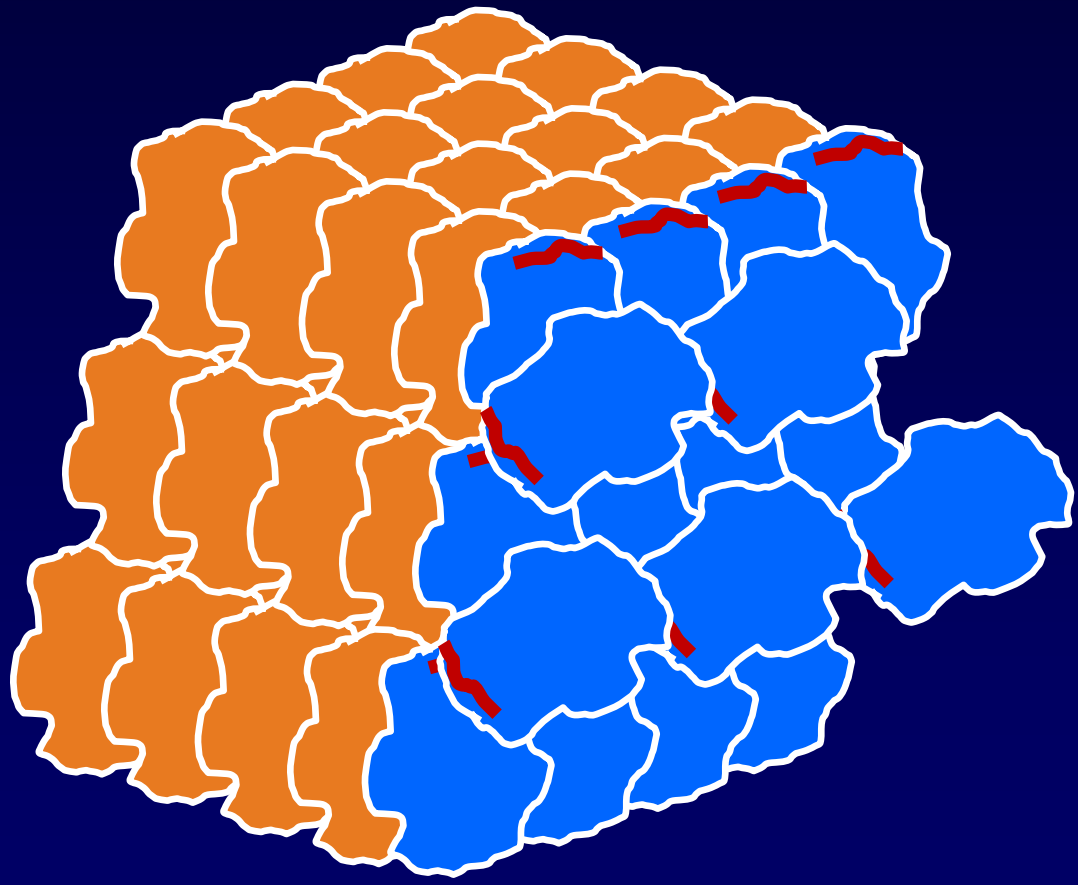
Cross-seeding

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



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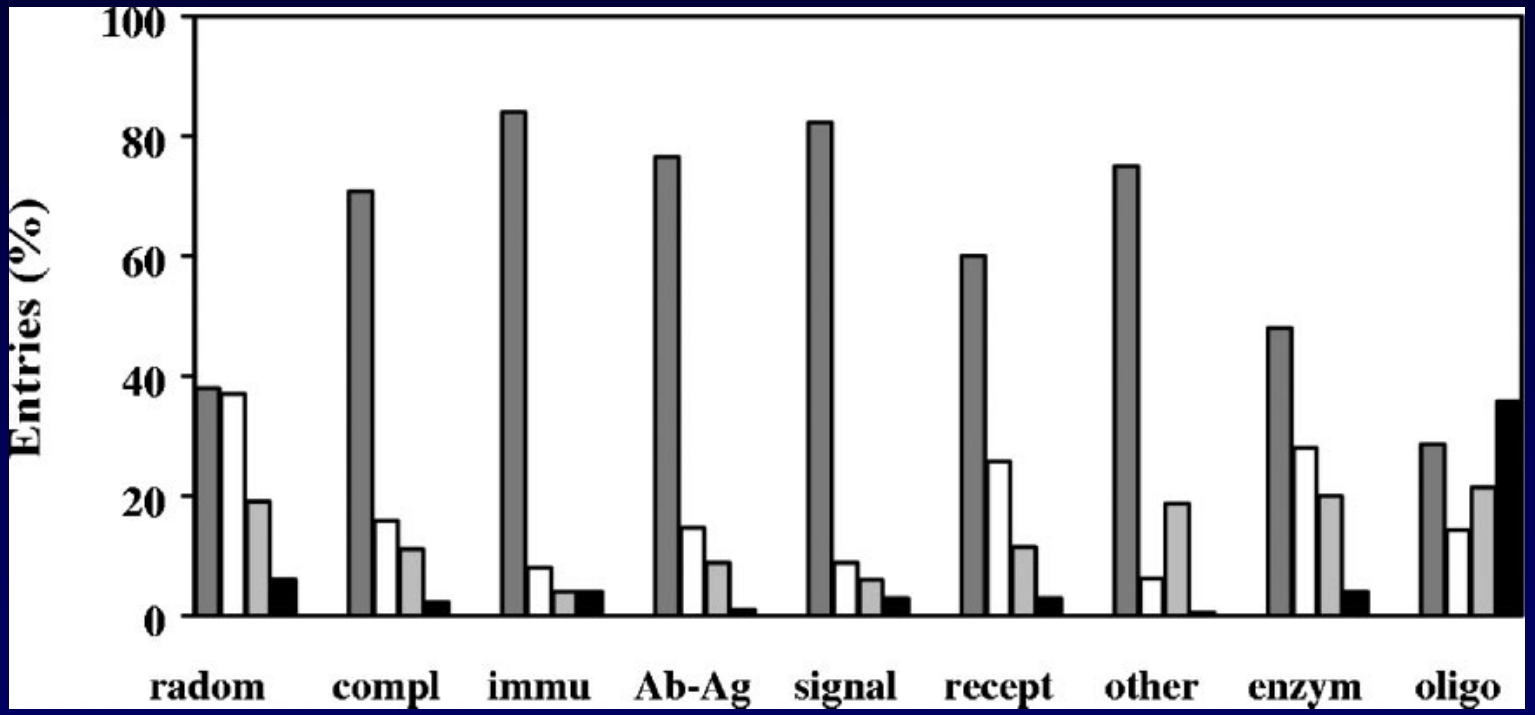
Crystallizing complexes

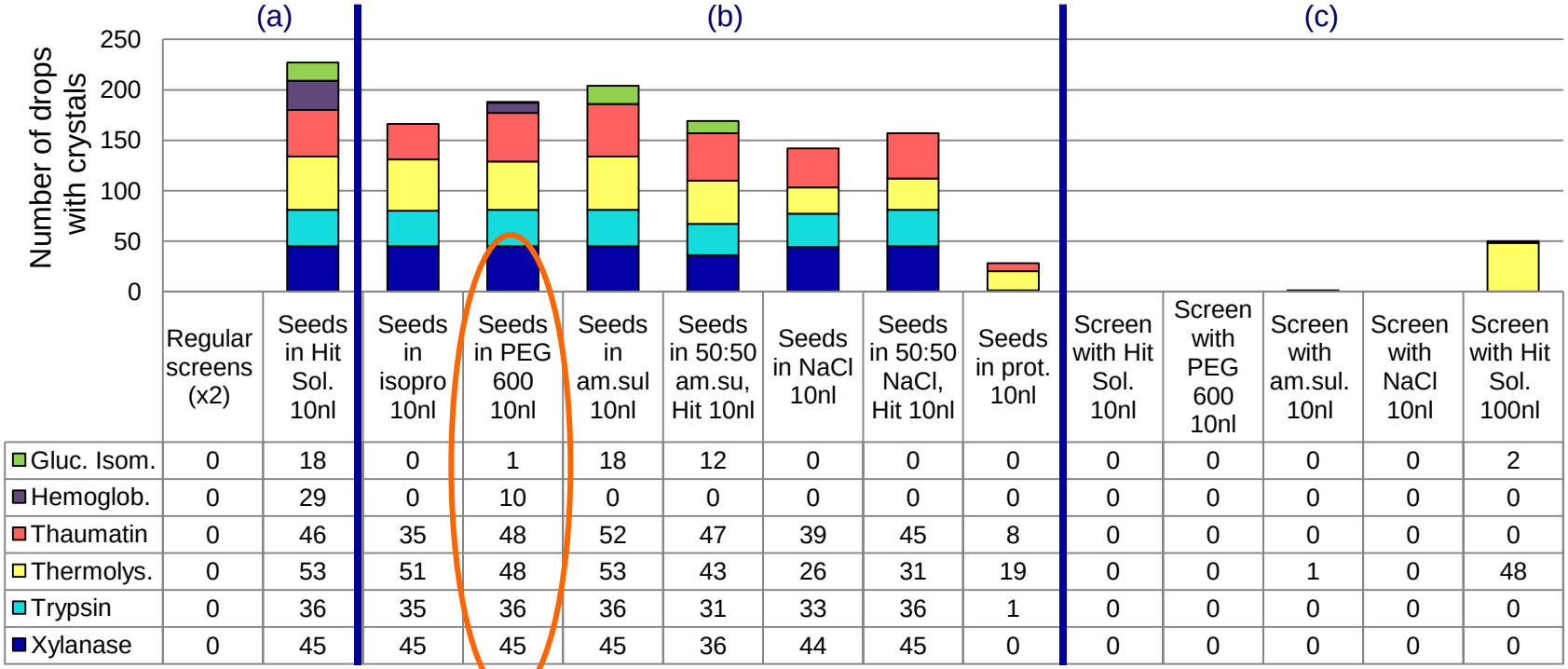
Radaev and Sun. Crystallization of protein-protein complexes.
J. Appl. Cryst. (2002). 35, 674-676



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

Random 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?



	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:*
(1) How can we get as many hits as possible?	*Stick to the 'hit solution' for suspending seed crystals for routine rMMS*
(2) How stable are the seed stocks?	*Not completely stable so use your seed stock quickly, then freeze. Or cross-link.*
(3) Is "preseeding" the protein stock helpful?	*Please read the paper!*
(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, including heavy atom, small molecule and peptide derivatives ?	*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 crystals after incubation for one day

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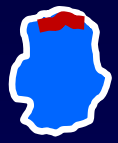
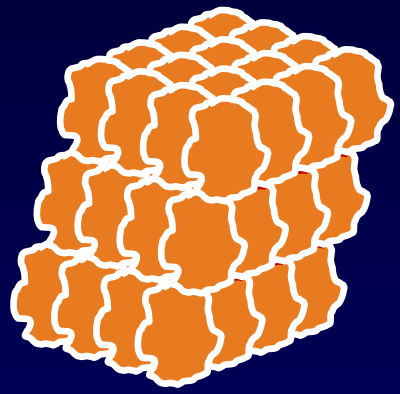
Protein	Crystals in Hit Sol.	Crystals in Isopropanol	Crystals in PEG 600	Crystals in Amm.sul.	Crystals in NaCl	Crystals in protein stock
Gluc. Isom.	**OK**	Cracked	Shattered	**Cracked**	Dissolved	Dissolved
Hemoglobin	**OK**	Cracked	**OK**	Dissolved	Dissolved	Dissolved
Thaumatin	**OK**	**Cracked**	**OK**	**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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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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(5) How can we get more diverse crystals?	*Please read the paper!*
(6) How can we stabilize protein complexes, including heavy atom, small molecule and peptide derivatives ?	*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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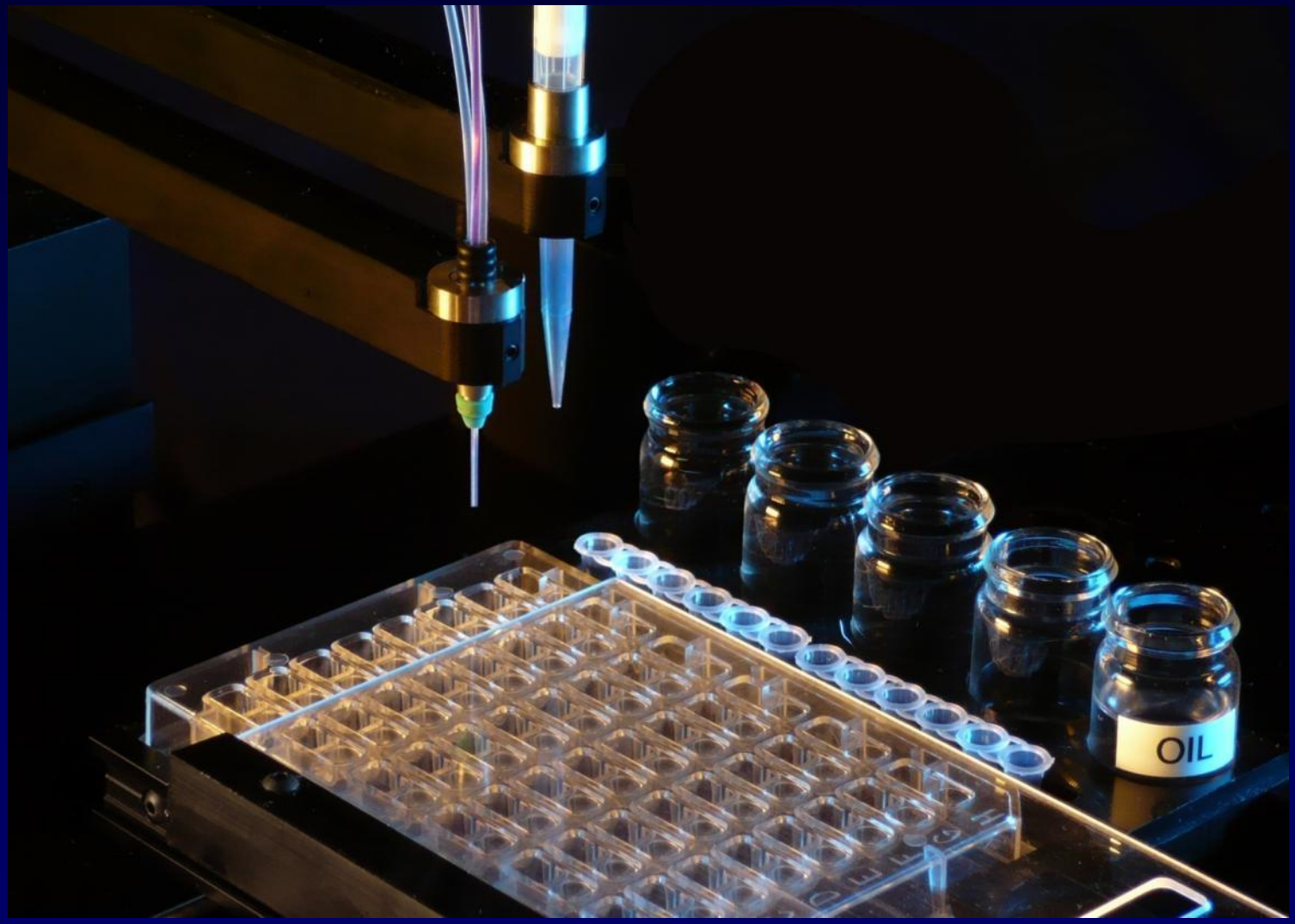
Soaking experiments

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

Seeding with diluted seed stock is “the only reliable way” to achieve this

New “combinatorial” experimental design



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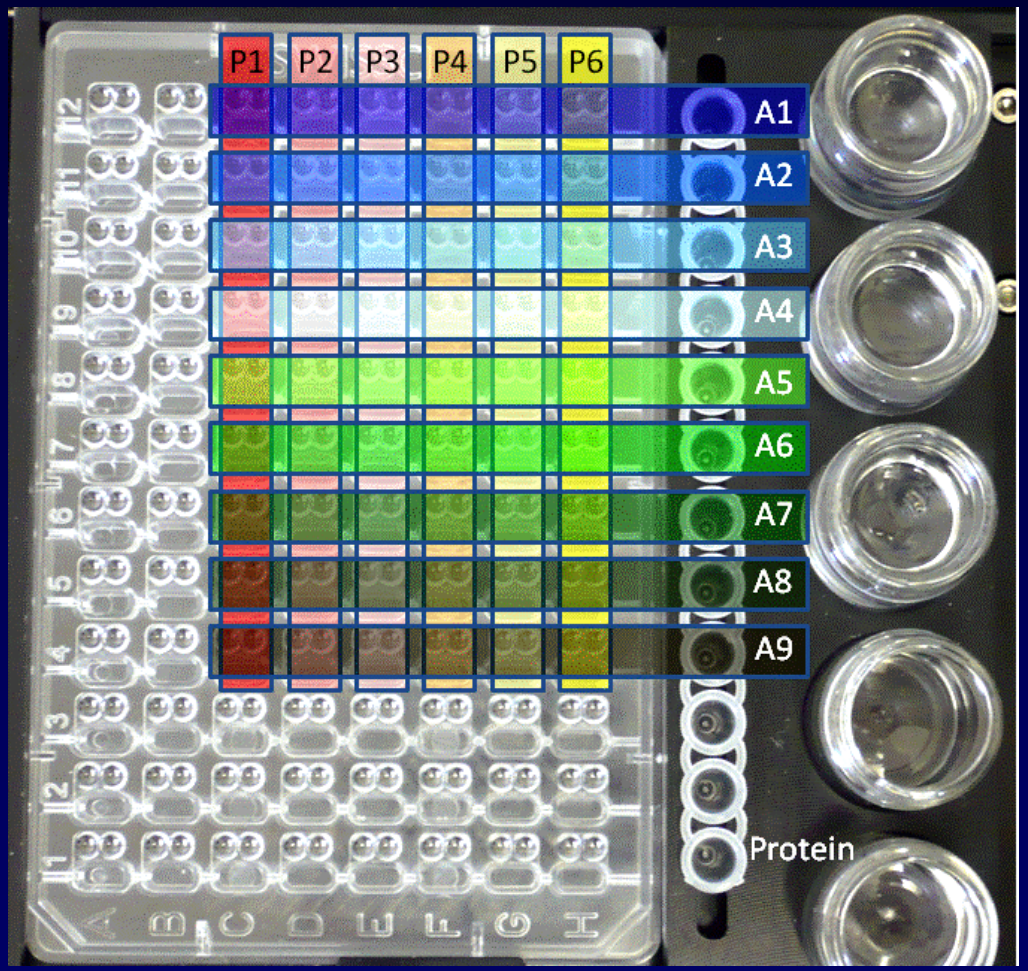
New “combinatorial” experimental design



Microseeding:

A1: 100% seed stock
A2: 25% seed stock
A3: 6.3% seed stock
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A9: 0.002% seed stock

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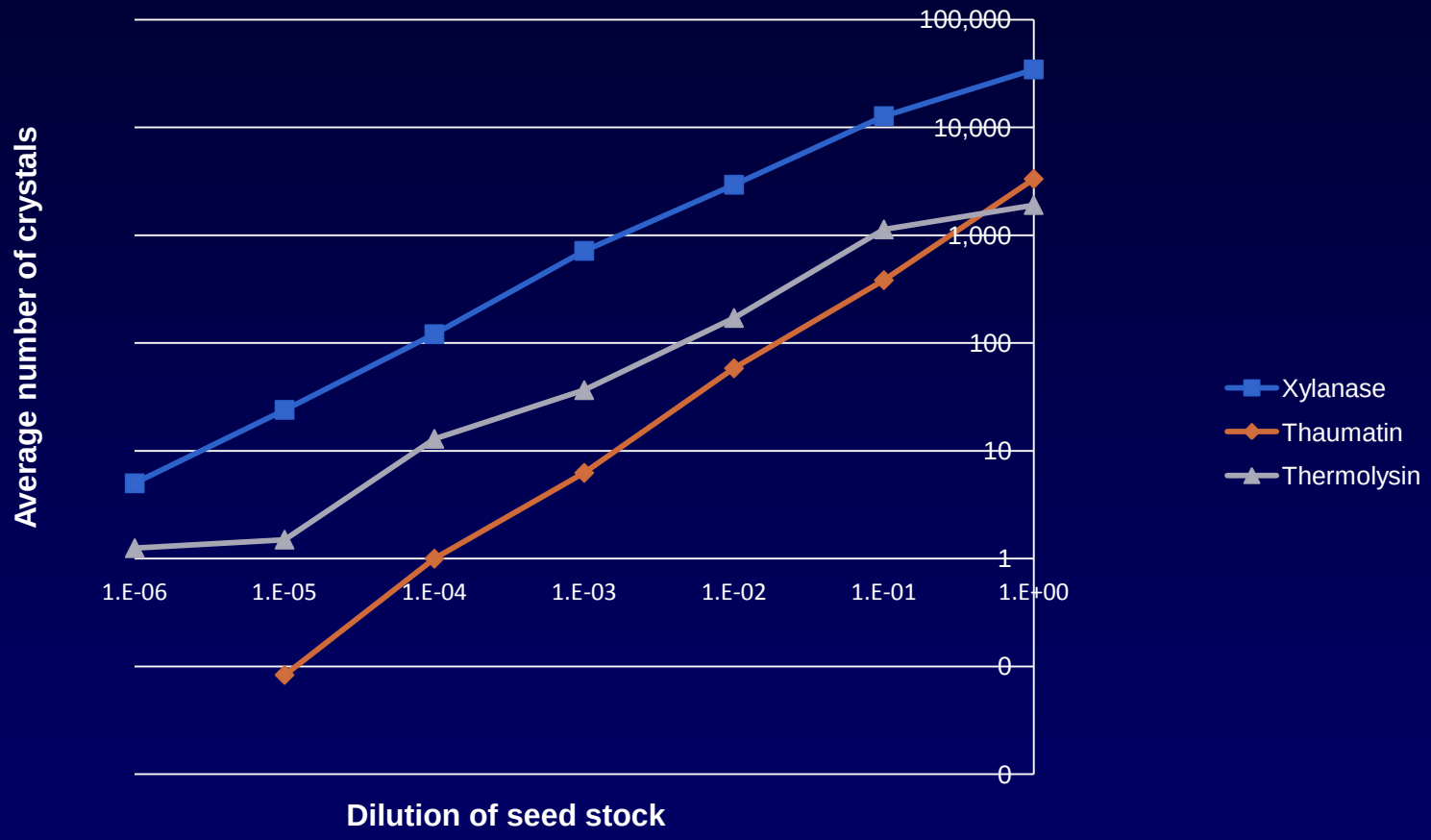
New “combinatorial” experimental design



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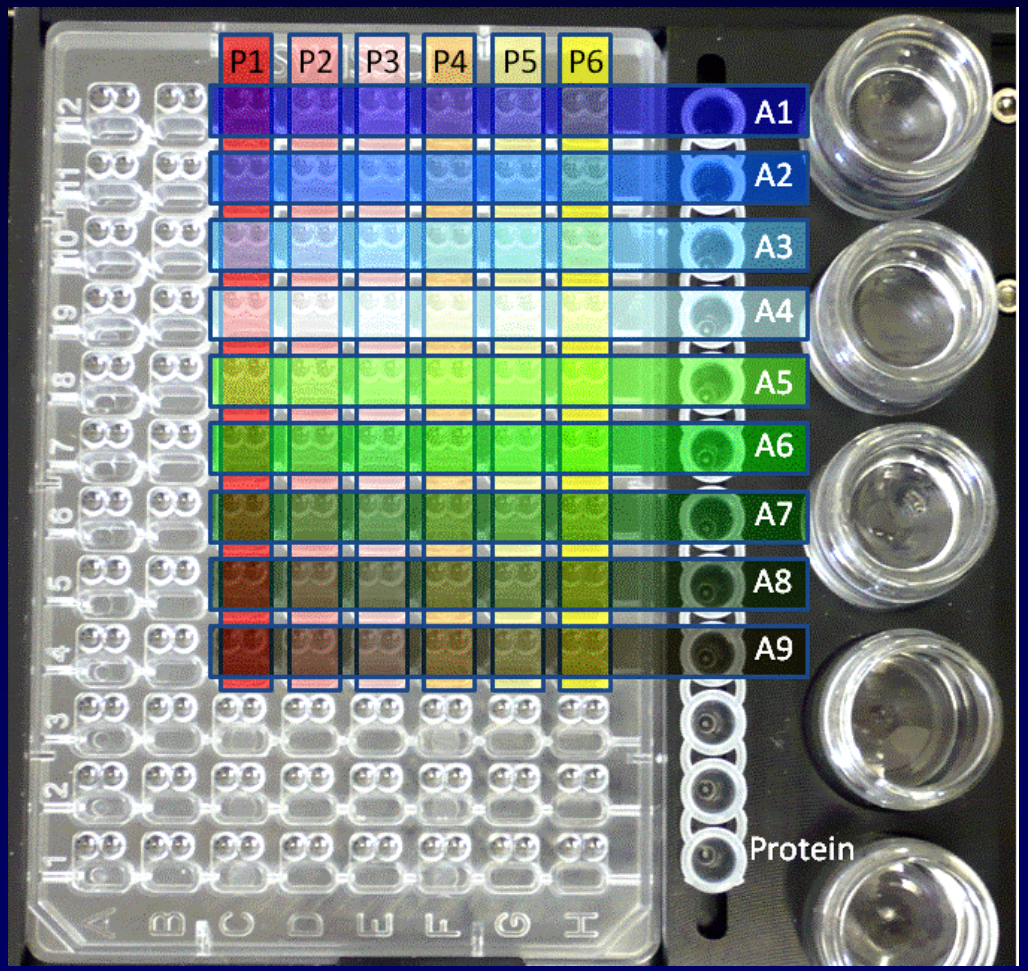
New “combinatorial” experimental design



Or test up to 12
inhibitors or ligands

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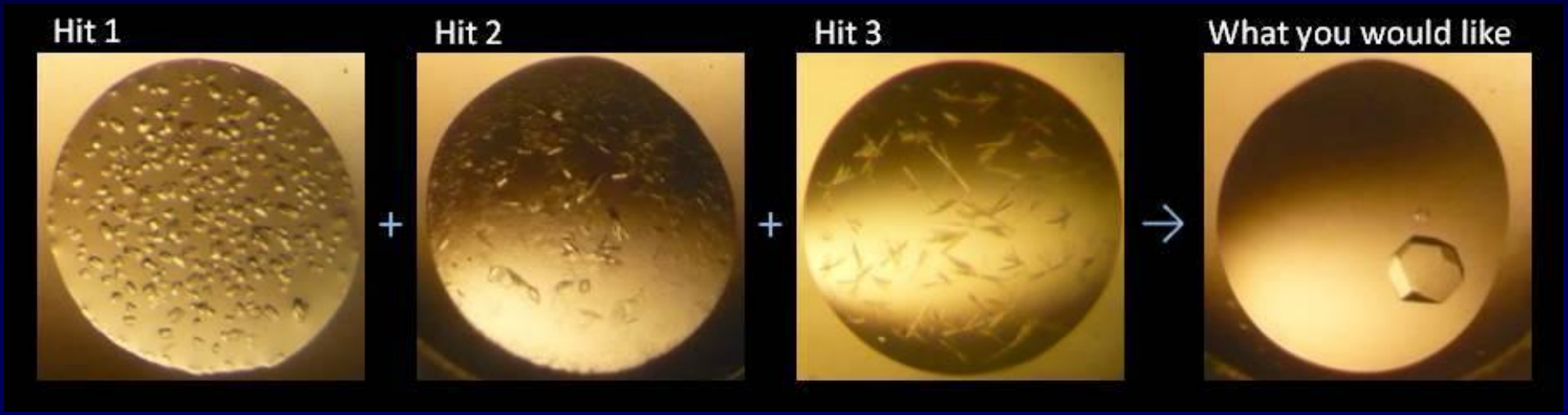
New “combinatorial” experimental design

A third use -



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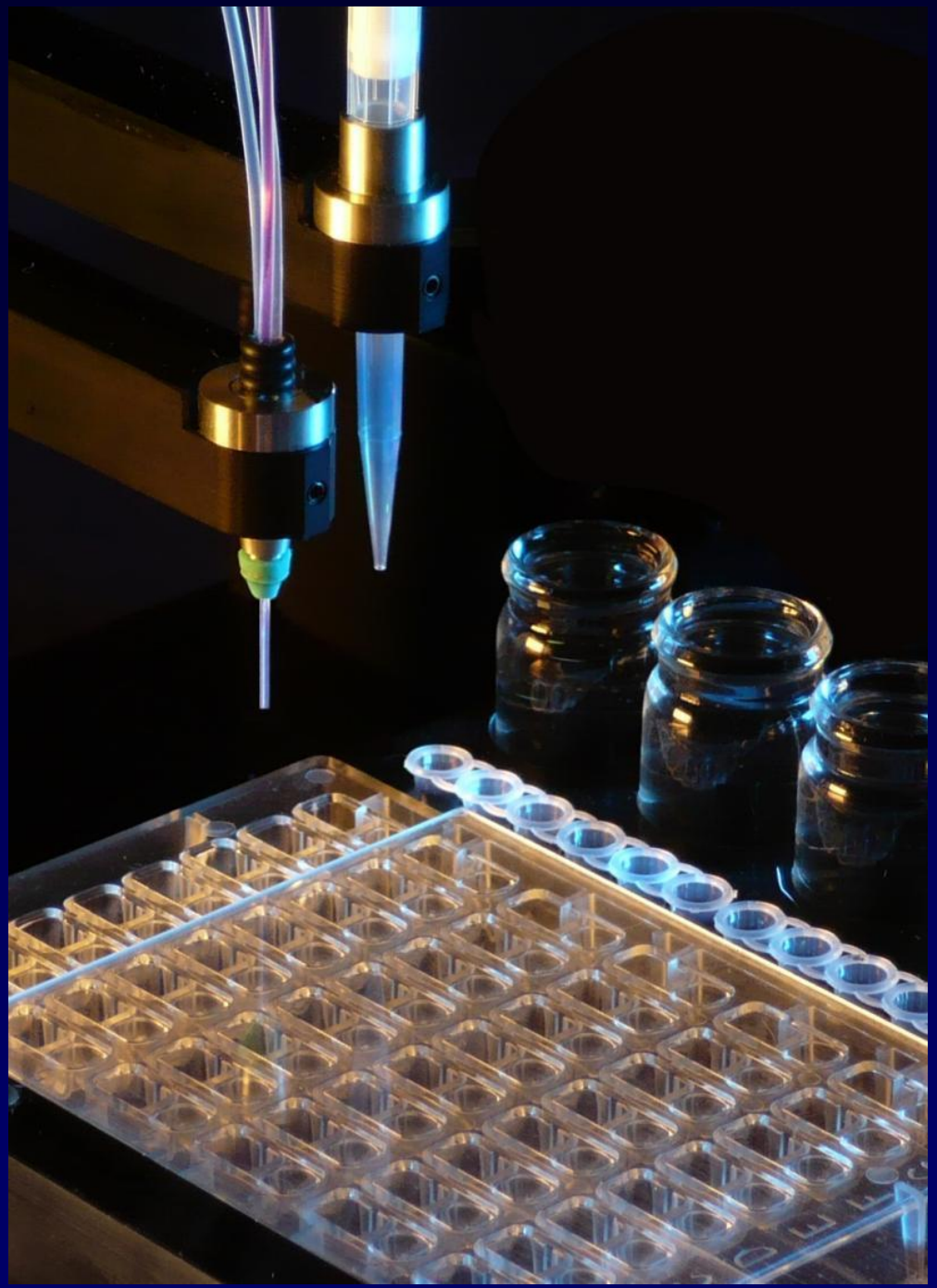
Thank you for listening!



Microseed it!

1. Scaling up
2. Microseeding with membrane proteins
3. Reshuffling ingredients

Patrick Shaw Stewart

Douglas Instruments Ltd

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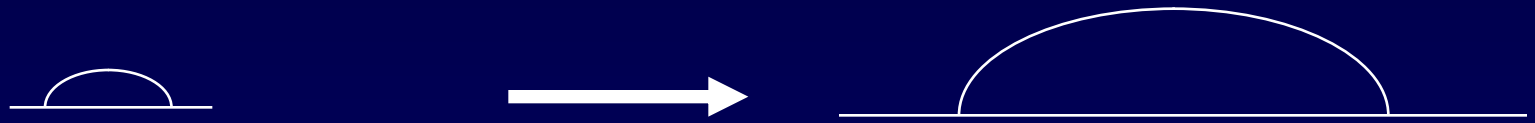
Scaling up



100 + 100 nl

1 + 1 µl

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Scaling up

100 + 100 nl

1 + 1 µl

Tartan indicates precipitation

(my family is Scottish)

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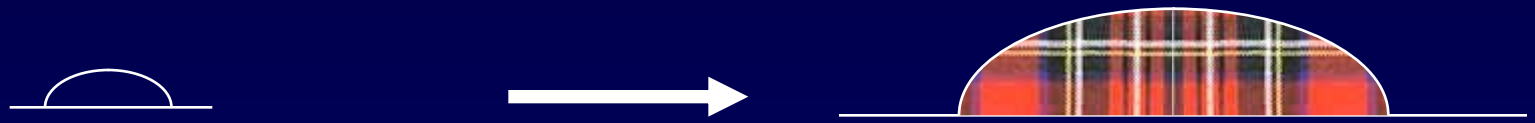
Scaling up

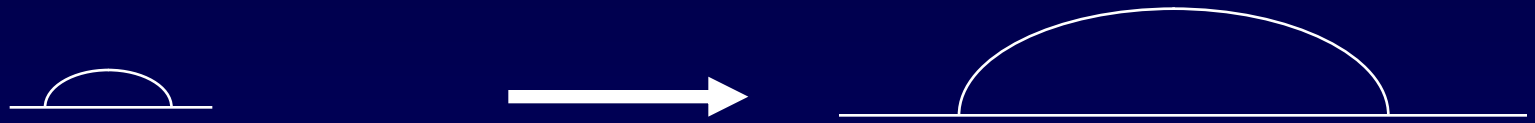


High surface to volume ratio

Low surface to volume ratio

- More protein is lost at the air/liquid interface
- Equilibration is faster

Scaling up



Try 200 nl (protein) +
100 nl (reservoir solution)

Scales up to 1 + 1 µl
(Heather Ringrose, Pfizer)

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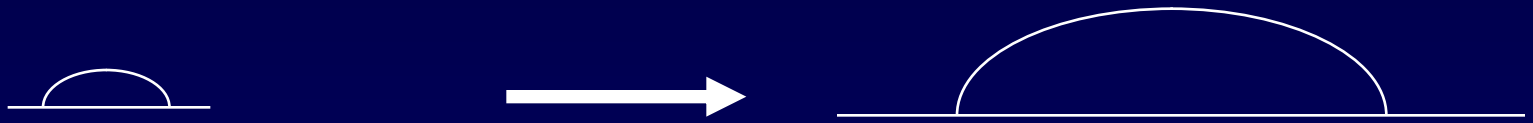
Scaling up



100 nl (protein) + 100 nl (reservoir solution)

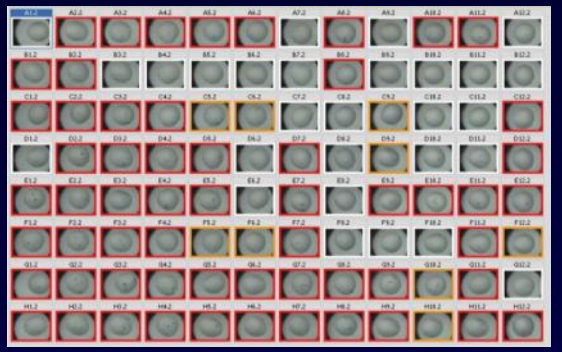
Scales up to 0.5 + 1 µl (Heather Ringrose, Pfizer)

Equilibrates faster

Increase the salt by 50 – 100%

Protein crystallization

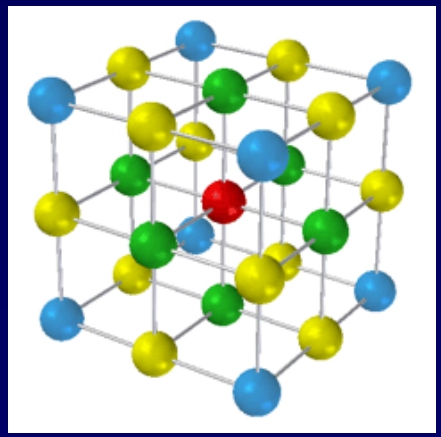
Step 1: screening with random solutions that have given crystals before x 96



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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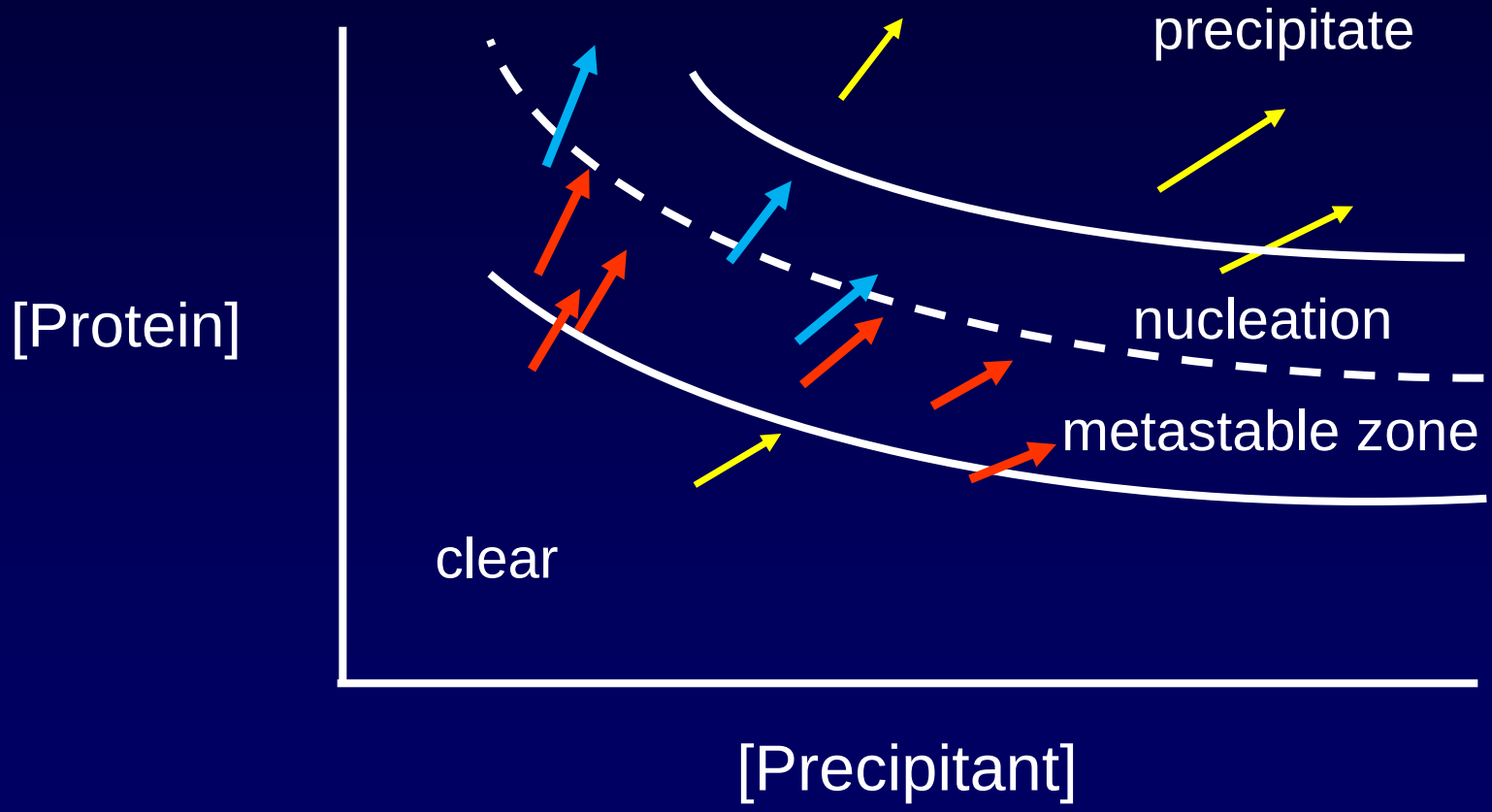
Phase diagram of a protein



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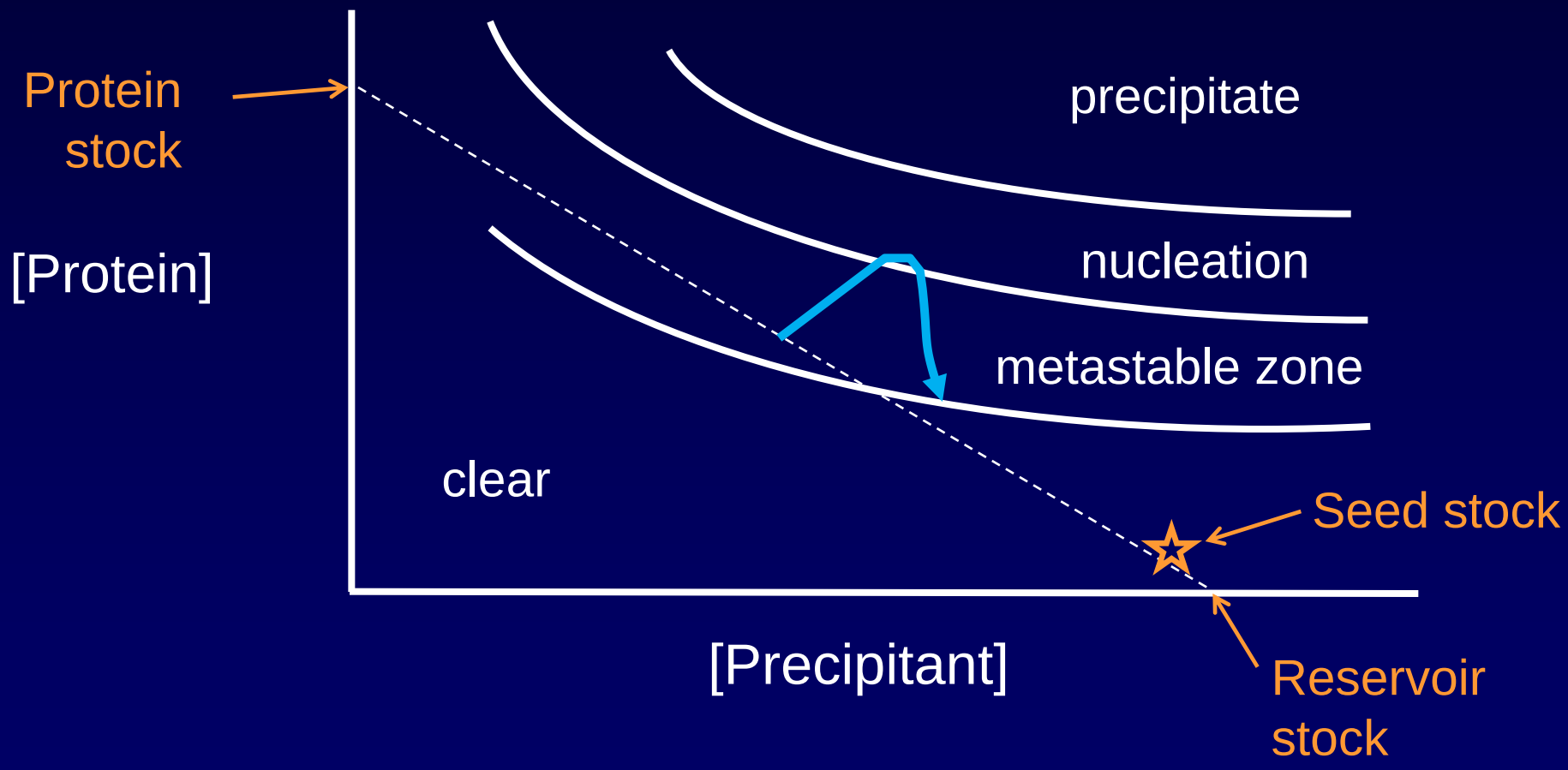
Phase diagram of a protein



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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

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Matrix seeding volumes:

0.3 µl protein

+ 0.2 µl reservoir solution

+ 0.1 µl seed stock

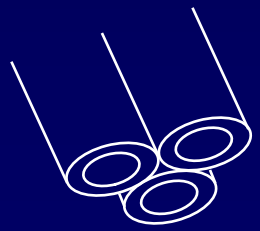
E.g. for membrane proteins:

0.3 µl protein

+ 0.29 µl reservoir solution

+ 0.01 µl seed stock

3-bore tip



Or: for membrane proteins:

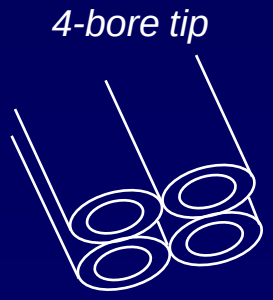
0.3 µl protein

+ 0.2 µl reservoir solution

+ 0.09 ul “hit solution” (additive)

+ 0.01 µl seed stock

4-bore tip



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Membrane proteins

Collaboration with MPL at Diamond

1. Several proteins showed no improvement
2. One protein showed a different crystal form in the same conditions
3. One protein showed greatly improved diffraction

Christina Oswald showed that microseeding (more than) doubled the number of hits for a well-known membrane protein

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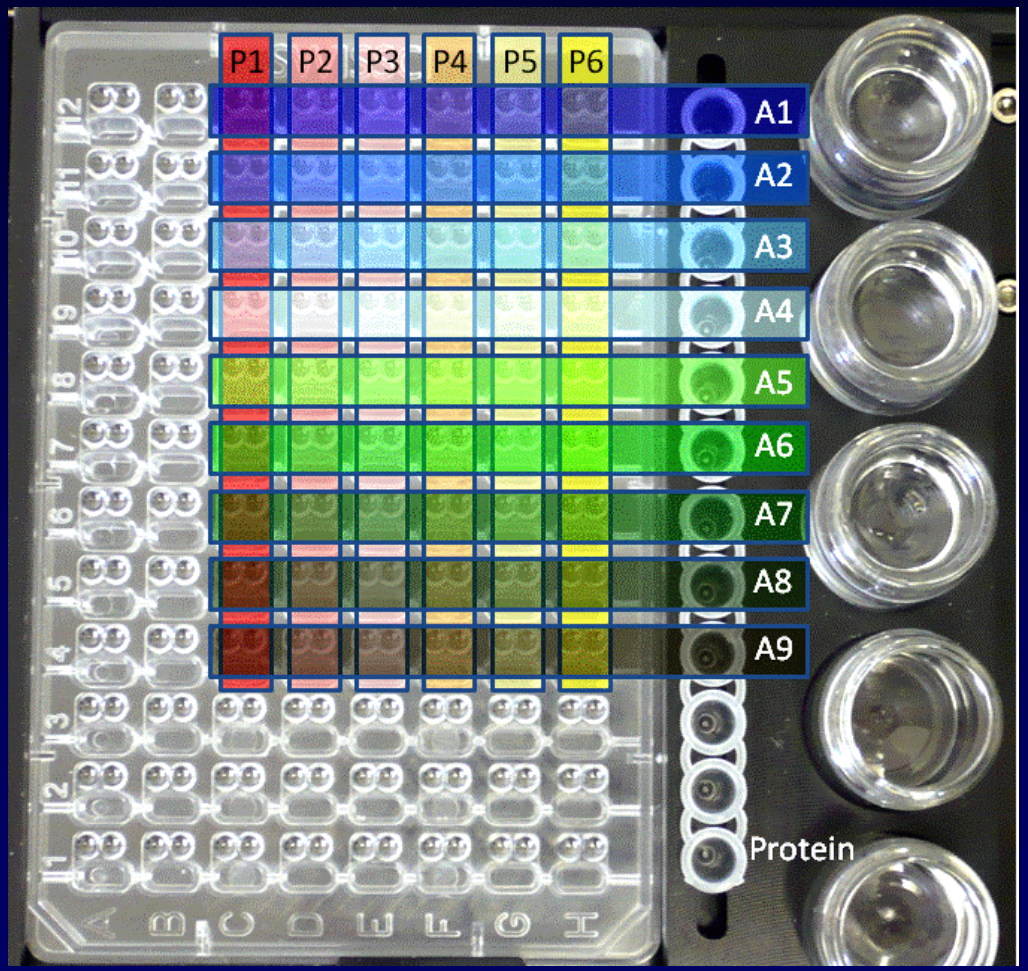
New “combinatorial” experimental design



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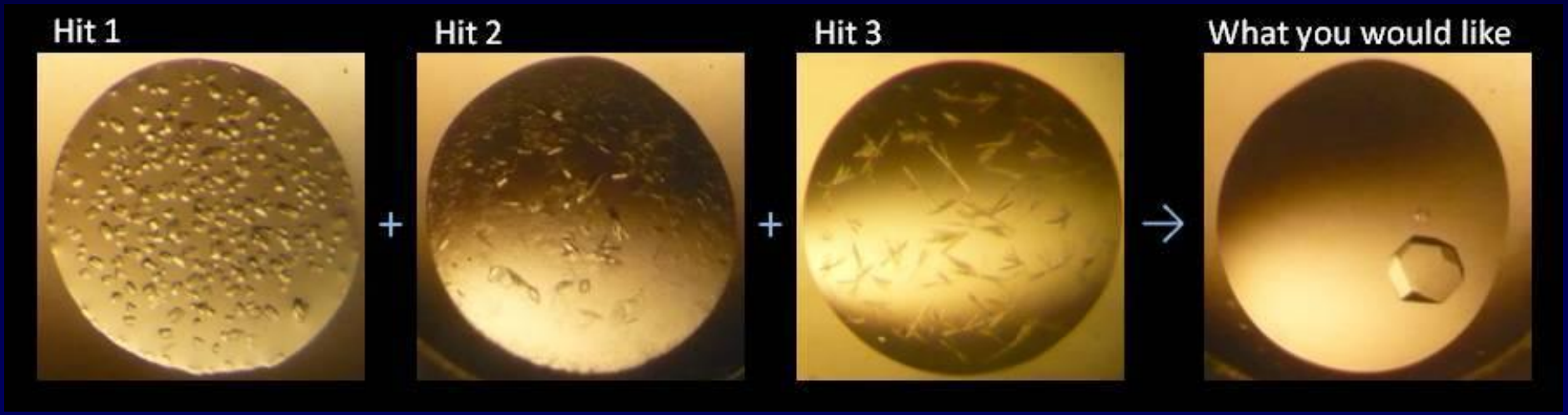
New “combinatorial” experimental design

A third use -



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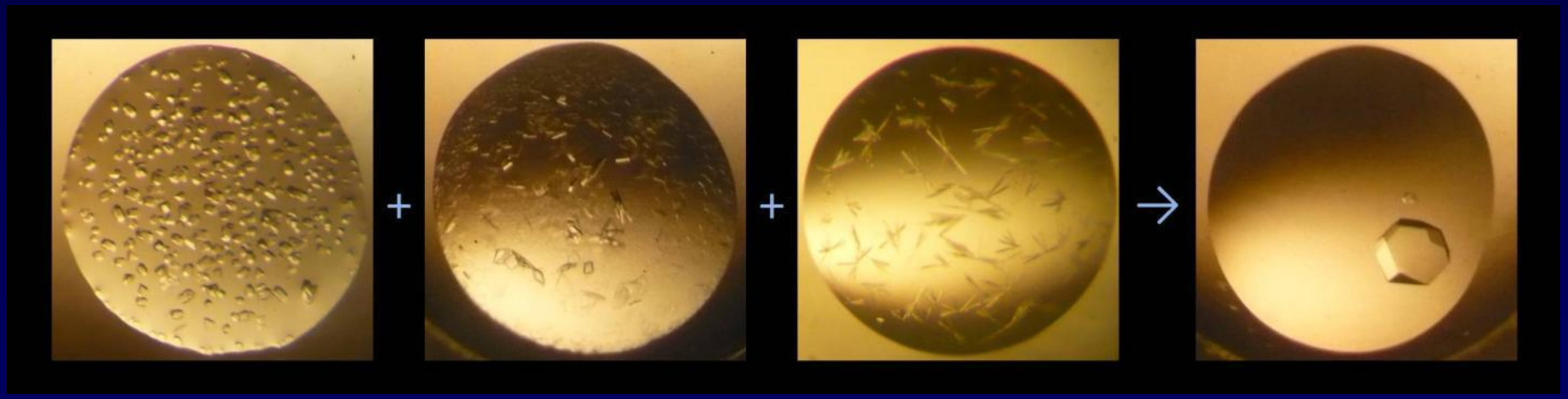
New “combinatorial” experimental design

1. PEG4000, MgCl2, citrate pH5
2. PEG600, CaCl2, TRIS pH8
3. NaCl, imidazole pH6



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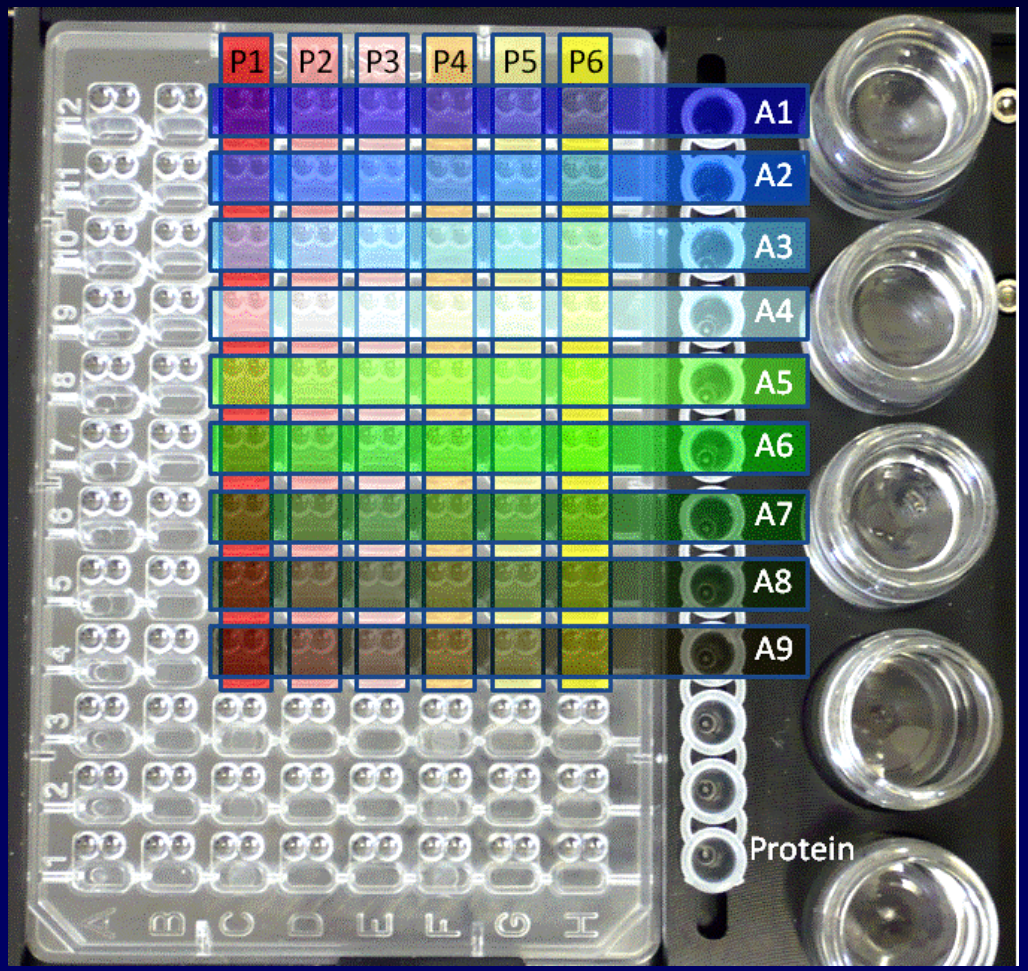

Original hits:

1. PEG4000, MgCl2, Citrate pH5
2. PEG600, CaCl2, TRIS pH8
3. NaCl, Imidazole pH6

P1, P2: PEG4000

P3, P4: PEG600

P5, P6: NaCl

A1: MgCl2

A2: MgCl2 + Citrate

A3: CaCl2 etc

Ingredients can be reshuffled! This is equivalent to a “targeted screen”. Yellow indicates the best combination above.

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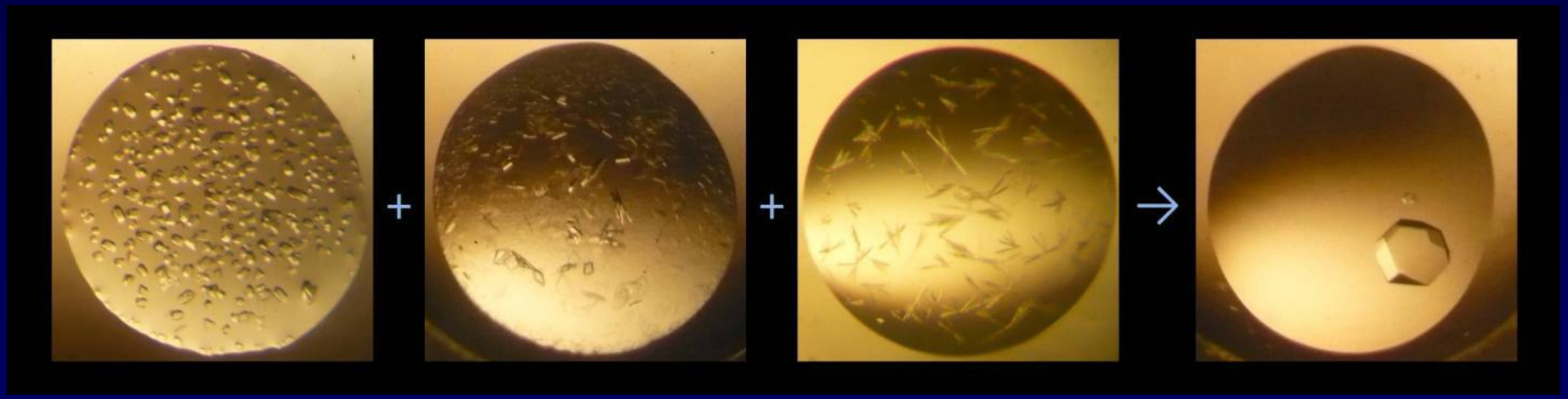
New “combinatorial” experimental design

1. PEG4000, MgCl2, citrate pH5
2. PEG600, CaCl2, TRIS pH8
3. NaCl, imidazole pH6

PEG 4000, CaCl2, imidazole pH6



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

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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. So always do it – unless you can solve the structure with crystals taken straight from your initial screens

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Thank you for listening!

Microseeding paper:

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