Experimental design for microbatch-under-oil optimization to establish batch crystallization conditions that are suitable for XFEL data collection.

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Introduction

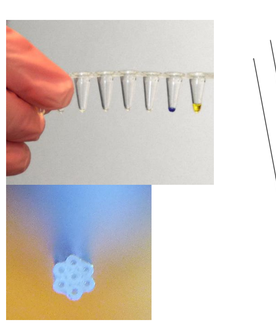
- Vapor diffusion experiments cannot easily be scaled up because the drop concentration changes during the vapor diffusion process.
- Also protein can be lost at the drop surface forming a skin and the drop volume : surface area ratio changes when drop volume increases. This points to "batch" crystallization.

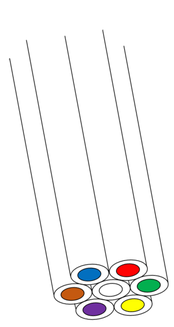
XFEL requires batch -> Screen in batch -> Optimize in batch

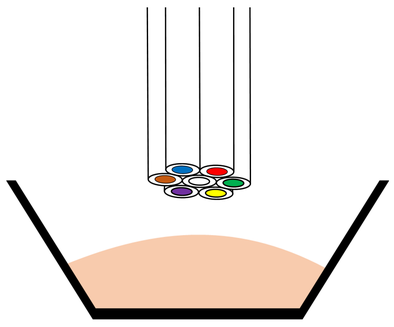
Microbatch-under-oil is a convenient and well-established crystallization method, and it is a true batch method when drops are covered with paraffin oil. Because the experiment is covered with a layer of oil the concentration of the drop remains constant. This means the condition can be scaled up without adjusting the concentration.

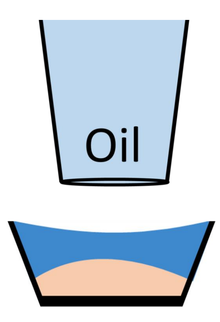
- Once a phase diagram of the protein is established, microseeding can be used allowing control over the number of nucleation sites.
- MMS microseeding and cross-seeding can be used to find new crystallization conditions under oil.

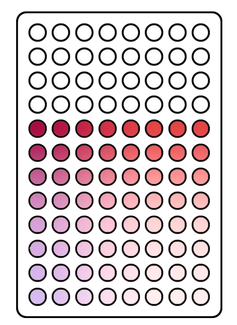
Microbatch-under-oil optimization – Automated with Oryx8











1. 7-Channel Microtip for simultaneous dispensing

- Each ingredient is aspirated from a low profile PCR tube (shown above).
- Practically no solution wasted. Ideal for precious solutions such as protein or seed stock.
- Viscous solutions are aspirated at a slower speed to ensure accurate dispensing.

2. The Aqueous drop is dispensed by the 7-channel microtip.

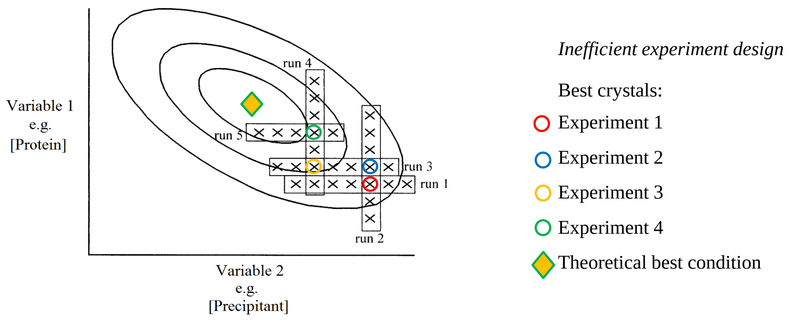
- Ingredients are dispensed simultaneously in different ratios to form the drops.
- The ingredients mix for the first time in the microbatch well
- Drop volumes from can vary from 200nL to 2µL or larger.

3. Oil is dispensed covering the drop. The remaining drops are dispensed until the experiment is completed.

- Paraffin oil is dispensed on the aqueous drop.
- The layer of paraffin oil prevents evaporation.
- This makes scaling up easier as the concentration remains constant.

Optimization strategy for XFEL

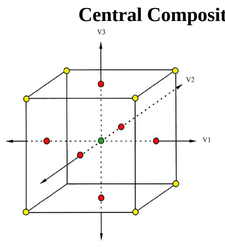
Designing experiments for optimization can be confusing and the results can be difficult to interpret. Whilst an experiment increasing e.g. precipitant concentration or protein concentration may be easy to design and setup, changes to these and other important variables often interact - that is to say, changes in the level of one variable often change the optimum settings of the others.



Rational experiment design -> Saves protein and precious materials -> cover more of the crystallization space

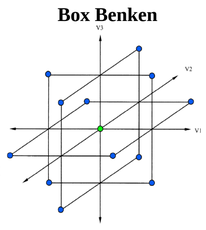
Confusion can be avoided by using appropriate experimental designs where all of the important variables are varied. The example below is a well known experimental design - the **Central Composite**. This design consists of one or more center points (where all variables are set to their mid levels), together with “axial” points (where all variables are set either to their low or their high levels).

Central Composite



- Center point
- Axial point
- Factorial point

Box Benken



- Center point
- Variables change two at a time

Other experiment designs such as **Box Benken** above or custom designs can be created. Such designs reduce the number of experimental points, saving time and materials, and help to identify trends. The general feature of multivariate designs is that all of the important variables are varied in each experimental run. Once a trend is identified a fine gradient or scale up experiment can be designed and dispensed.

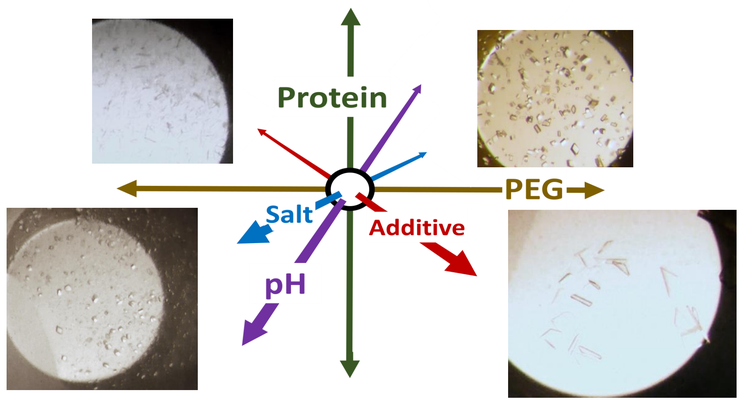
Identify Trends:

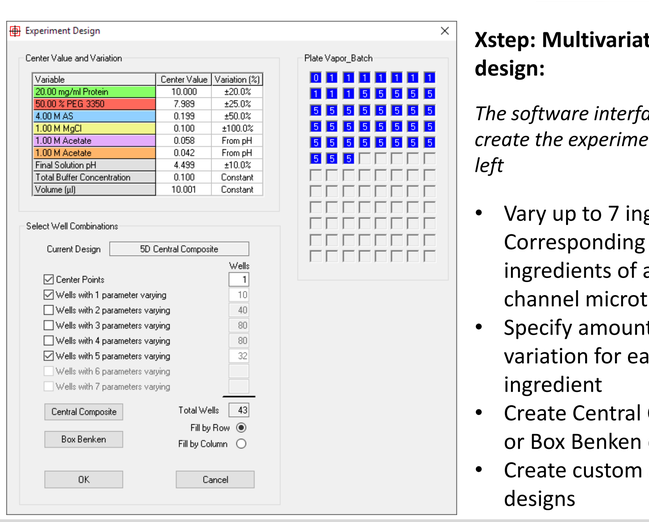
Multivariate auto design

An efficient strategy for a typical protein:

- Identify the best condition from screen
- Optimize best condition: Create Central Composite varying e.g. protein, precipitant, salt, additive, seed concentration and pH
- Experiment should be designed to cover as much crystallization space as possible but use as little protein as possible

The diagram below is an example of a 5 dimensional auto design experiment





Xstep: Multivariate auto design:

The software interface used to create the experiment is shown left

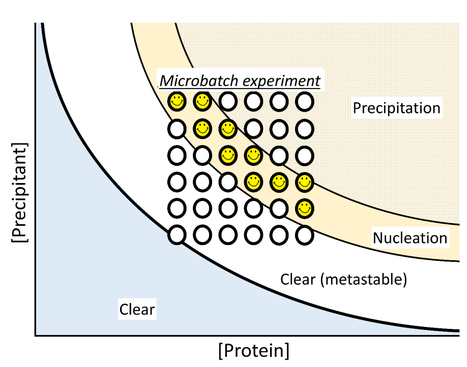
- Vary up to 7 ingredients – Corresponding to the 7 ingredients of a 7-channel microtip.
- Specify amount of variation for each ingredient
- Create Central Composite or Box Benken designs
- Create custom auto designs

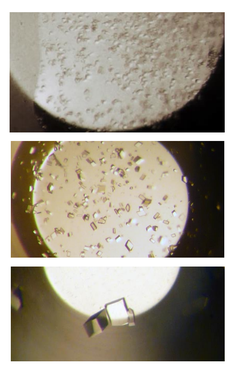
Fine gradient or scale up:

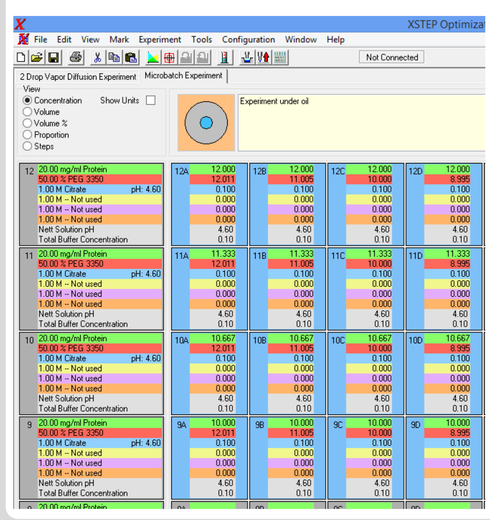
Gradient optimization design

Make small changes

- The most promising condition from the auto design can be investigated in greater detail by creating gradient experiments
- Pairs of ingredients such as precipitant concentration and pH can be varied







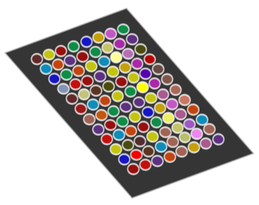
Xstep: Gradient design

- A spread sheet interface (shown left) allows full control over the conditions of each well
- Gradients can be created using the interpolate tool
- Multiple gradients can be dispensed to one plate
- Drop volume can be varied from 200 nL to 0.2 mL+

Microbatch seeding for XFEL

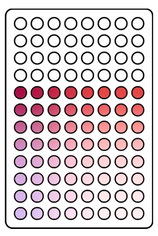
- Highly concentrated (undiluted) seed stock contains ~10^10 crystals.
- This can produce the right number of crystals for XFEL in 1 mL.
- The seed stock should be thoroughly crushed with a probe then seed bead. It is important that the seeds are all of similar size. This will increase the number of seeds in the stock.
- If possible the seed stock should be prepared using only the liquid from the drop. This will increase the stability of the seed stock and number of nucleation sites.
- Work in the metastable zone. Identify conditions where crystals only grow with seeds added. Here the seeds are more powerful and more control over nucleation can be achieved.
- rMMS can be used under oil to identify new conditions where crystals only grow with seeds.
- Xstep can be used to dispense seed stock volumes to drops as small as 5nL. A microbatch-under-oil gradient can be prepared using Xstep with to create the phase diagram shown right.

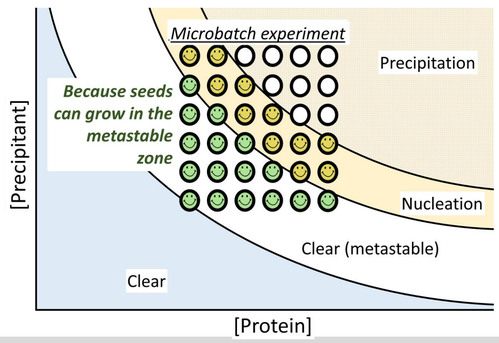
1. Identify crystals for seeding.
2. Crush crystals with crystal crusher.
3. Vortex using seed bead.



Add seeds stock to random screen. (rMMS) Identify new crystallization conditions.

Optimize using seed stock. Identify metastable wells to control number of crystals.





Microbatch-under-oil gradient with seeds stock added.

Key Points

- Batch screening and optimization are most suiable for XFEL sample preparation.
- Crystallization is a multivariate problem.
- Powerful multivariate experimental design and dispensing are available
- Microseeding is suitable for XFEL sample preparation

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