Random microseeding: a theoretical and practical exploration of seed stability and seeding techniques for successful protein crystallization

Patrick Shaw Stewart

Douglas Instruments Limited (near Oxford, UK):
Peter Baldock, Patrick Shaw Stewart, Richard Briggs, Stefan Kolek

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random Microseed Matrix-Screening

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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")
3. Automate!

To get:

(1) more hits

(2) better crystals

Microseeding in *screening* experiments

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

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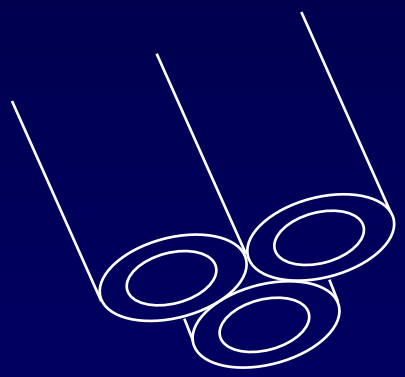
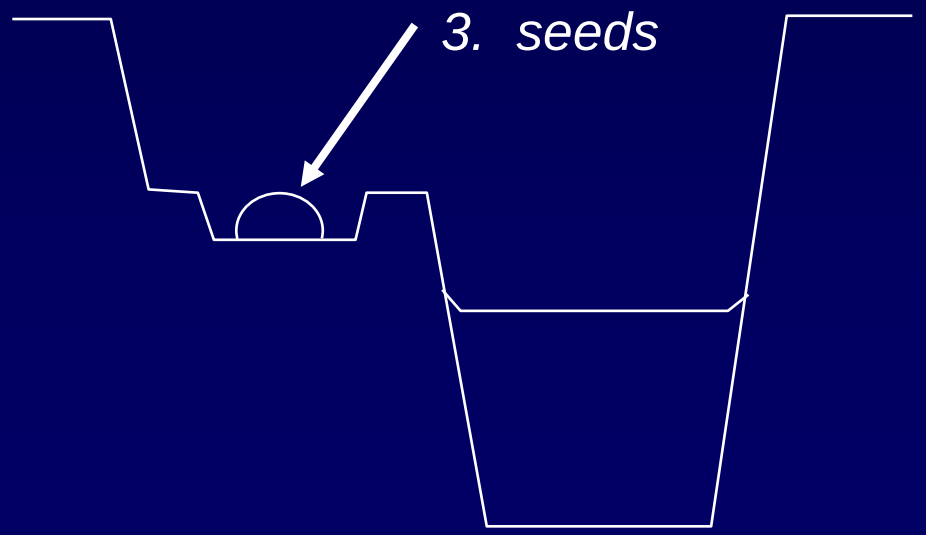
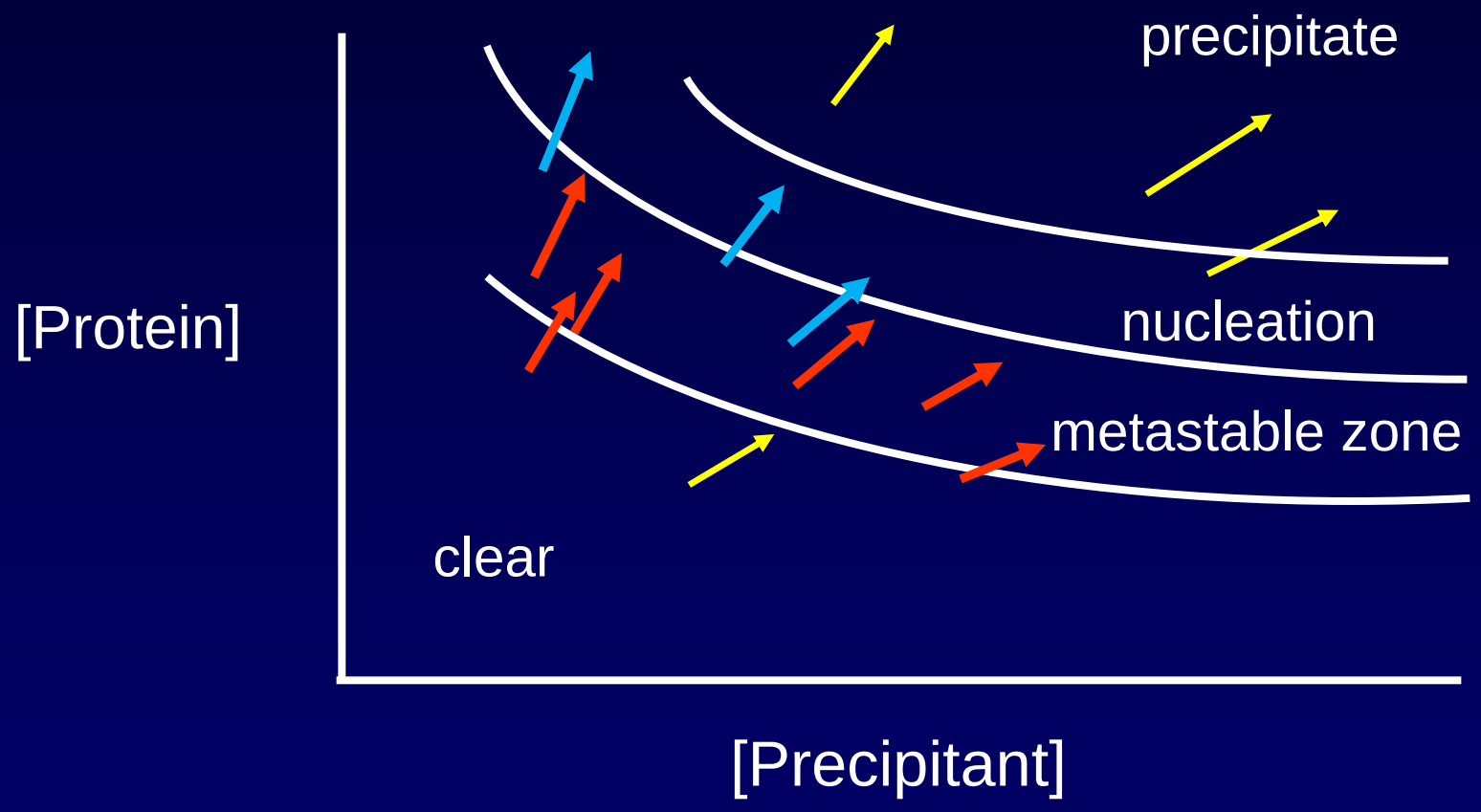
3-bore tip



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



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 microlitres are enough!

MPL (Diamond Light Source) / Douglas Instruments: 2 of 5 projects worked very well

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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. 'Getting the most out of the random microseed matrix-screening method in protein crystallization'.

Cryst. Growth Des., 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.

Microseeding

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

Microseeding

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Microseeding

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

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(4) How can we avoid salt crystals?	
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(7) Can we harvest seed crystals from microfluidic devices?	
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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.

“Receptive” conditions

Conditions where:

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

25 receptive conditions were found

1	Glucose Isomerase	JCSG+	2-2	2 M (NH4)2SO4, 0.2 M NaCl, 0.1 M Na MES, PH 6.5
2	Glucose Isomerase	JCSG+	2-43	25%(w/v) PEG 3350, 0.2 M (NH4)2SO4, 0.1 M bis-tris
3	Hemoglobin	JCSG+	2-25	30%(w/v) Jeffamine ED-2001, 0.1 M Na HEPES, PH 7.0
4	Hemoglobin	JCSG+	2-33	30%(w/v) PEG 2000 MME, K thiocyanate
5	Hemoglobin	JCSG+	2-34	30%(w/v) PEG 2000 MME, K bromide
6	Hemoglobin	JCSG+	2-44	25%(w/v) PEG 3350, 0.2 M NaCl, 0.1 M bis-tris, PH 5.5
7	Thaumatin	Structure screen 1	7	30%(w/v) PEG 4K, 0.2 M ammonium acetate, 0.1M Na citrate, PH 5.6
8	Thaumatin	Structure screen 1	9	20%(v/v) IPA, 20%(w/v) PEG 4K, 0.1 M Na citrate, PH 5.6
9	Thaumatin	Structure screen 1	14	30%(w/v) PEG 8K, 0.2 M (NH4)2SO4, 0.1 M Na cacodylate, PH 6.5
10	Thaumatin	Structure screen 1	15	20%(w/v) PEG 8K, 0.2M magnesium acetate, 0.1 M Na cacodylate, PH6.5
11	Thaumatin	Structure screen 1	32	2 M (NH4)2SO4, 0.1 M tris, PH 8.5
12	Thaumatin	Jena Bioscience Membrane screen3	D5	1.5 M Li2SO4, 0.1 M Na HEPES, PH 7.5
13	Thermolysin	JCSG+ (2:1 water)	1-2	20%(w/v) PEG 3K, 0.1 M Na citrate, PH 5.5
14	Thermolysin	JCSG+ (2:1 water)	1-21	20%(w/v) PEG 6k, 0.1 M citric acid, PH 5.0
15	Thermolysin	JCSG+ (2:1 water)	2-18	10%(v/v) MPD, 0.1 M bicine, PH 9.0
16	Thermolysin	JCSG+ (2:1 water)	2-19	0.8 M succinic acid, PH 7.0
17	Thermolysin	JCSG+ (2:1 water)	2-21	2.4 M Na malonate, PH 7.0
18	Thermolysin	JCSG+ (2:1 water)	2-22	0.5%(w/v) Jeffamine ED-2001, 1.1 M Na malonate, 0.1 M Na HEPES, PH 7.0
19	Trypsin	Jena Bioscience Membrane screen3	D3	1.5 M NaCl, 0.1M Na acetate, PH 4.6
20	Trypsin	Jena Bioscience Membrane screen3	D3	1.5 M NaCl, 0.1M Na acetate, PH 4.6
21	Trypsin	Jena Bioscience Membrane screen3	D6	2 M NaCl, 0.1 M Na citrate
22	Trypsin	Jena Bioscience Membrane screen3	D6	2 M NaCl, 0.1 M Na citrate
23	Xylanase	Structure screen 1	32	2 M (NH4)2SO4, 0.1 M tris, PH 8.5
24	Xylanase	Structure screen 1	37	30%(w/v) PEG 4K, 0.2 M Na acetate, 0.1 M tris, PH 8.5
25	Xylanase	Structure screen 1	45	4 M Na formate
26	Xylanase	Jena Bioscience Membrane screen3	B5	3.5 M (NH4)2SO4, 0.25M NaCl, 50mM Na/K phosphate, PH 7.5
27	Xylanase	Jena Bioscience Membrane screen3	D4	1.5 M K phosphate, PH 7.0

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11	Thaumatin	Structure screen 1	32	
12	Thaumatin	Jena Bioscience Membrane screen3	D5	
13	Thermolysin	JCSG+ (2:1 water)	1-2	
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"Hit Solution"

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

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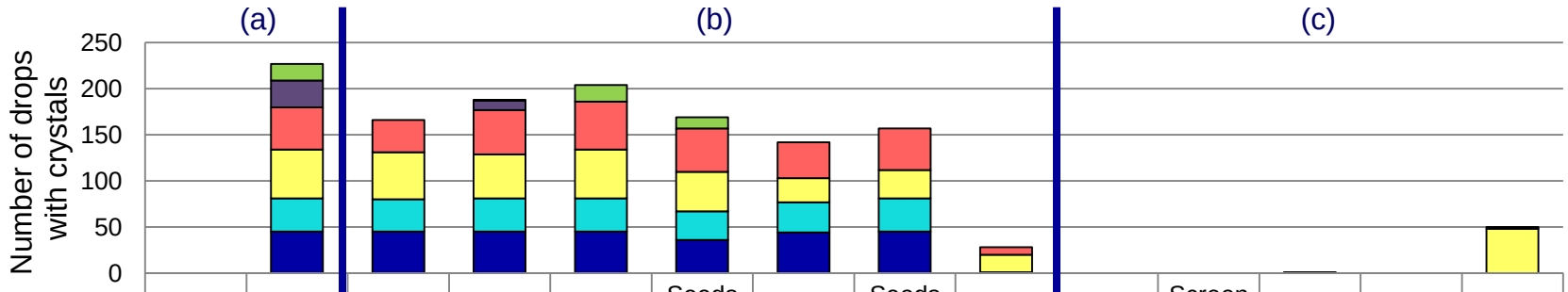
Focusing on "pregnant" conditions



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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?	
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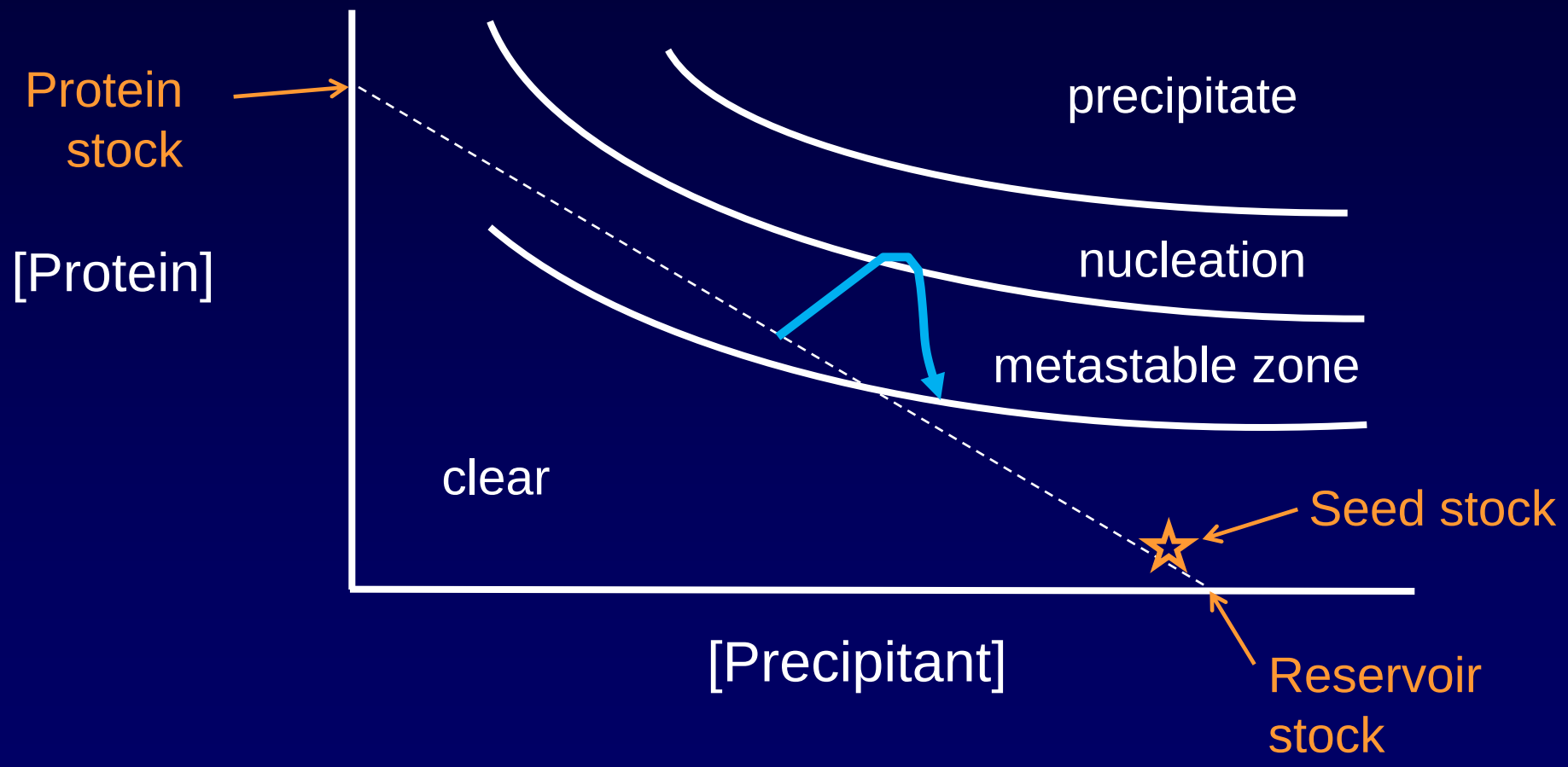
Phase diagram of a protein



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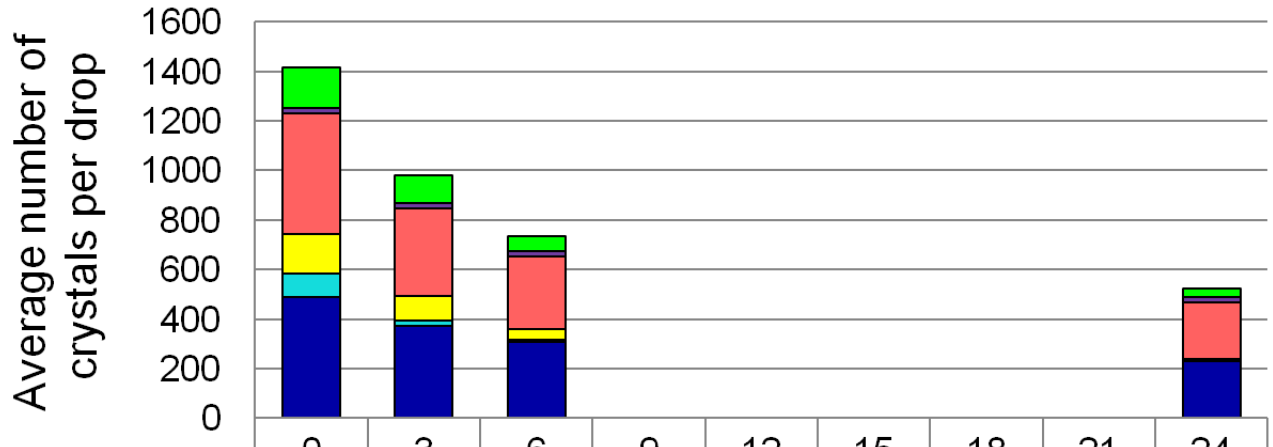

	0 hrs	3 hrs	6 hrs	9 hrs	12 hrs	15 hrs	18 hrs	21 hrs	24 hrs
Gluc. isom.	166	113	63						36
Hemoglob.	19	18	19						20
Thaumatin	489	357	293						228
Thermolysin	158	96	46						11
Trypsin	95	23	6.0						0.1
Xylanase	490	372	309						229

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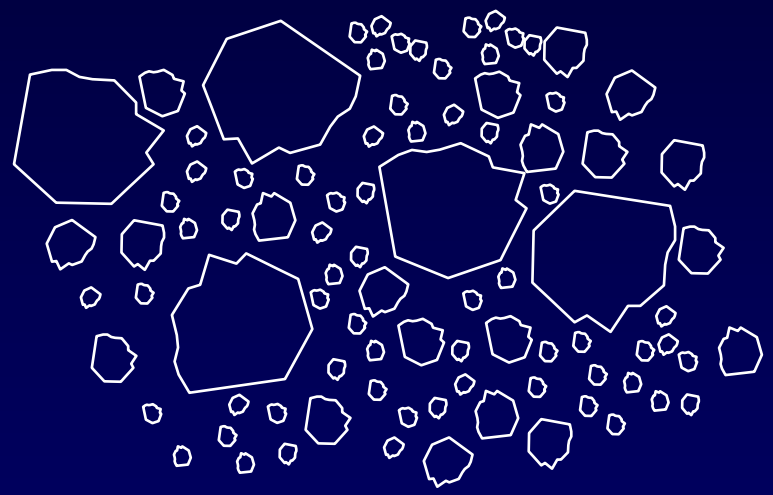
Our interpretation: there is a wide range of crystal particle sizes



We believe that people are losing seed crystals but don't realize it

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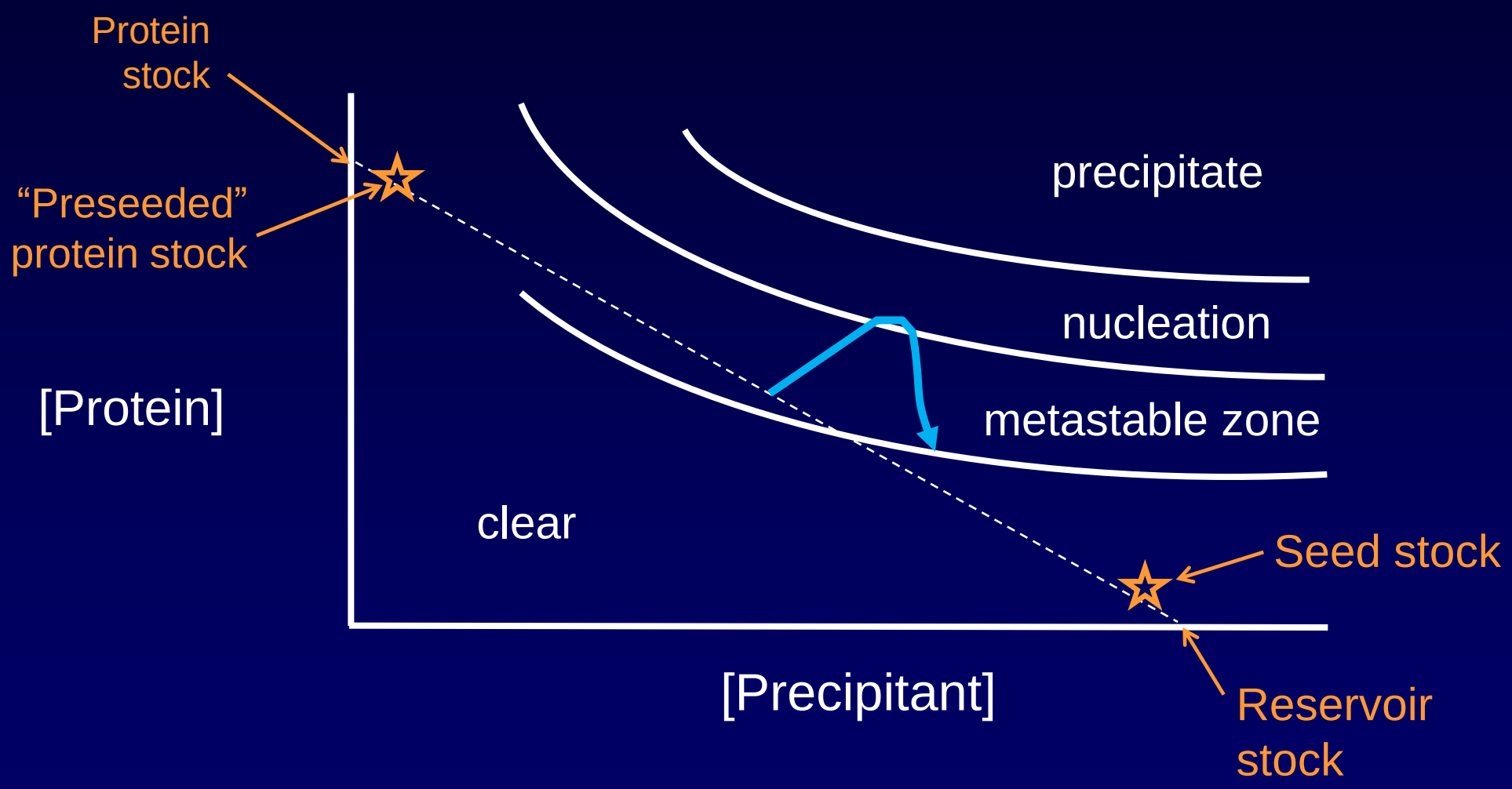
Phase diagram of a protein



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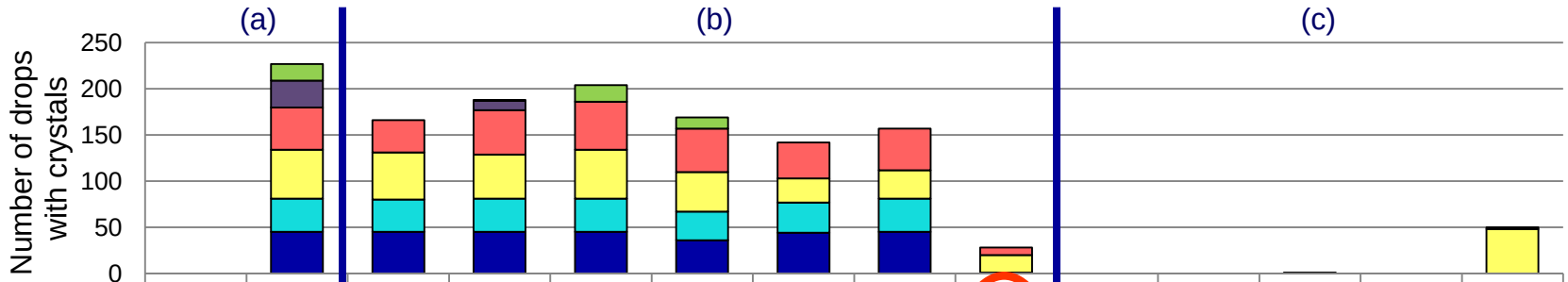
Preseeding the protein



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Why would you want to avoid the Hit Solution?

1. To avoid salt crystals

   Sulfate + Ca^{2+} = gypsum (plaster-of-Paris)

   Phosphate + Mg^{2+}, Ca^{2+}, Zn^{2+}, or Cd^{2+} = salt crystals

Matrix seeding volumes:

0.3 µl protein

+ 0.2 µl reservoir solution

+ 0.1 µl seed stock

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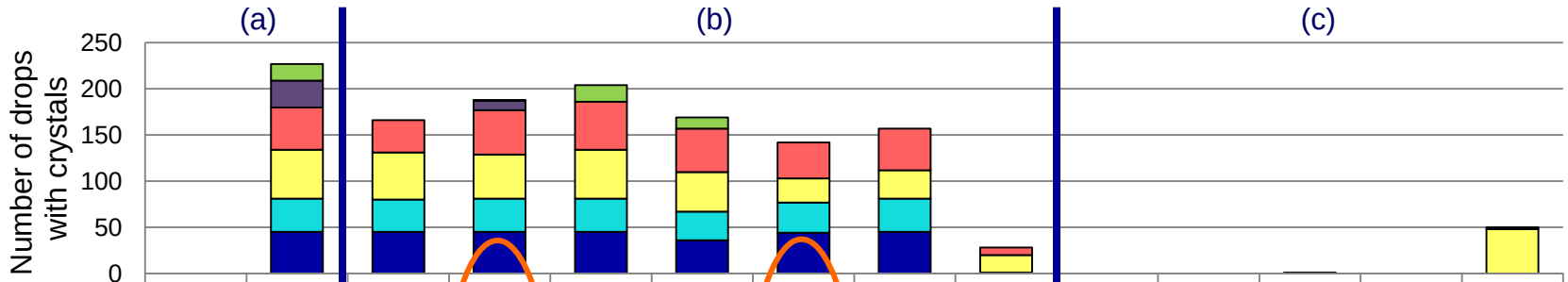
What can we replace the Hit Solution with?



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2. To avoid the additive effect, thereby increasing the diversity of crystals

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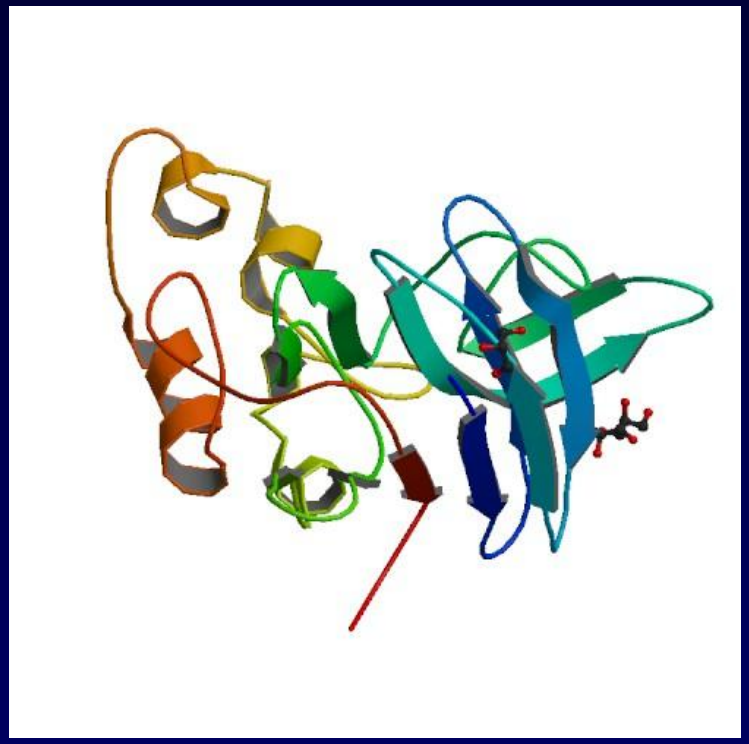
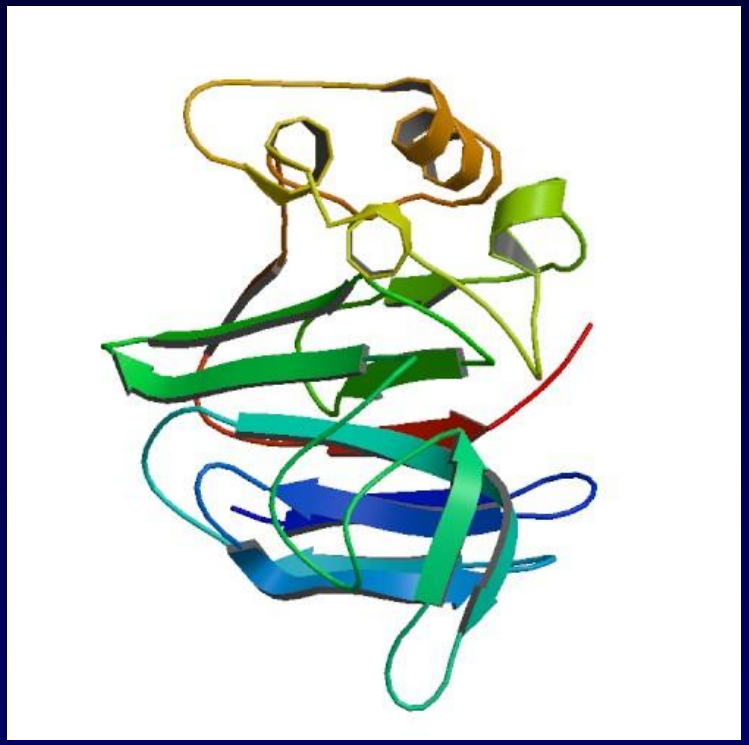
Increasing the diversity of crystals



PDB code 2VU6: thaumatin with tartrate. $P4_12_12$ bipyramid crystal form.



Thaumatin crystallized in the absence of tartrate. $P6_1$ hexagonal crystal form.

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(4) How can we avoid salt crystals?	*Suspend the seed crystals in PEG or NaCl*
(5) How can we get more diverse crystals?	*Remove ingredients that you suspect may be interacting with your protein from the seed stock*
(6) How can we stabilize protein complexes, including heavy atom, small molecule and peptide derivatives ?	
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2. To avoid the additive effect, thereby increasing the diversity of crystals

3. For crystallizing complexes, including heavy atom, small molecule and peptide derivatives

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Suggested by Lesley Haire

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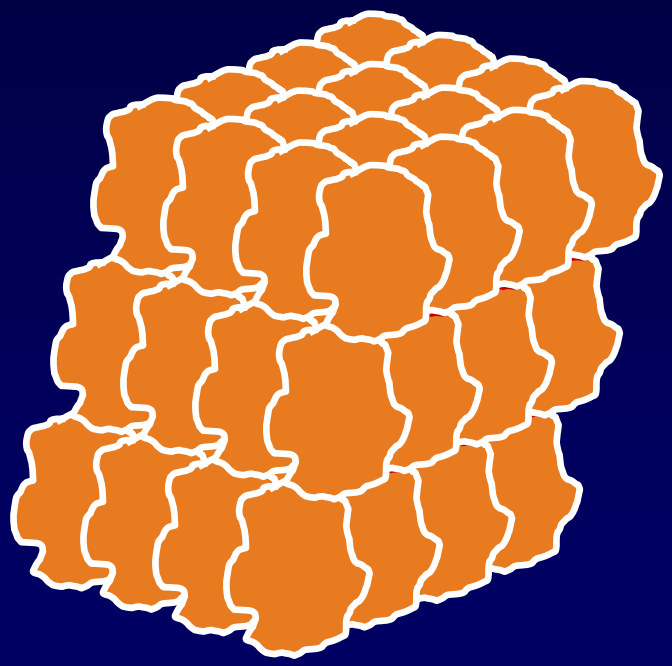
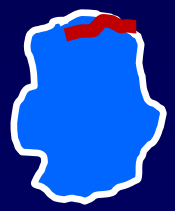
Cross-seeding

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



Uncomplexed protein crystals



Complex

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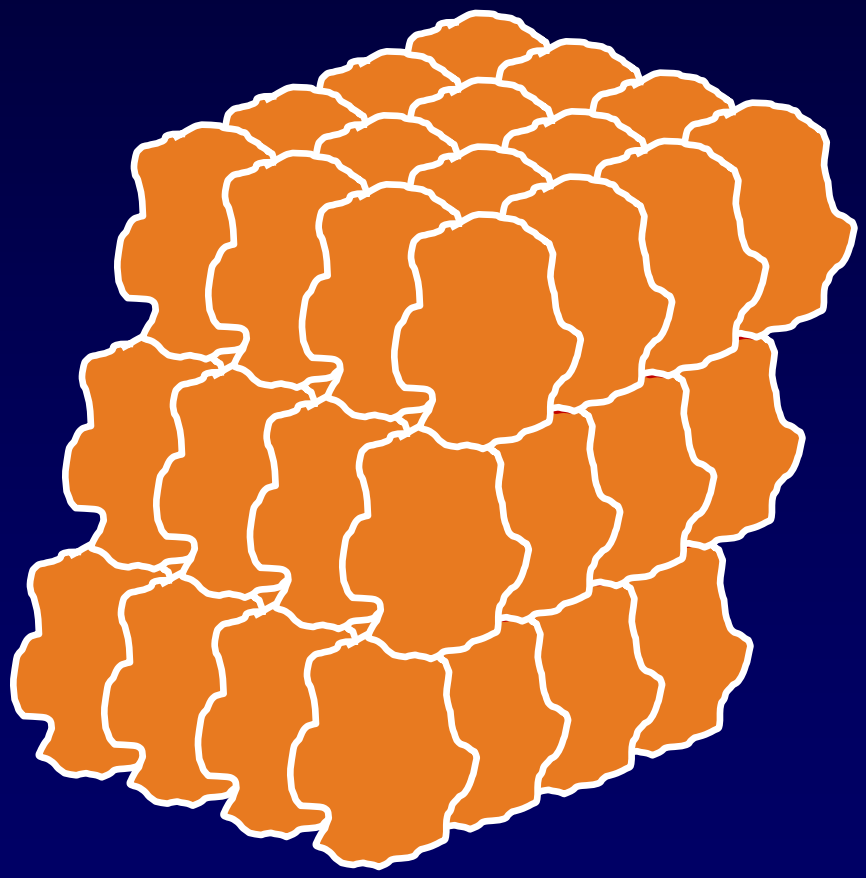
Cross-seeding

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



Douglas Instruments

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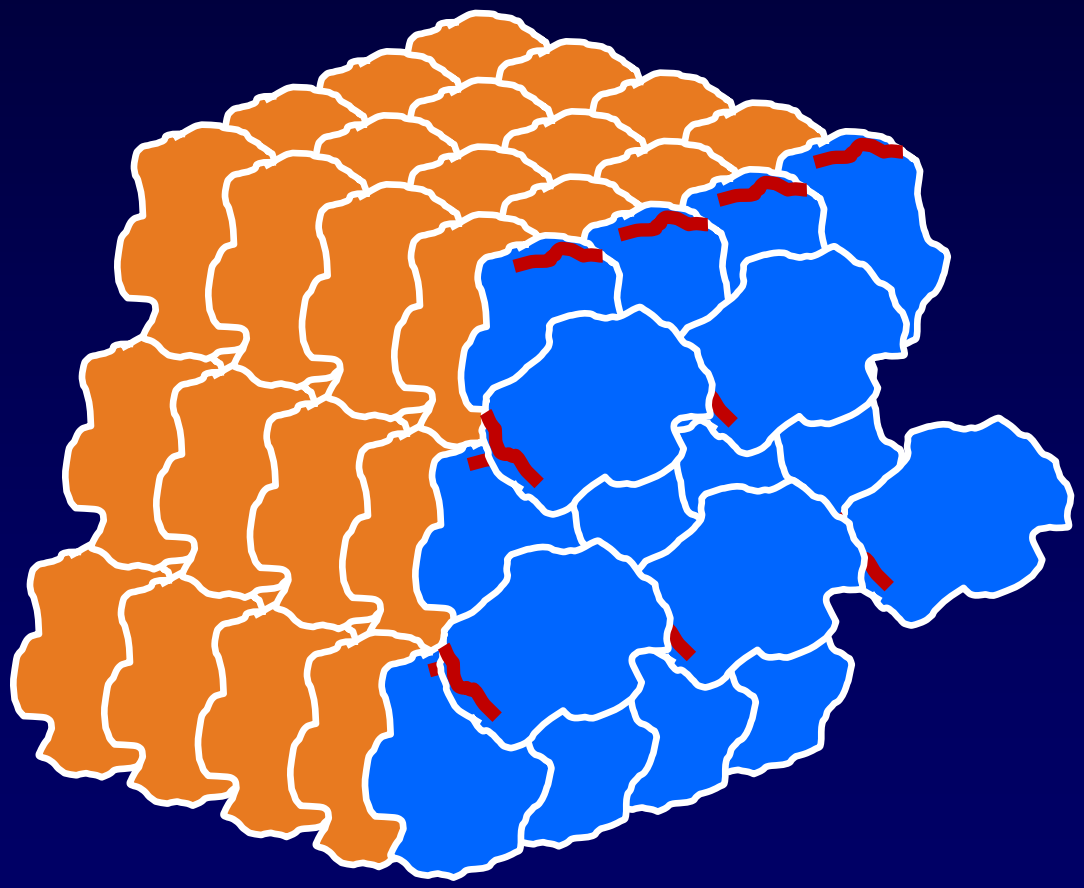

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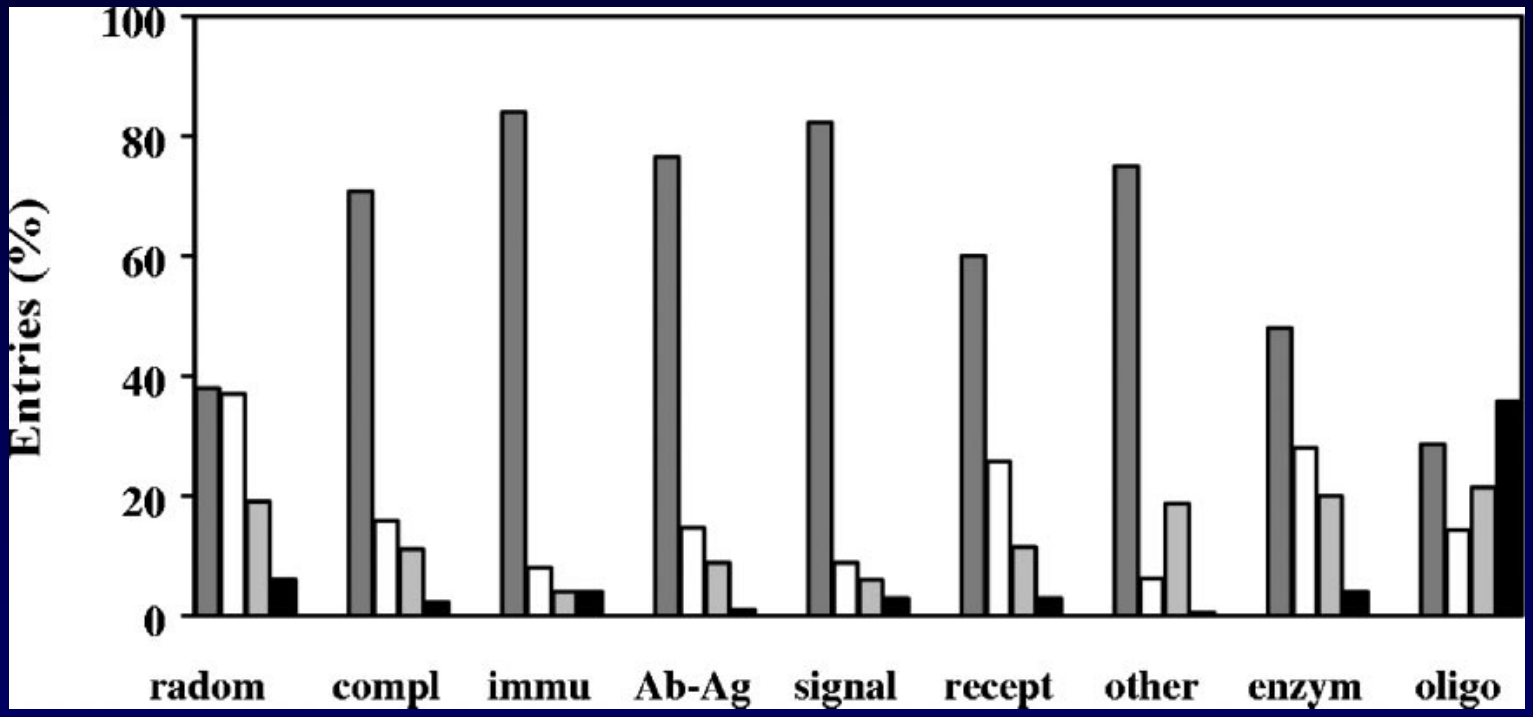
Crystallizing complexes

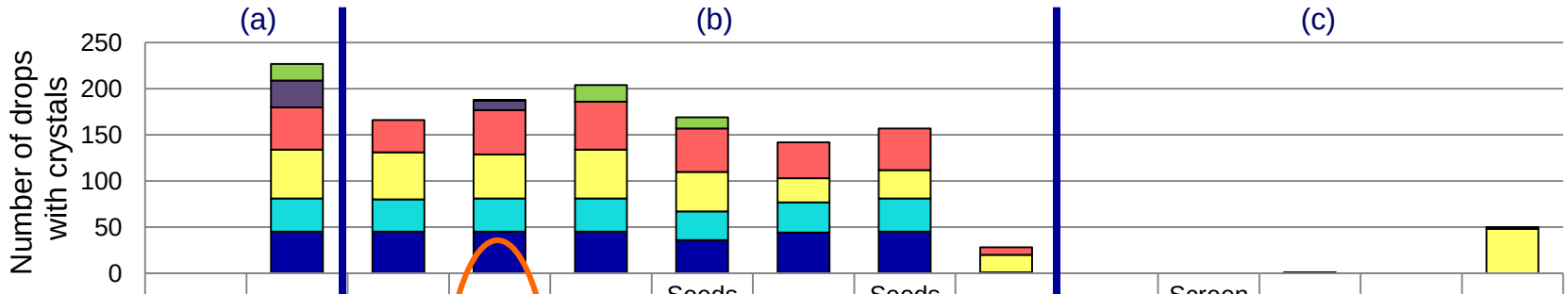
Radaev and Sun. Crystallization of protein-protein complexes.
J. Appl. Cryst. (2002). 35, 674-676



- 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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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*
(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?	*Not so good! (Better than nothing. It's free!)*
(4) How can we avoid salt crystals?	*Suspend the seed crystals in PEG or NaCl*
(5) How can we get more diverse crystals?	*Remove ingredients that you suspect may be interacting with your protein from the seed stock*
(6) How can we stabilize protein complexes, including heavy atom, small molecule and peptide derivatives ?	*Avoid high salt in your seed stock; remove ingredients ....*
(7) Can we harvest seed crystals from microfluidic devices?	
(8) What can you do if you have no crystals?	

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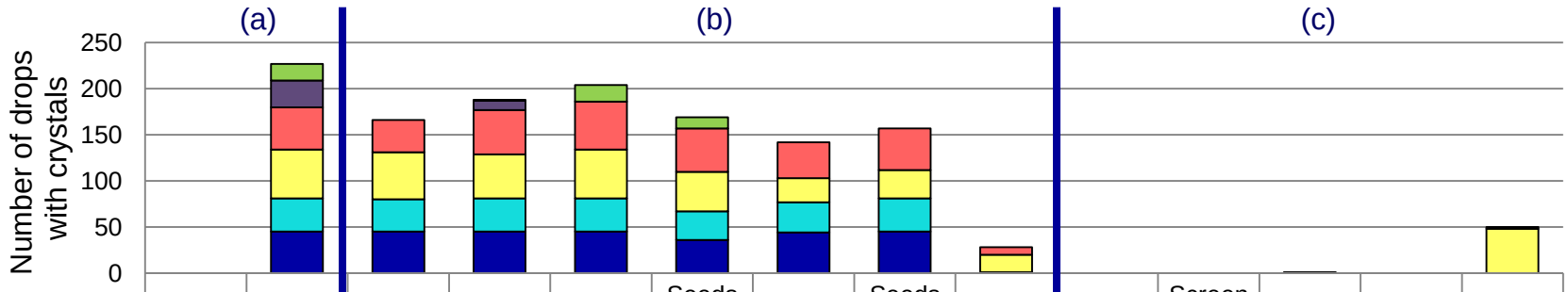
Can we predict which solutions the seed crystals will be stable in?

Focusing on "pregnant" conditions



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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

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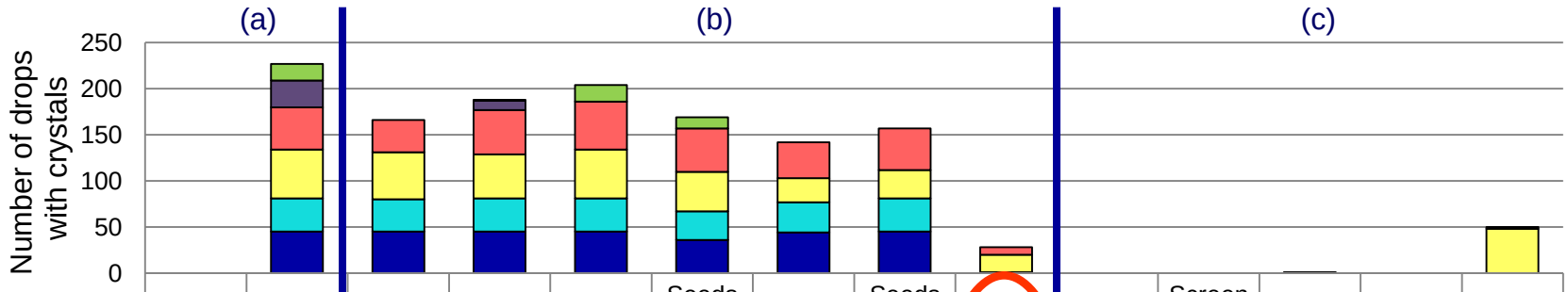
Thermolysin	**OK**	**OK**	**Shattered**	**OK**	**Dissolved**	**Grew**
Trypsin	**OK**	**OK**	**Dissolved**	**OK**	**OK**	Dissolved
Xylanase	**OK**	**OK**	**Cracked**	**OK**	**OK**	Dissolved

Glucose isomerase	JCSG+	2-43	25%(w/v) PEG 3350, 0.2 M (NH4)2SO4, 0.1 M bis-tris
Hemoglobin	JCSG+	2-33	30%(w/v) PEG 2000 MME, K thiocyanate

Preseeding the protein



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Douglas Instruments

random Microseed Matrix-Screening

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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 microlitres are enough!

MPL (Diamond Light Source) / Douglas Instruments: 2 of 5 projects worked very well

Douglas Instruments

random Microseed Matrix-Screening

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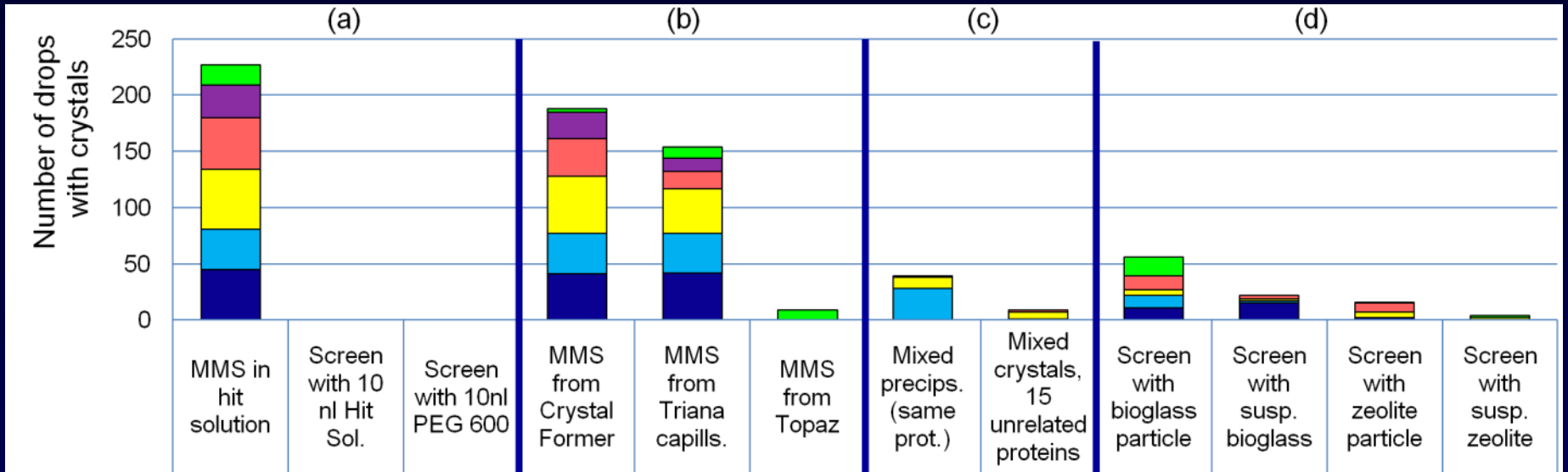
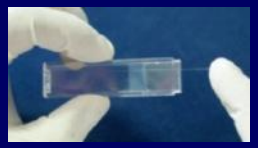
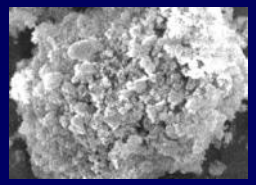


	MMS in hit solution	Screen with 10 nl Hit Sol.	Screen with 10nl PEG 600	MMS from Crystal Former	MMS from Triana capills.	MMS from Topaz	Mixed precips. (same prot.)	Mixed crystals, 15 unrelated proteins	Screen with bioglass particle	Screen with susp. bioglass	Screen with zeolite particle	Screen with susp. zeolite
Gluc. Isom.	18	0	0	3	10	9	0	0	17	0	1	2
Hemoglob.	29	0	0	24	12		0	0	0	0	0	0
Thaumatin	46	0	0	33	15		1	2	12	3	8	0
Thermolysin	53	0	0	51	40		10	6	5	2	5	2
Trypsin	36	0	0	36	35		28	0	11	2	0	0
Xylanase	45	0	0	41	42		0	1	11	15	2	0





Douglas Instruments

Thank you for listening!

Douglas Instruments

Thank you for listening!

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

Douglas Instruments

Crosslinking seed crystals after crushing

Protein	Seeds in Hit Sol	Seeds in PEG 600	X-linked seeds in PEG, used immediately	X-linked seeds in PEG, 1wk 20°C	Seeds in NaCl	X-linked seeds in NaCl, used immediately	X-linked seeds in NaCl, 1wk 20°C
Gluc. isom.	18	1	9	9	0	9	9
Hemoglobin	10	10	7	0	0	0	0
Trypsin	36	36	36	13	33	36	25

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