

Fast optimisation: 2-D grid seeding method.

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Introduction:

The optimisation of initial hits obtained by automated screening in nanodrops can often prove time-consuming and laborious.

A semi-automated seeding technique using sitting drop vapour diffusion method has been described by Walter *et al.*¹

A recent paper² describes the use of automated seeding using the microbatch method for optimising crystal quality. However, harvesting and freezing crystals from under oil is often regarded as difficult and messy.

The aim of this work was the development of a fast automated method to optimise initial hit conditions using vapour diffusion.

Initially, microbatch (*X-step*) software (Douglas Instruments) was used to generate a spreadsheet, varying protein concentration and precipitant concentration in the drops. After dispensing, the drops were equilibrated against a common dehydrant, either the 'hit' condition or NaCl³ in SwissCI 96-well 2-drop vapour diffusion plates. One drawback with this method is that seeding has to be performed in a separate experiment.

Subsequent optimisation experiments were carried out using a 2-dimensional grid script available in the Oryx software for vapour-diffusion. This is a script where, for example, protein concentration can be varied across the plate (X), and additive concentration (or seed-stock) up and down the plate (Y).

Growing crystals of SelenoMethionine-substituted (SeMet.) protein is often problematic, as the derivatised protein may have lower solubility than the native protein in the crystallisation conditions. Cross-seeding^{5,6} SeMet. crystallisation droplets with native microseeds using the 2-D grid method has produced SeMet. crystals for 5 in-house projects resulting in structure solution (unpublished results).

Advantages of 2-D grid seeding method

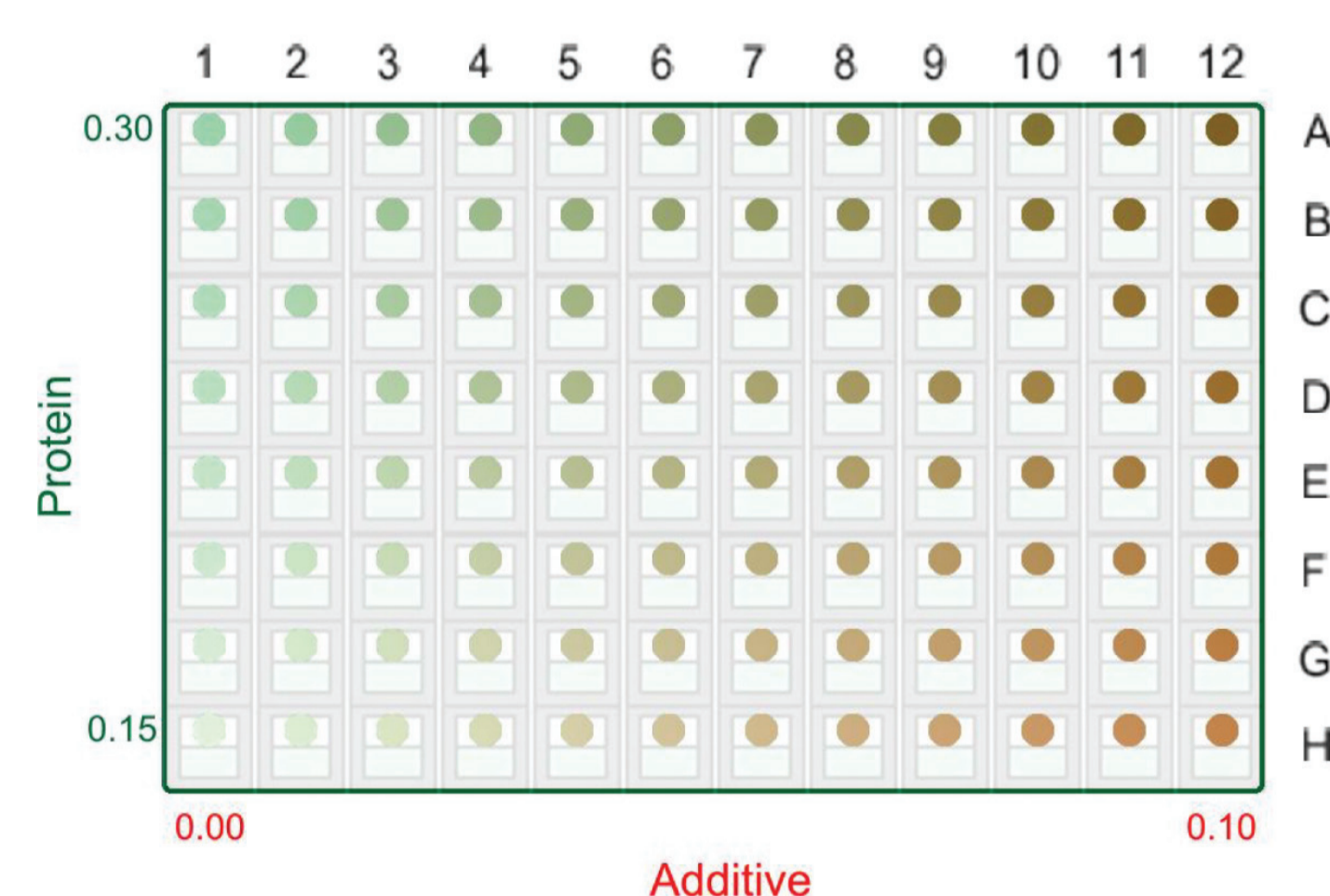
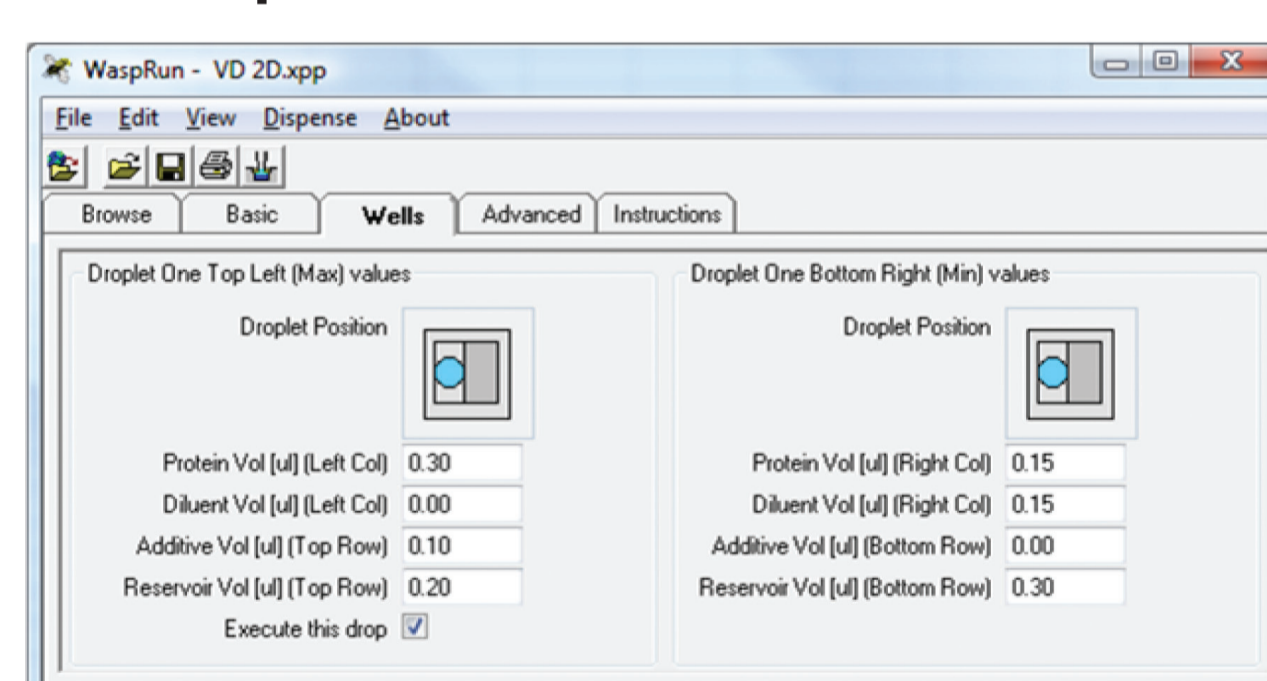
1. Only one reservoir solution needs to be prepared.
2. Drop volume is increased from 200nl to 600nl, allowing growth of larger crystals and easier harvesting of crystals from drop.
3. Optimisation using a fine gradient of protein concentration versus variable amounts of seed is a very effective method of improving crystal quality.
4. Cross-seeding SelenoMet. protein with native microseeds is now used routinely in our laboratory.

Preparation of seed solution

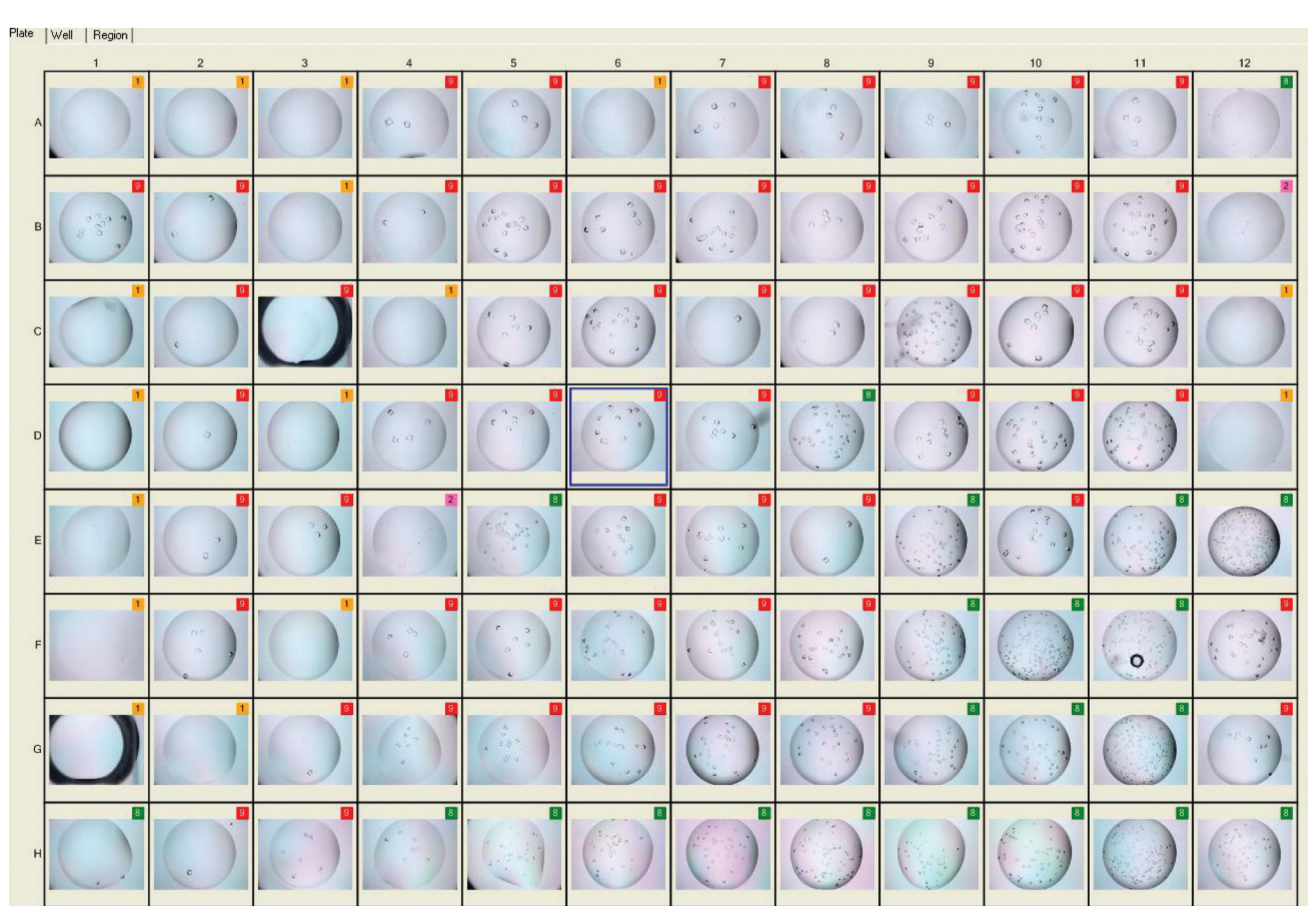
The reservoir solution of the 'hit' condition is used for the seed stabilising solution, using the method of D'Arcy *et al.*⁴

1. Add 1µl reservoir to drop containing crystals
2. Transfer entire drop contents into 50-100µl of reservoir solution
3. Vortex with Hampton "Seed Bead" approx. 90 seconds
4. Store at -80°C after use.

Method for 2D Grid Experiment



Droplets consisted of a final volume of 0.6µl and were dispensed with a 4-channel tip. Protein varied from 2.5 to 5mg ml⁻¹ in the X axis and volume of seed solution from 0 to 0.1µl in the Y axis. Protein buffer was used as the diluent in this experiment. All reservoirs contained 75µl of hit solution.



A thumbnail of images for a typical 2-D grid experiment.

Results

Example 1: Protein S

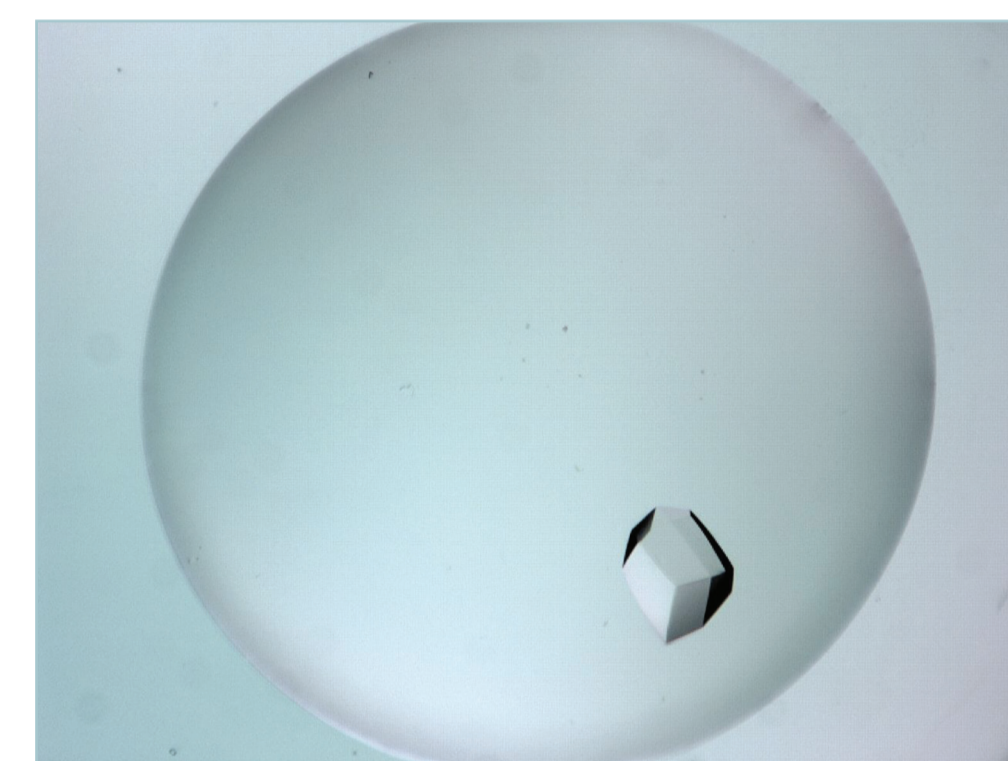
A. Native crystals

Crystals were only obtained from one condition, despite extensive screening. These crystals were X-rayed to confirm they were protein and then reused as seed in a 2-D grid experiment, varying protein and seed concentration. Native crystals grew within 1-3 days and gave diffraction to 2Å.

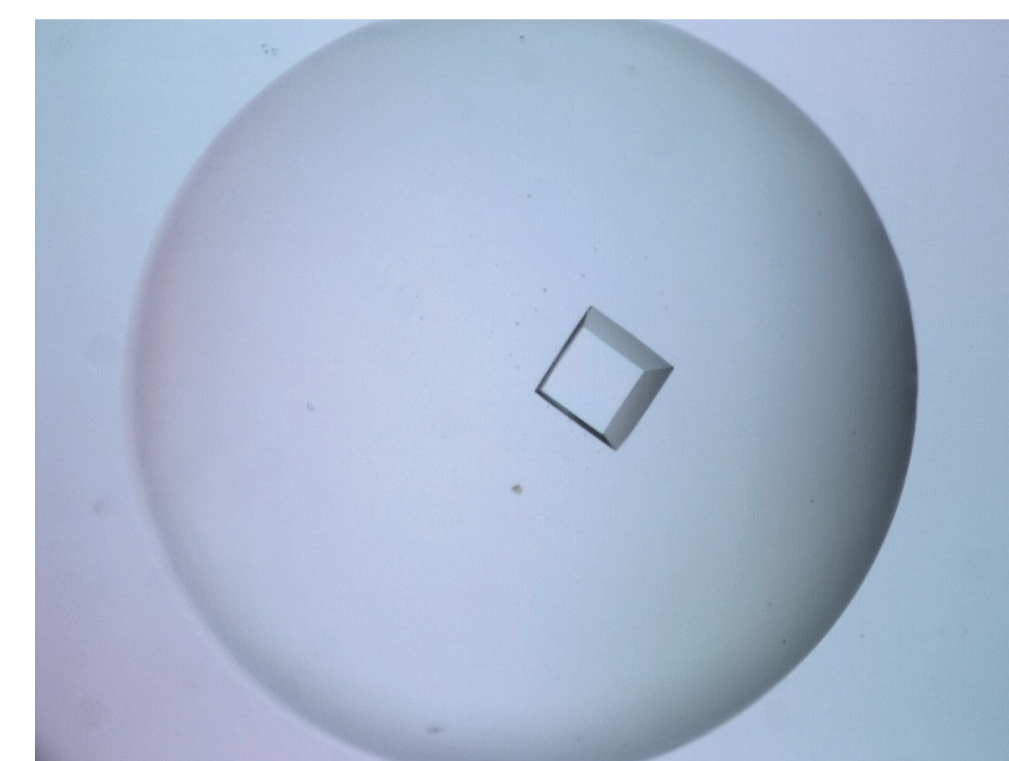
A control experiment using stabilising solution instead of seed solution did not result in nucleation.

B. SeMet. crystals

A 2-D grid experiment was dispensed using native crystals as seed. Crystals were frozen after 4 days growth (120µm x 100µm) and used for structure solution.

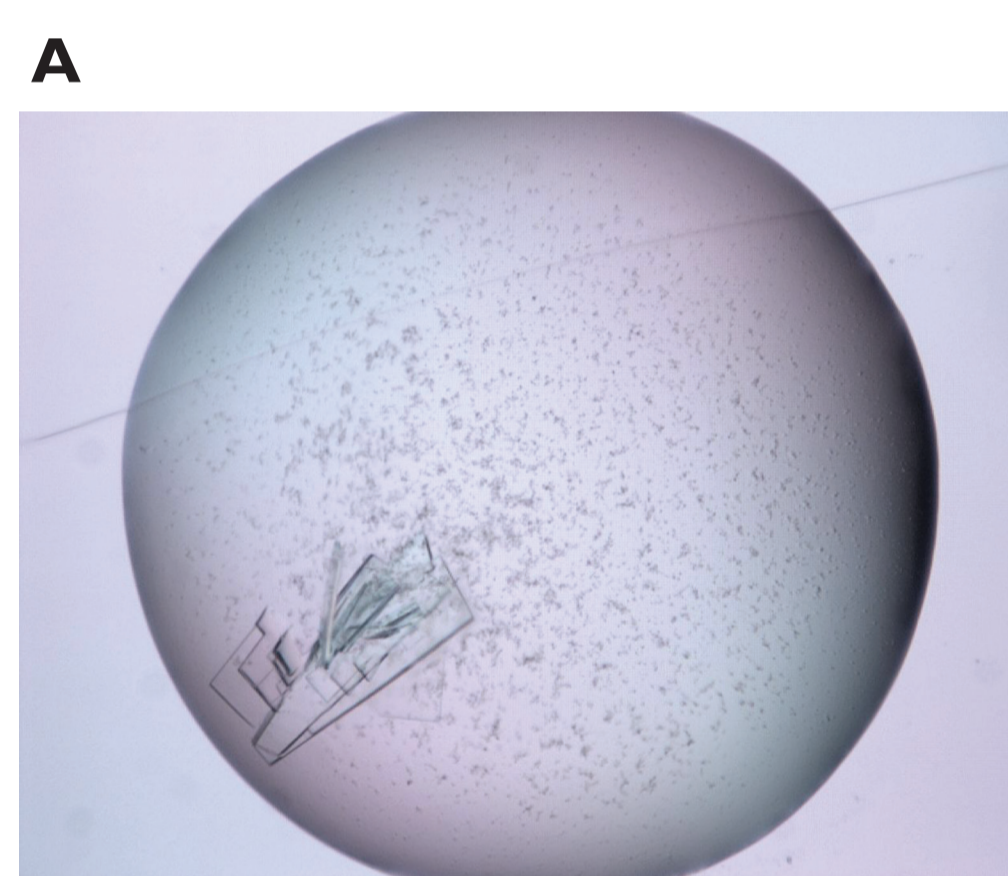


A. Native protein S crystals.

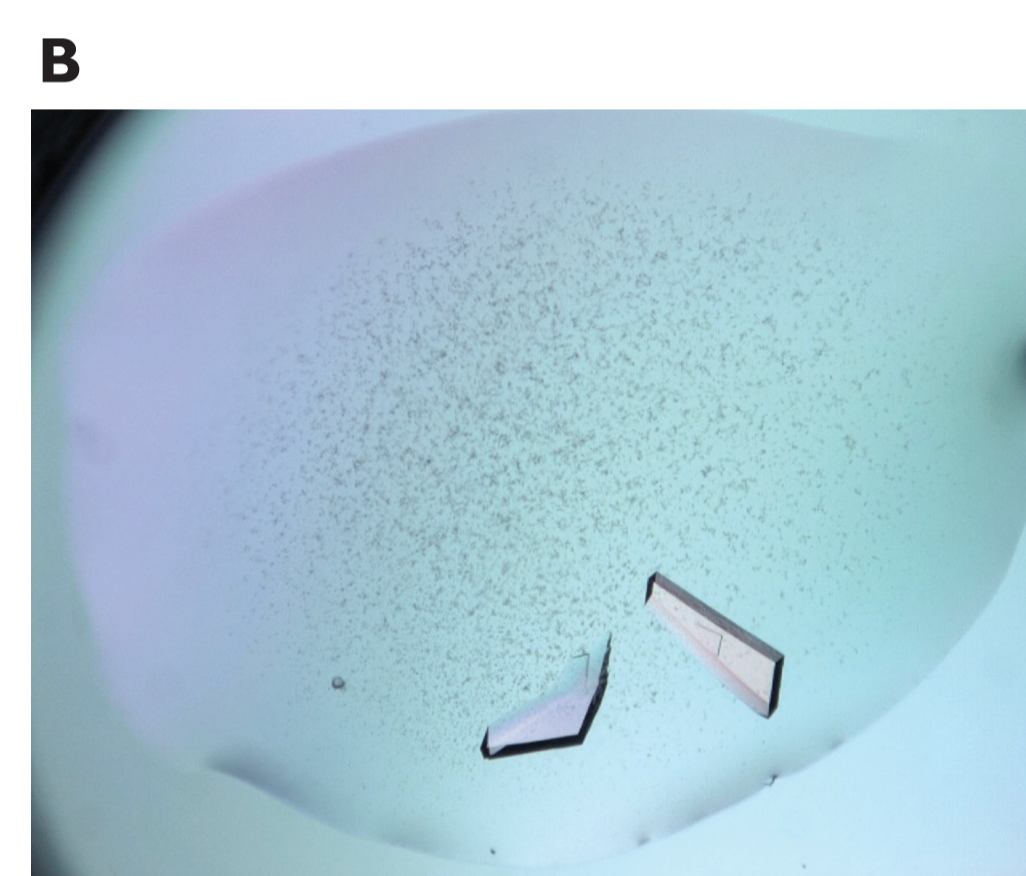


B. SeMet Protein S crystals.

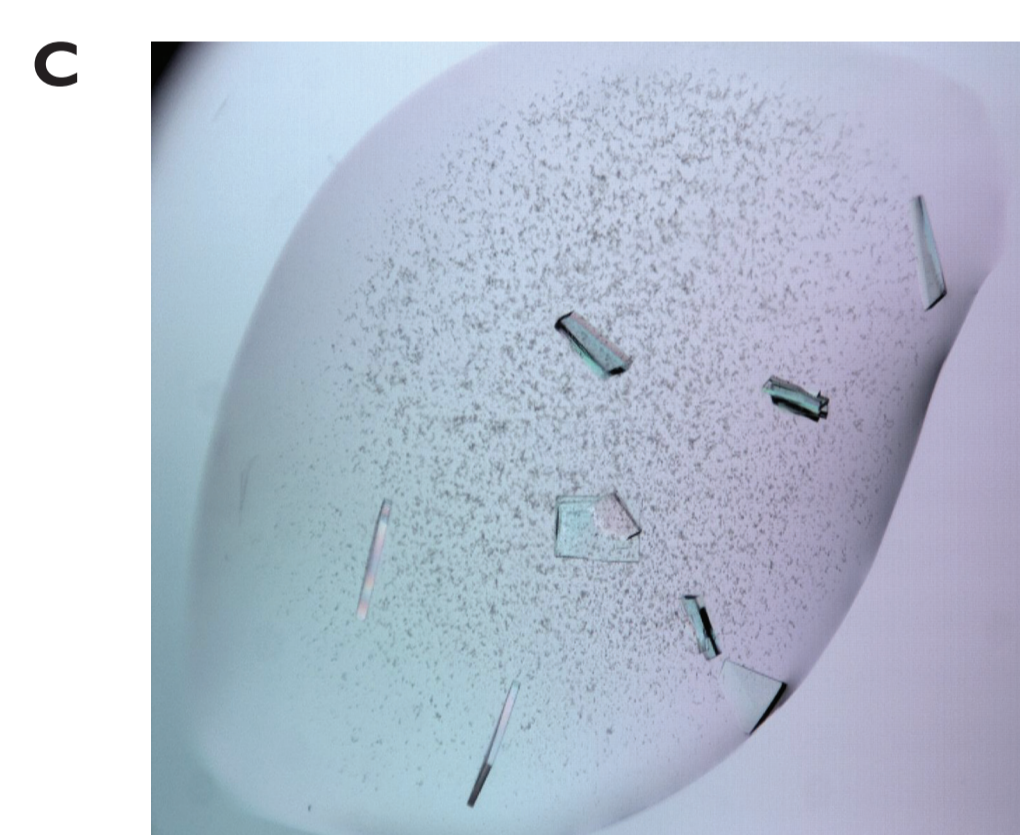
Example 2: SPNIB (N-terminal region of fission yeast Nbs I)



A



B



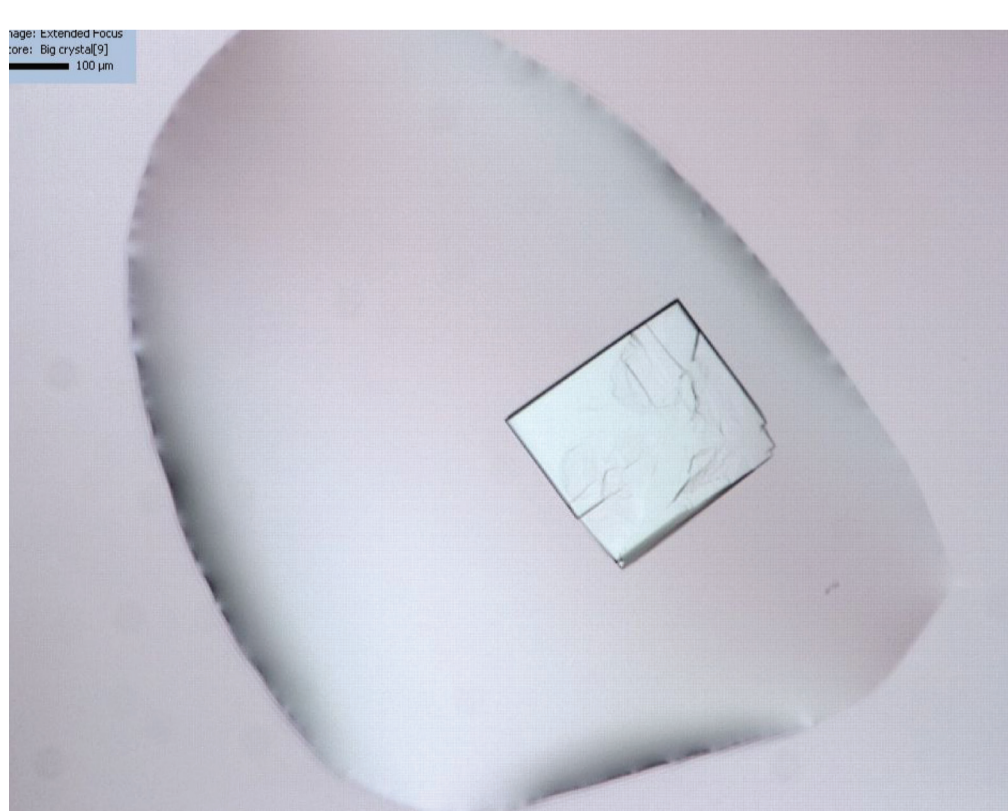
C

A. Crystals grew as stacks of plates from 10% Peg 6K, 0.1M MgCl₂, 0.1M MES pH 6.5 at a protein concentration of 8mg ml⁻¹. These crystals were used to prepare a seed solution for a 2-D grid experiment.

B. 2-D grid experiment. Protein varied from 2-4mg ml⁻¹ in the drops, seed solution 0-0.1µl. Thicker crystals grew in drop G11 and were used for data collection (2.3Å)⁷.

C. 2-D grid experiment. Drop H9 illustrates crystals obtained with lower protein concentration and more seeds.

Example 3: Protein X

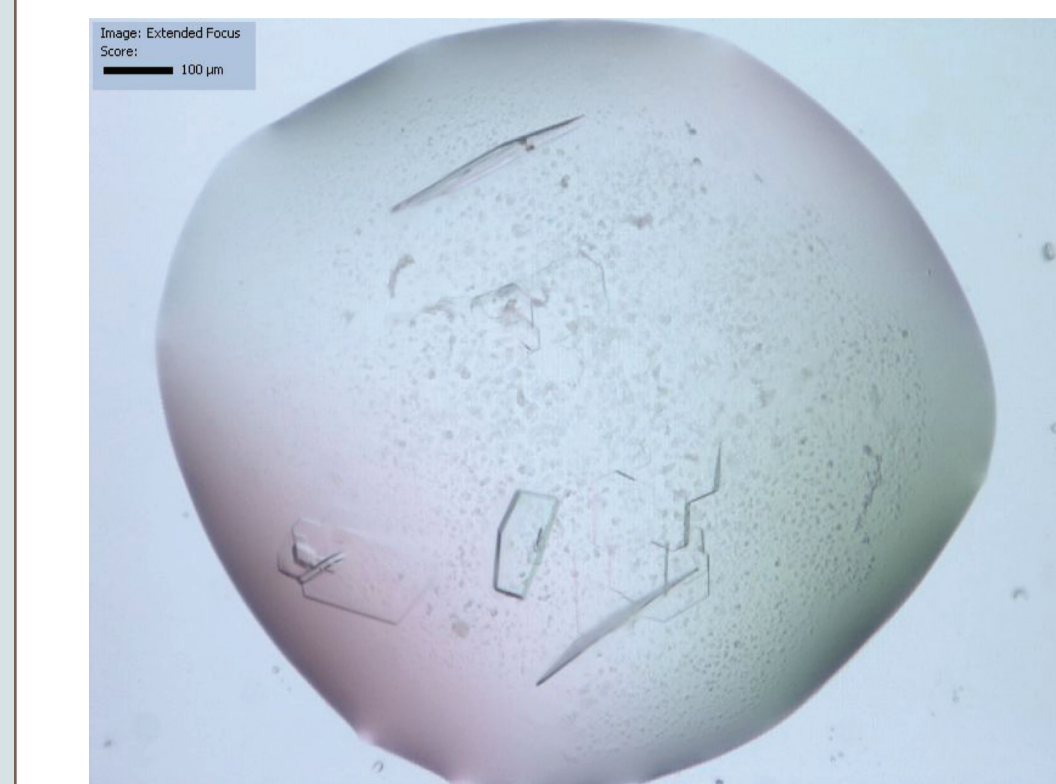


A. Initial hit used for seed.

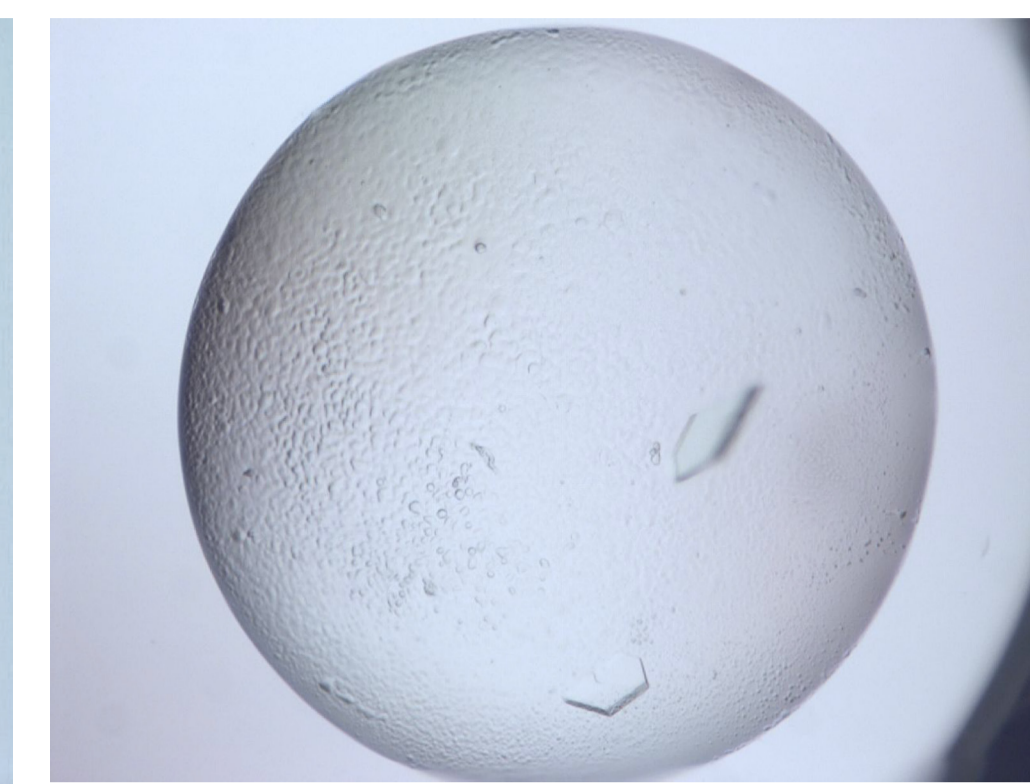


B. Crystals grown using 2-D grid diffracted to 1.6 Å.

Example 4: Protein H

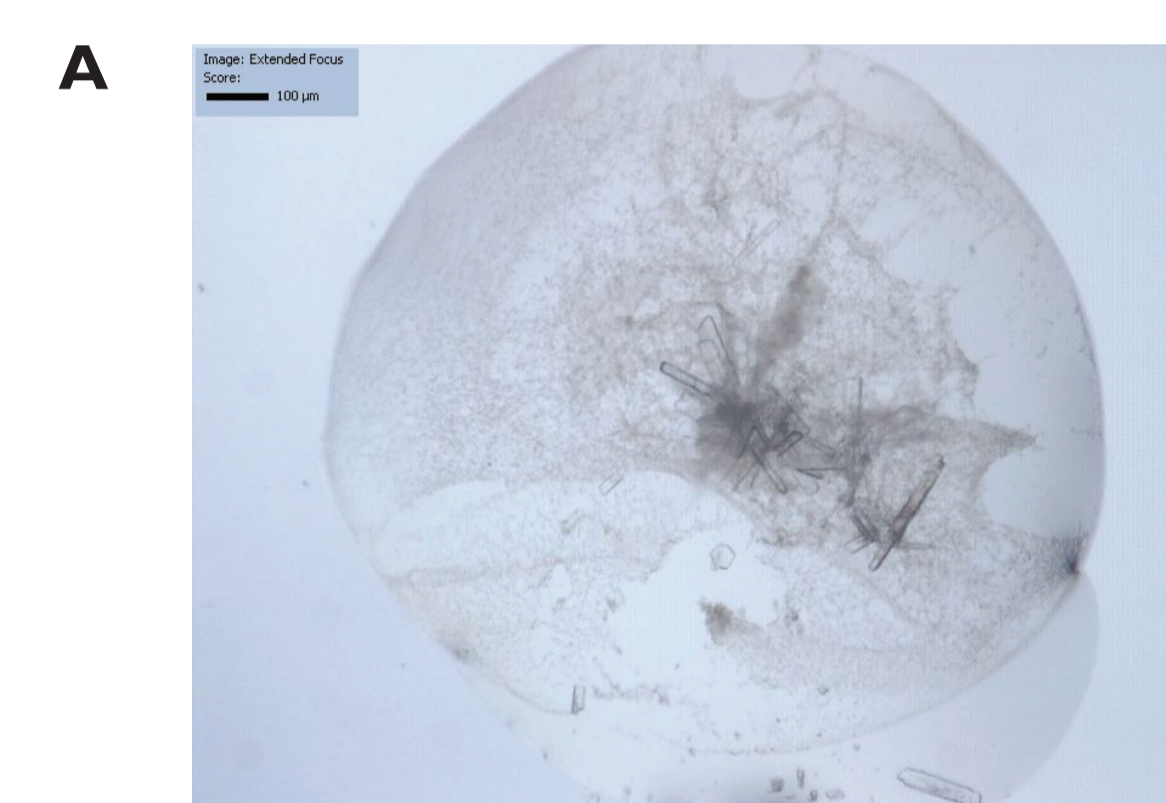


A. Native crystals
Multiple crystals grew from phase separation without seeding.



B. SeMet. crystals
2-D grid method with seeding gave crystals used for data collection.

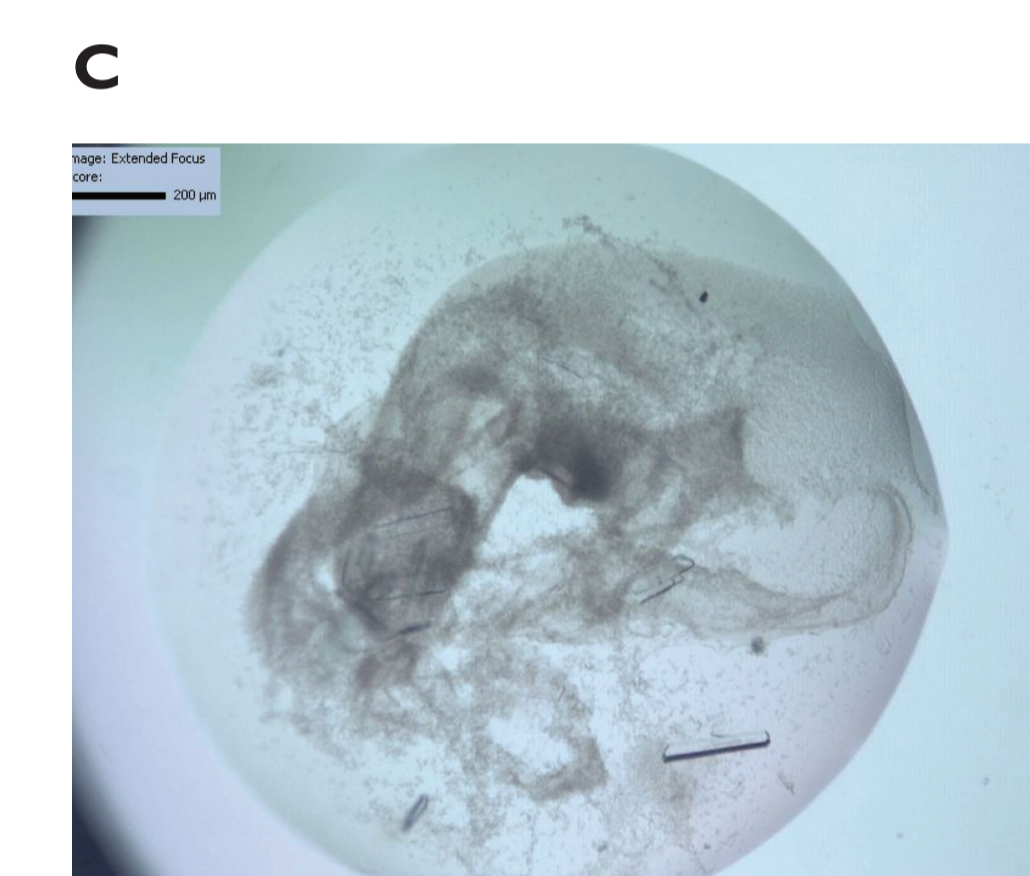
Example 5: Protein H – shorter construct



A



B



C

A. Initial hit

B. Optimisation of hit using dilutions of the screen solution (50-100% (v/v) in reservoir) and 5, 10, 15 mg/ml protein, without seeding.

C. Crystals grown by the 2-D grid method were thicker and gave useful diffraction.

Comparison of standard vapour diffusion optimisation with 2-D grid method and microbatch

	Increase drop volume	Pre-equilibrate drop before seeding	Preliminary experiment to find optimal seed dilution	Preliminary experiment to find optimal protein concentration	Prepare reservoirs with varying precipitant concentration
Classical vapour diffusion	✓	✓	✓	✓	✓
2D grid method	✓	✗	✗	✗	✗
Microbatch	✓	✗	✓	✗	✗

Summary

A fast optimisation method has been developed where protein concentration and quantity of seed are simultaneously varied in an experiment using an Oryx-8 robot (Douglas Instruments). This has proved very successful for obtaining native crystals for data collection and also for cross seeding into Selenomethionine-substituted protein for structure solution.

References:

- ¹Walter, T.S. *et al.* (2008) *Acta Cryst.* F64, 14-16.
- ²Khurshid, S., Haire, L.F. & Chayen, N.E. (2010) *J. Applied Cryst.* 43, 752-756.
- ³Newman, J. (2005) *Acta Cryst.* D51, 490-493.
- ⁴Allan D'Arcy, *et al.* (2007) *Acta Cryst.* D63, 550-554.
- ⁵Stura, E.A. & Wilson, I.A. (1992). *Crystallization of Nucleic Acids and Proteins: A Practical Approach*, edited by A. Ducruix & R. Giegé, Oxford University Press pp.99-126.
- ⁶Bottomley, M. J. *et al.* (1994). *J. Mol. Biol.* 244, 464-468.
- ⁷Lloyd J. *et al.* (2009) *Cell.* 139, 100-111.

Acknowledgements

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