Detecting and visualizing crystals

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What contributes to reflection intensity?

The Intensity of a reflection

$$I(h,k,l) = \frac{\lambda^3}{\omega \cdot V^2} \times \left(\frac{e}{mc^2} \right)^2 \times V_{cr} \times I_o \times L \times P \times A \times |F(hkl)|^2$$

Incident intensity

X-ray scattering from unit cell – multiplier for electrons

Squared to get intensity

Scattering due to a single electron

The Intensity of a reflection

$$I(h,k,l) = \frac{\lambda^3}{\omega \cdot V^2} \times \left(\frac{e}{mc^2} \right)^2 \times V_{cr} \times I_o \times L \times P \times A \times |F(hkl)|^2$$

Volume of crystal

Absorption

Polarization

Lorentz correction

For a fixed position of the crystal the scattering occurs at the intersection of the diffracting region and Ewald sphere. The intensity scattered by an electron into all directions toward the complete surface of the sphere surface $4\pi(1/\lambda)^2$ has a fixed value and is independent of λ. The λ^2 dependence is multiplied by a l-dependent term related to the time it takes for the complete diffracting region to pass through the Ewald sphere.

The $1/V^2$ component comes from the number of unit cells per given volume. Larger unit cells, fewer per volume.

The intensity of a reflection (or the average intensity of the data set) is directly related to:

- Volume
- Incident intensity

Doubling intensity allows data collection from a crystal of half the volume (note, not half the size)

Going from an incident brilliance of approximately 10^{8}-10^{9} photons s^{-1} $mrad^{-2}$ 0.1% $bandwidth^{-1}$ for a laboratory system to values approaching 10^{20} for a third generation undulator source considerably decreases the volume of the crystal required to provide useable *I(hkl)s*.

If we consider X-ray Free Electron Laser sources (XFELs) with a brilliance of ~10^{30} the volume required decreases still further even for samples where a jet of crystals are sprayed through the beam.

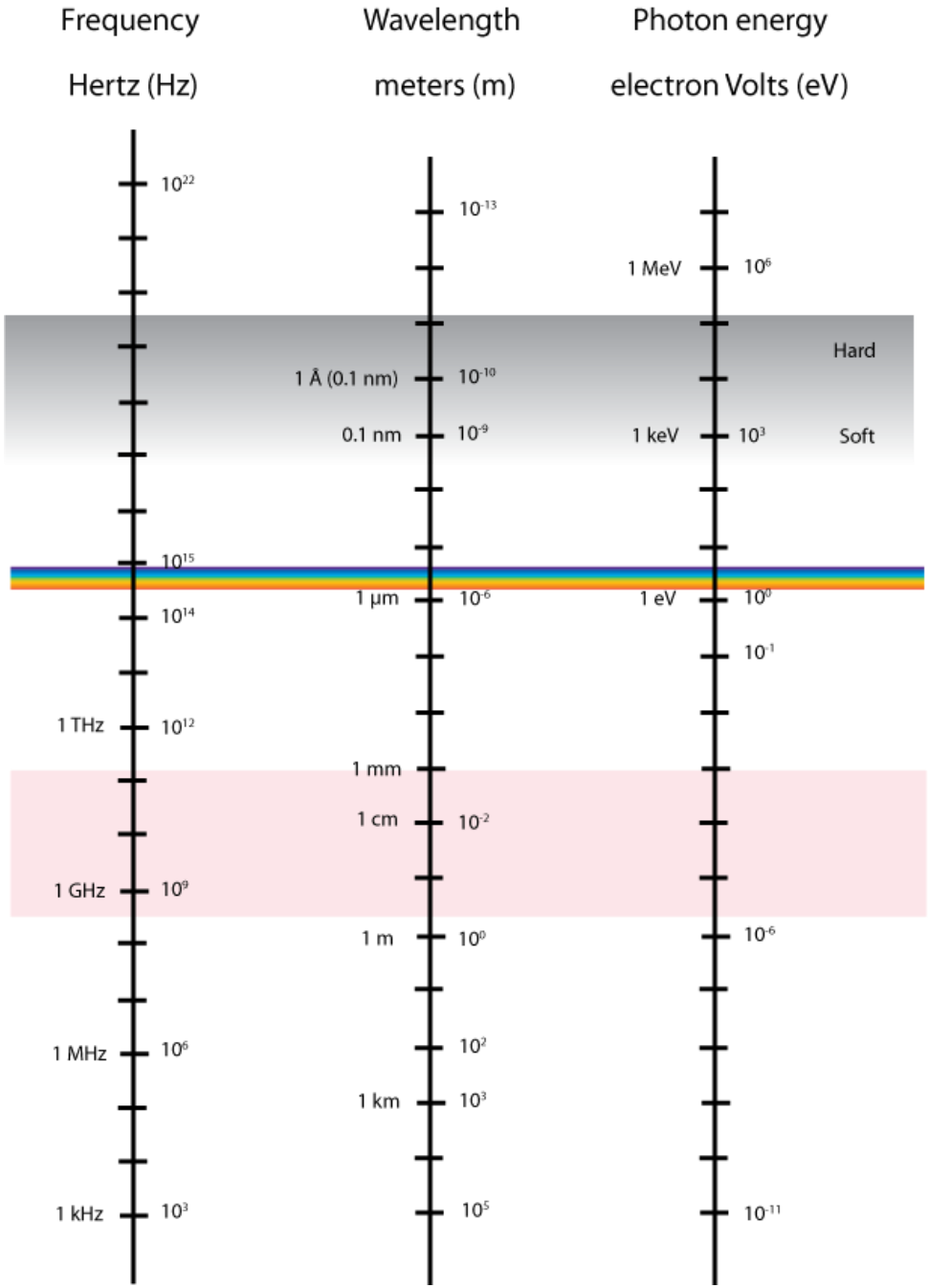
Tiny crystals become useable

Microscope resolving power

$$R = \frac{0.61\lambda}{NA}$$

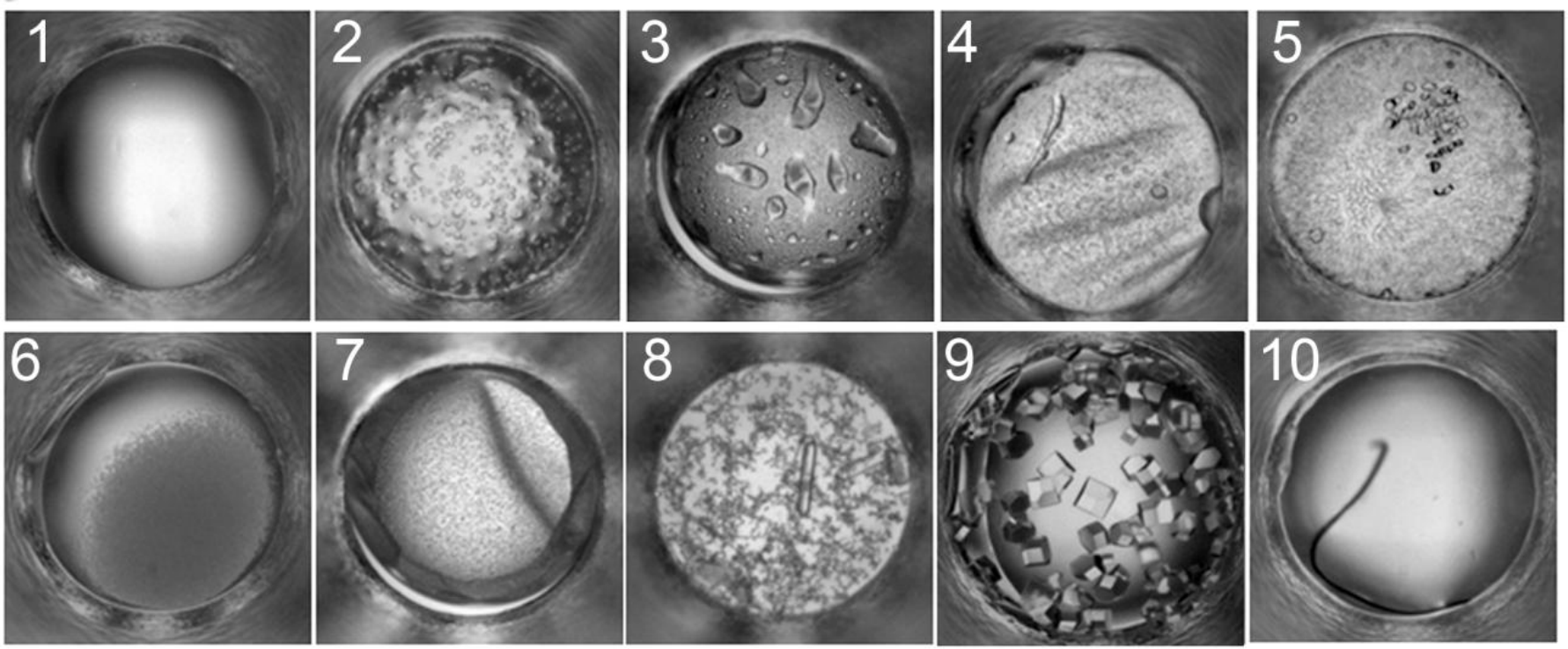
Where NA is the numerical aperture (typically a maximum of 0.95 in air for visible light) and λ is the wavelength.

Using this, the effective maximum resolution of an optical microscope *i.e.* the ability to distinguish two objects, is ~250 nm at the violet end of the visible spectrum, in practice it is much larger than this, on the order of microns.


Frequency
Hertz (Hz)
Wavelength
meters (m)
Photon energy
electron Volts (eV)
Name
Size
10^{22}
10^{-13}
1 MeV
10^{6}
Gamma rays
Atomic nuclei
Hard
1 Å (0.1 nm)
10^{-10}
X-rays
0.1 nm
10^{-9}
1 keV
10^{3}
Soft
Atoms
Proteins
Ultraviolet
Viruses
10^{15}
Visible light
E. coli
1 µm
10^{-6}
1 eV
10^{0}
10^{14}
Cell - basal cell
10^{-1}
Infrared
Pollen
1 THz
10^{12}
Tissue - Epidermis
1 mm
Organ - Skin
1 cm
10^{-2}
Microwaves
Insects
1 GHz
10^{9}
1 m
10^{0}
10^{-6}
Organism - Humans
Radio
1 MHz
10^{6}
10^{2}
Buildings
1 km
10^{3}
1 kHz
10^{3}
10^{5}
10^{-11}

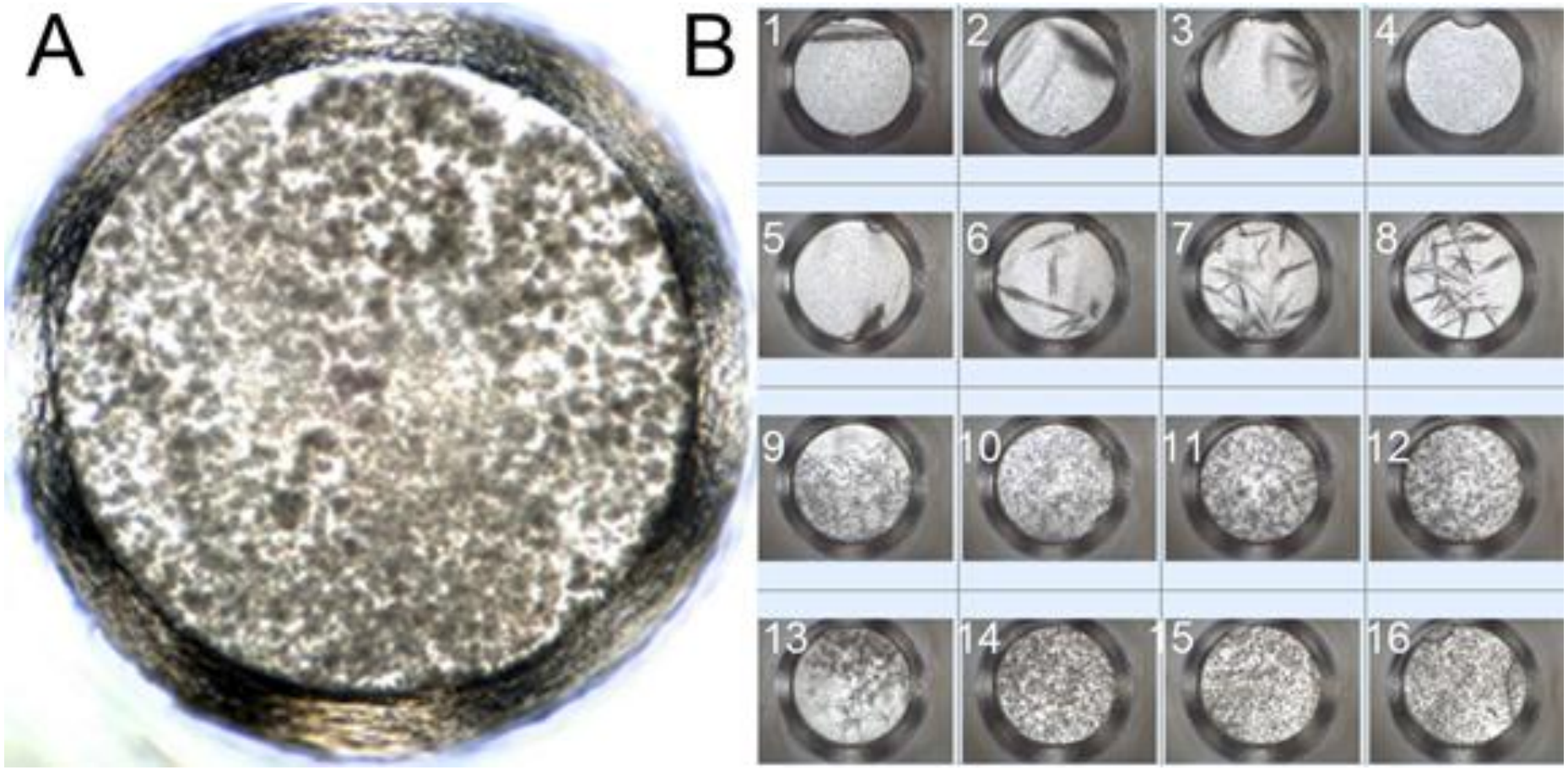
Monitoring Crystallization

Outcomes



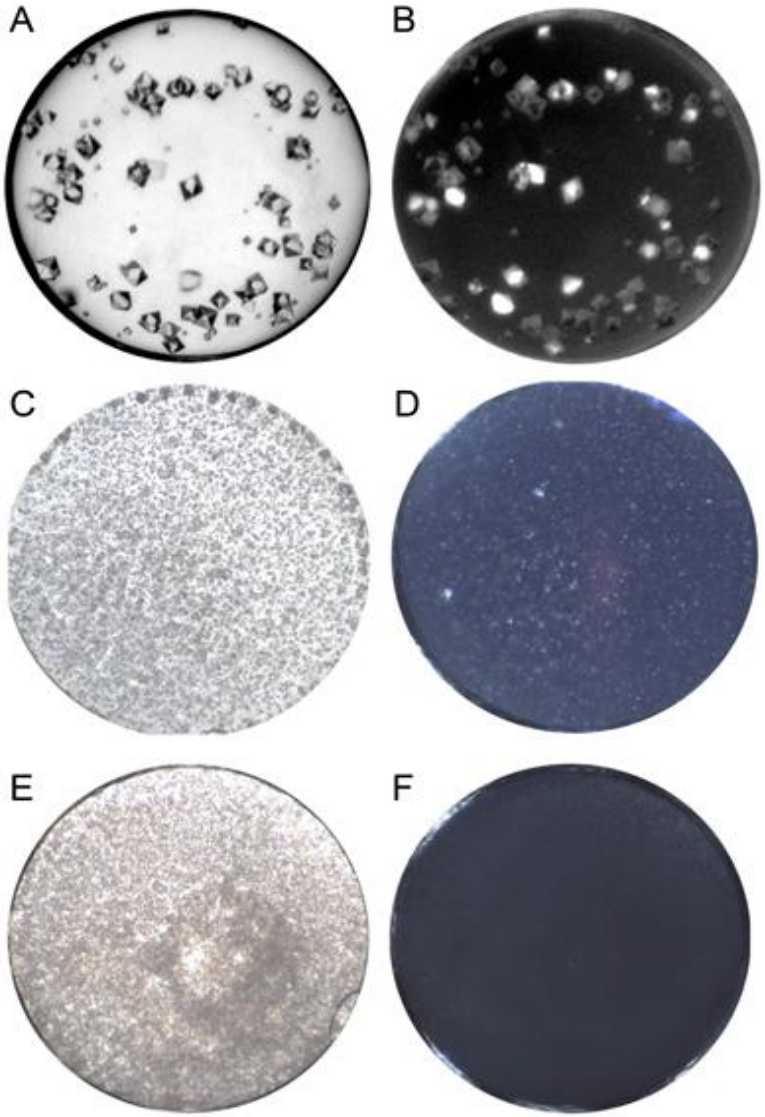
The 10 most common outcomes are shown with 1 being clear, 2 showing phase separation, 3 with phase separation and precipitate, 4 with phase separation and skin, 5 with phase separation and crystals, 6 with precipitate, 7 with precipitate and skin, 8 with precipitate and crystal, 9 with a crystal and 10 for anything that is undefined or possible contamination

A non-crystal result can lead to a crystal



Optical microscopy

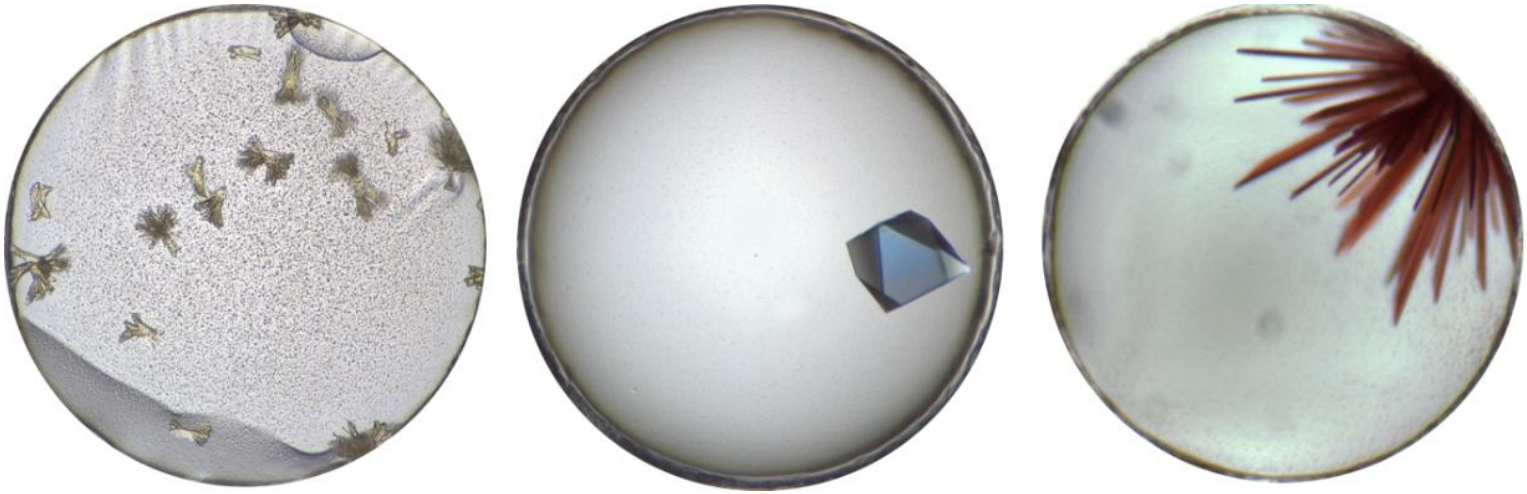
- Typically visible microscopy or digital images are characterized by human observation, The fewer the number of possible annotations, the more likely to be consistent.
- “Crystal” or “not crystal” would appear to be the minimum requirement and can be a successful strategy in many cases, but even this is non-trivial.
- other outcomes can guide optimization. microcrystalline.
- Visible microscopy can be used repeatedly to follow the course of crystallization and identify changes over time manually or with more sophisticated imaging systems,
- Given enough sample, time, careful experimental design, and consistent observation, a phase diagram of the sample can be constructed allowing rational crystallization from microscopic studies alone.



Use of polarization

- Many crystals can be birefringent.
- A crystalline as opposed to amorphous precipitate can be identified by the birefringent properties, a soft glow as the analyzer is rotated.
- Unfortunately, this property is linked to both salt and macromolecular crystals and is not present in cubic systems.
- The disadvantages of missing a small number of cases or a false positive is outweighed by the advantage, in that once a good microscope is available, the addition of a polarizer and analyser is a fraction of the initial investment.

Importance of color



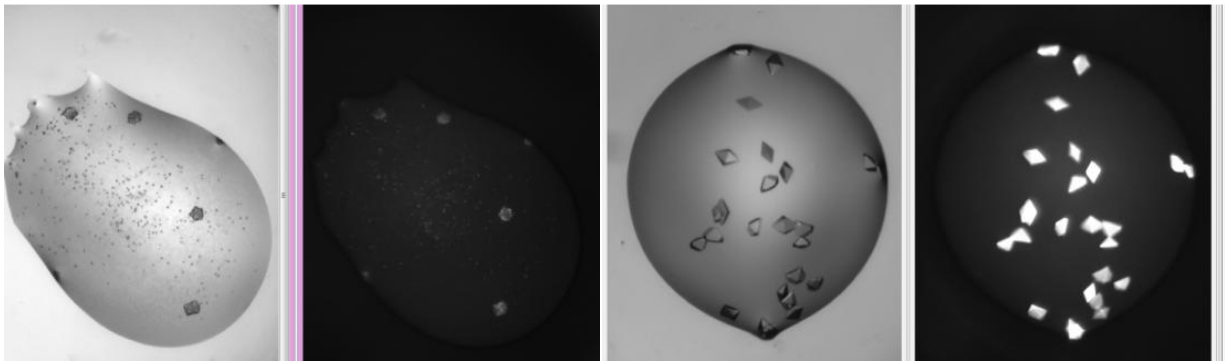
- The number of colored proteins is small but color can indicate the presence of metal ions, or other ligands bound to the protein.
- It can also, through the oxidation state of the metal, inform the crystallographer on the functional state of the protein under particular chemical conditions.
- In the case of a colored protein, the color can sometimes help to distinguish microcrystalline precipitates from denatured precipitates.

Use of other wavelengths (UV)

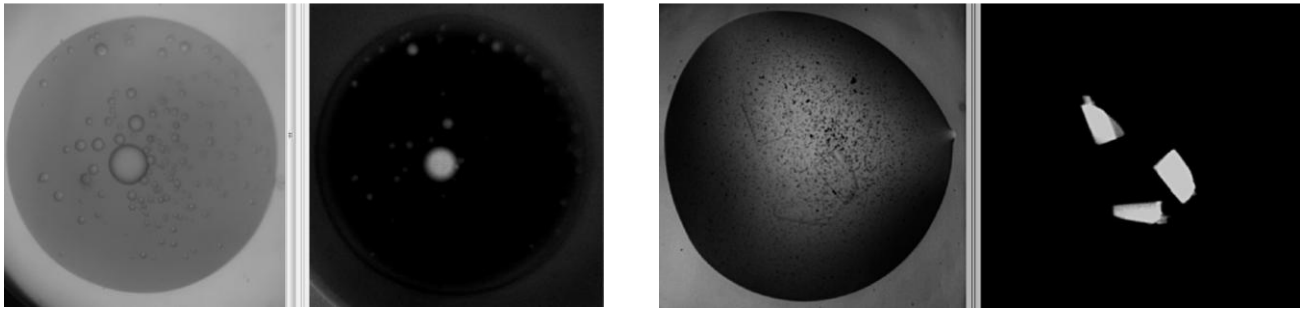
- The amino acid tryptophan contained in many proteins absorbs ultraviolet light in the range 260-320 nm, and fluoresces from approximately 300 to 450 nm, with peak emission at 340-360 nm.
- Protein crystals containing tryptophan can be identified in the presence of precipitate and distinguished from salt crystals.
- The technique relies on the assumption that the local concentration of protein is greatest in a crystal and if tryptophan fluorescence is imaged, crystals should glow more strongly than the background
- However, the signal can be quenched from other structural features, e.g. the presence of a haem group in the protein, or of some metal centers
- Tryptophan fluorescence is highly sensitive to the local environment of the tryptophan side chain, so that proteins of similar size and containing the same number of tryptophans may respond quite differently to UV light.

Use of other wavelengths (UV)

- Salt crystals may emulate fluorescing protein crystals if protein adheres to the surface.
- The crystallization cocktail itself can influence the success of the technique; e.g. the presence of the nitrate ion completely quenches the fluorescence from tryptophan.
- Tryptophan makes up an average of 1.09% of the residues in proteins but there are macromolecues that do not contain this amino acid. Fluorescent dyes can be added by covalently modifying the macromolecule or the addition of non-specific dyes during the crystallization process allowing detection of crystals as small as 1 μm in dimension

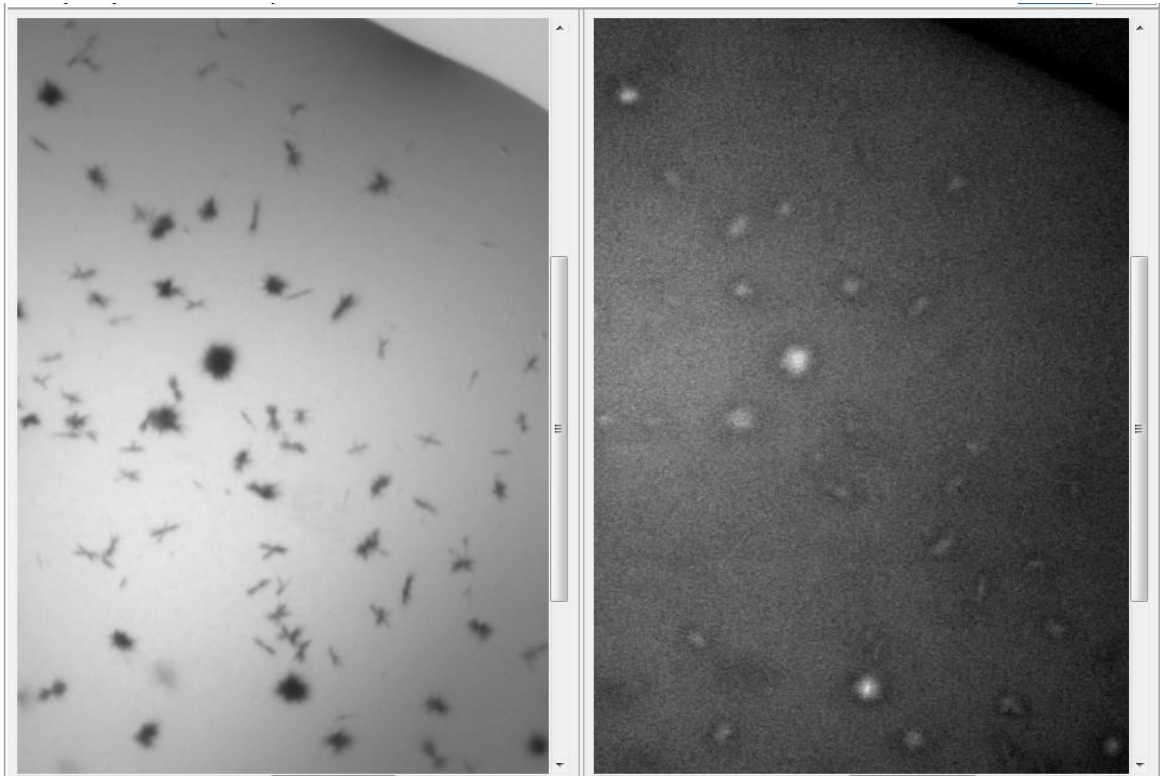


(a) (b)



(c) (d)

Examples of UV imaging outcomes with the associated brightfield (visible) image: (a) the good, protein crystals fluorescing strongly (Proteinase K) also easily identified visually, (b) the bad, salt crystals (calcium sulfate) with adsorbed protein, (c) the ugly, non-crystal information showing phase separation (also a potential lead condition for optimization) in a myoglobin containing trial – myoglobin is excluded from one phase and (d) the beautiful, an example of the identification of protein crystals that may have been easily missed visually.



Despeckling (median filtering) applied by default to UV images can remove the signal from small protein crystals. During the process of median filtering, a pixel's value is replaced by the median value of the pixels around it. This image was taken at a magnification such that one pixel is approximately 1 µm, so that the very thin needles are approximately 1-2 µm wide, and thus get removed during the median filtering step.

Other optical techniques

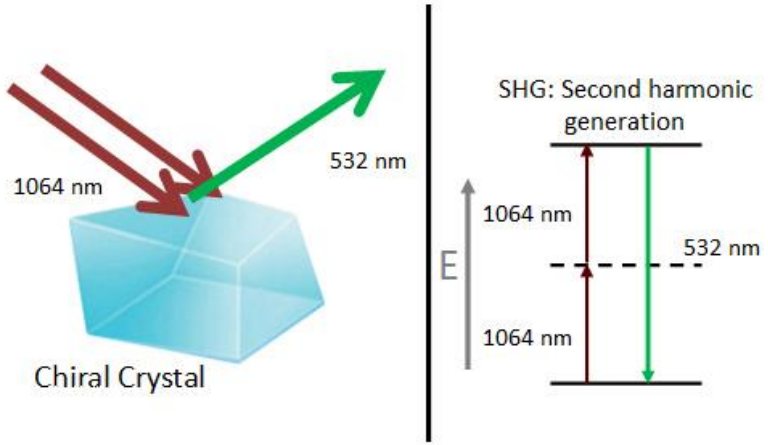


Figure 1. Two photons of IR (1064 nm) interact with a chiral crystal to generate SHG (532 nm).

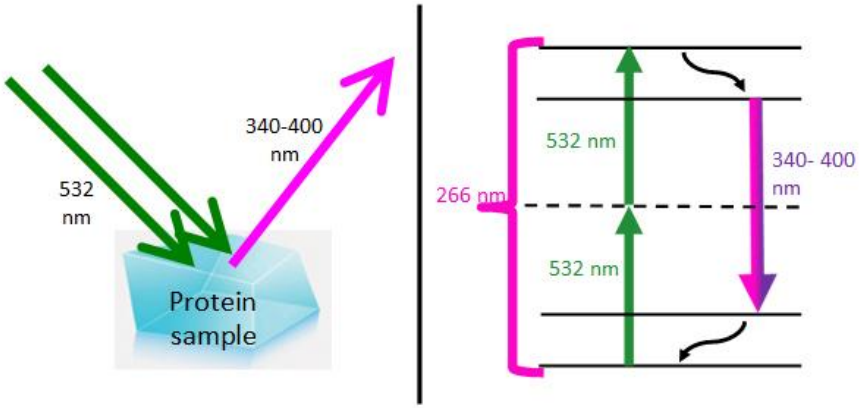


Figure 2. Depiction of UV-TPEF where two photons of green interact with a protein sample to generate UV excited fluorescence

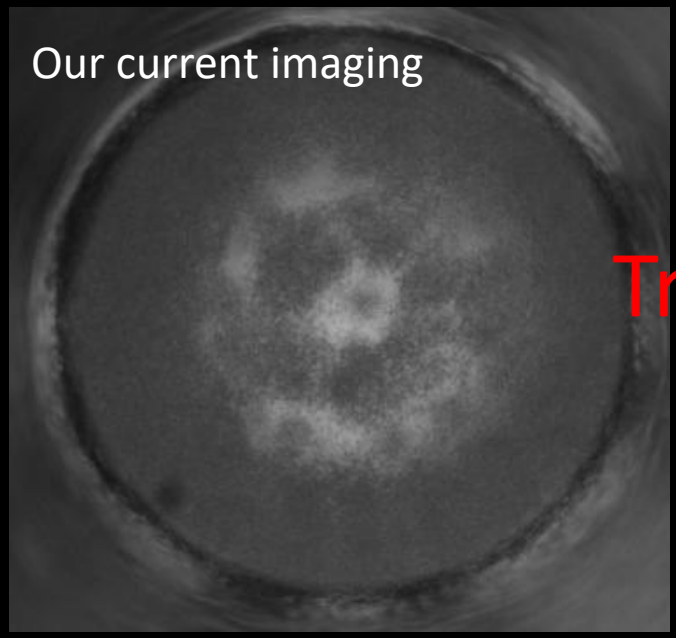
- Using SONICC and UV-TPEF we can observe and verify protein crystals < 1 micron in size.
- ~80% of proteins in PDB low-symmetry generate SHG

http://www.formulatrix.com/products/protein-crystallography-tools/sonicc/how.html

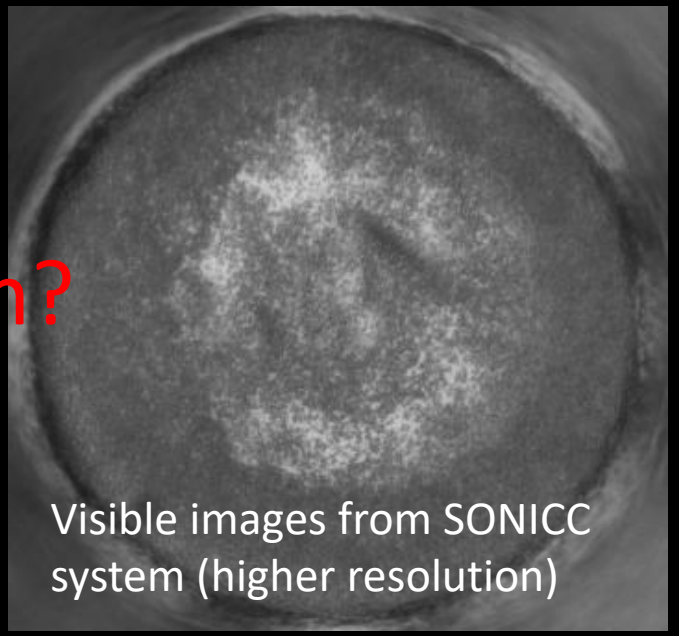
One protein tested

Protein 1, part of the pyruvate dehydrogenase protein complex

Hampton Research PEGRx HT-F4, 4% (v/v) 2-Methyl-2,4 pentanediol, 0.1 M Citric Acid pH=3.5 20% (w/v) PEG 1500 produced the following:



Try again?

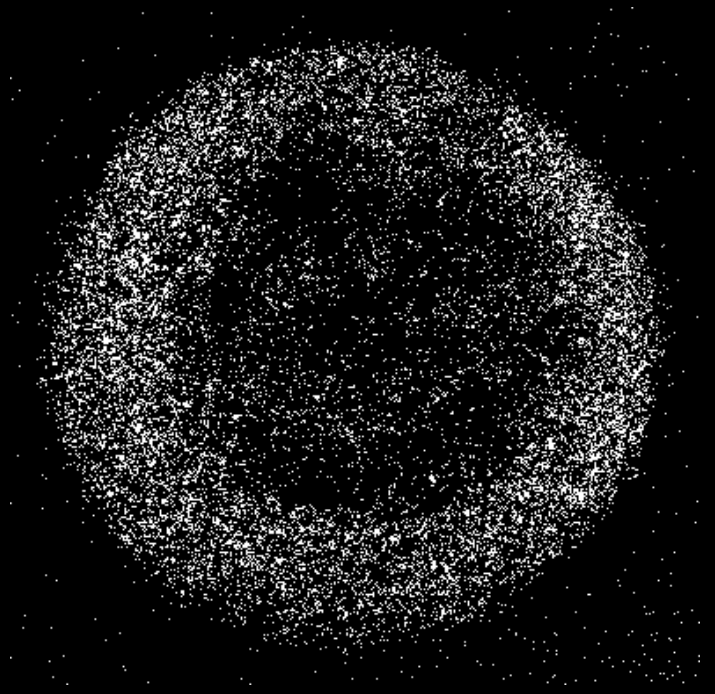


Immediately after the protein is added to the cocktail

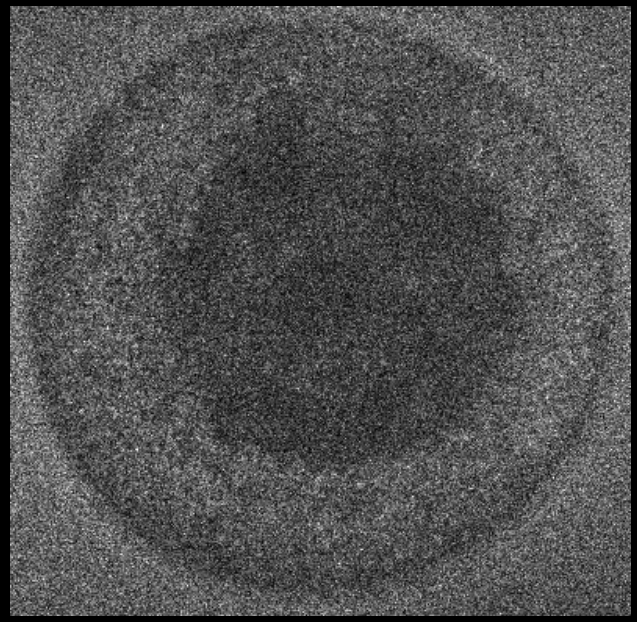
Initial use of SONICC and UV imaging

Protein 1, part of the pyruvate dehydrogenase protein complex

Hampton Research PEGRx HT-F4, 4% (v/v) 2-Methyl-2,4 pentanediol, 0.1 M Citric Acid pH=3.5 20% (w/v) PEG 1500 produced the following:



SONICC SHG image



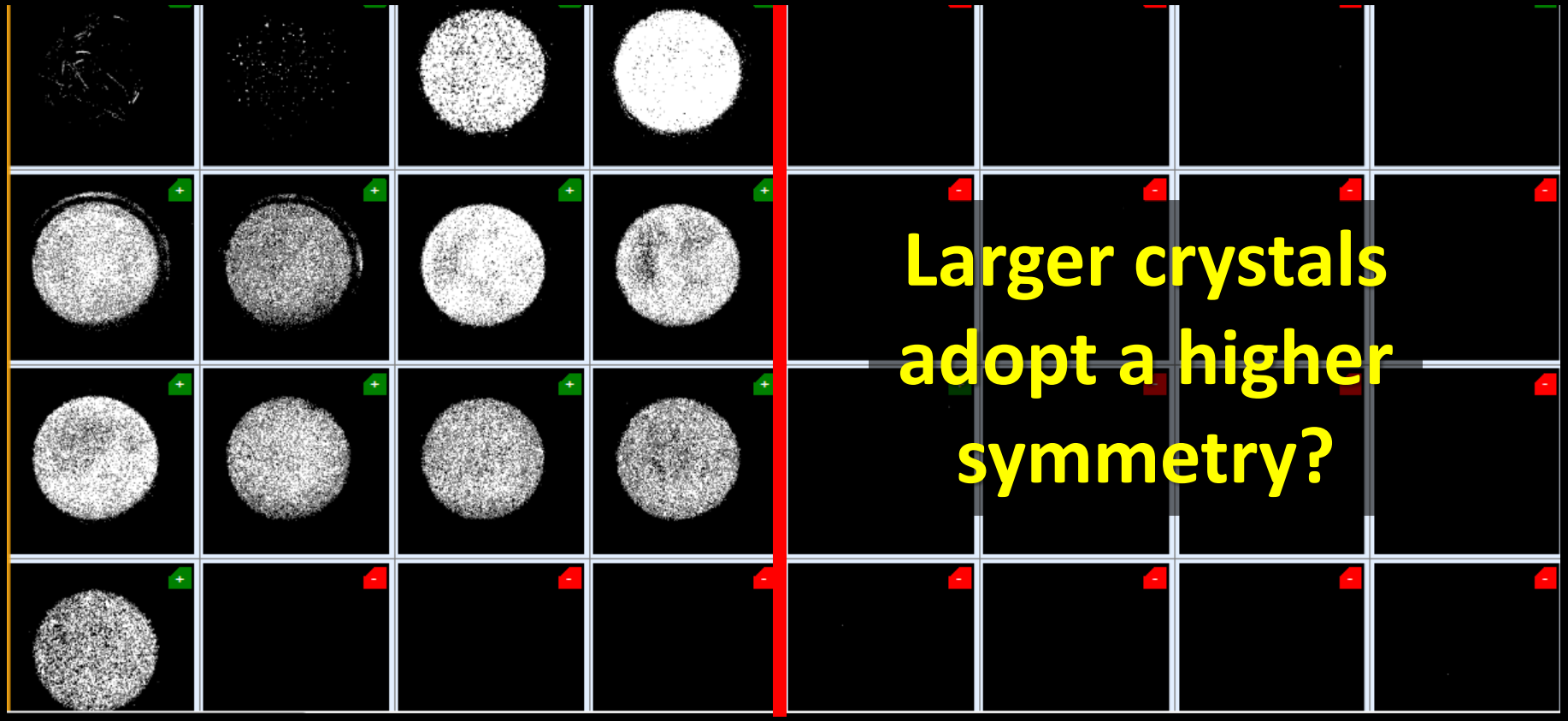
UV-TPEF image

Protein 1, part of the pyruvate dehydrogenase protein complex

Hampton Research PEGRx HT-F4, 4% (v/v) 2-Methyl-2,4 pentanediol, 0.1 M Citric Acid pH=3.5 20% (w/v) PEG 1500

X14163- Full[P]- 10mg/ml

X14163- [P]/2- 5mg/ml



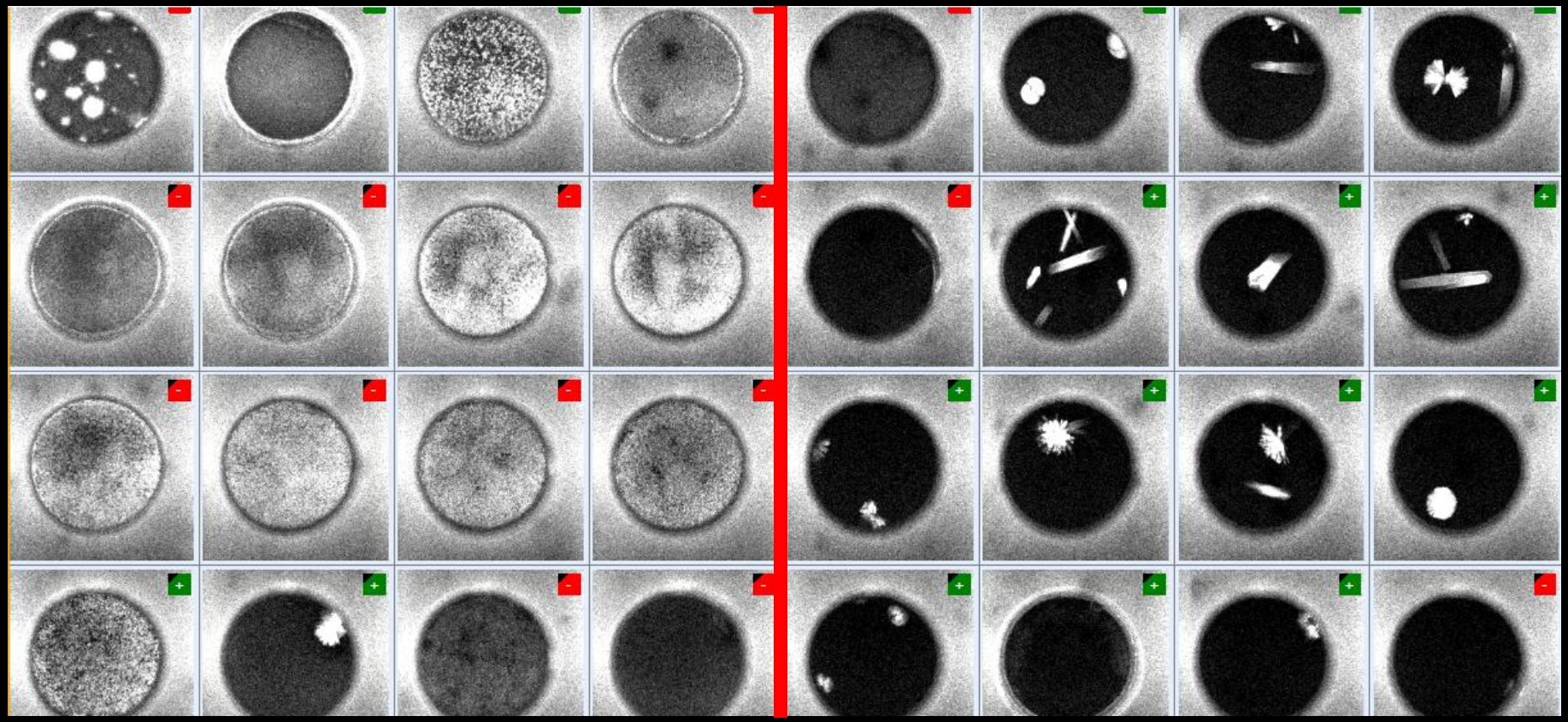
SHG at 4wk

Protein 1, part of the pyruvate dehydrogenase protein complex

Hampton Research PEGRx HT-F4, 4% (v/v) 2-Methyl-2,4 pentanediol, 0.1 M Citric Acid pH=3.5 20% (w/v) PEG 1500

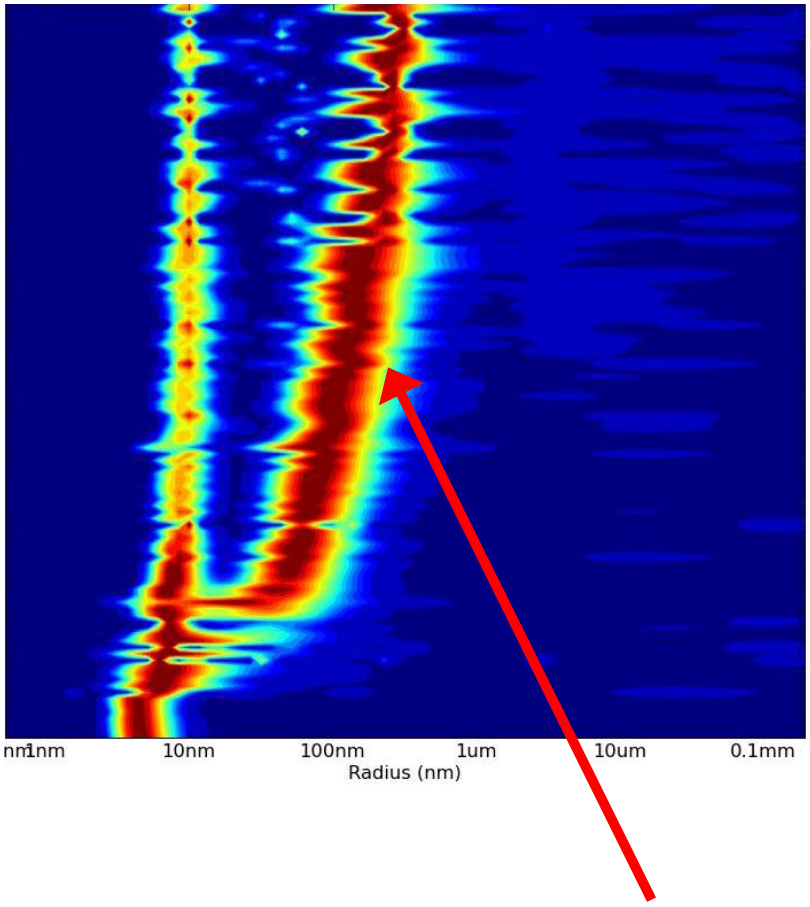
X14163- Full[P]- 10mg/ml

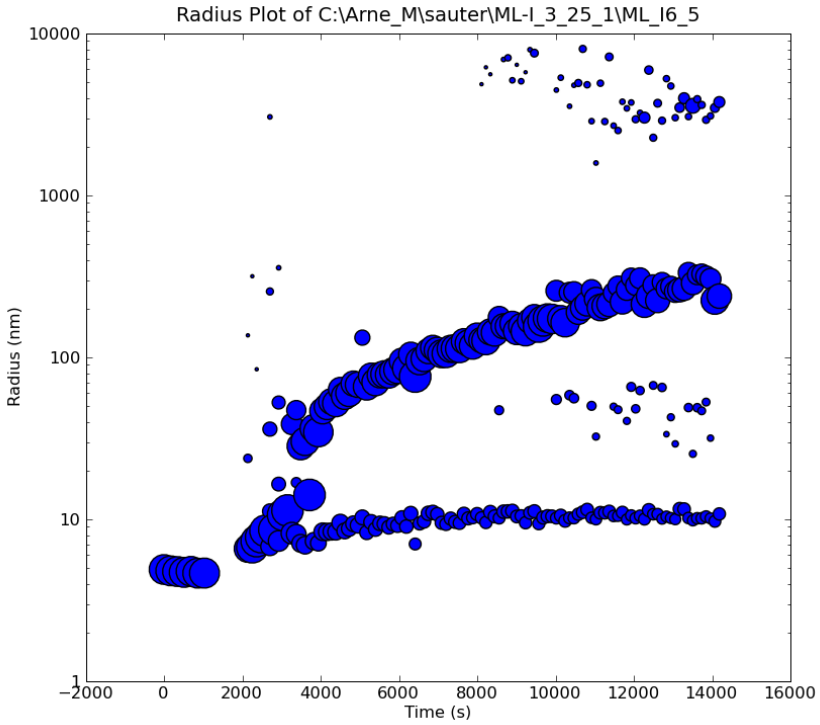
X14163- [P]/2- 5mg/ml



UV-TPEF at 4wk

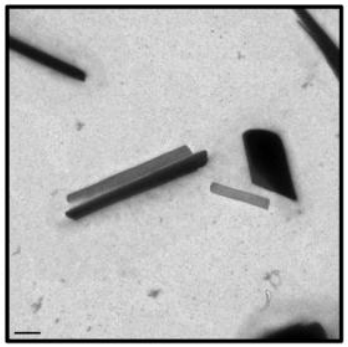
Other techniques (Light scattering)



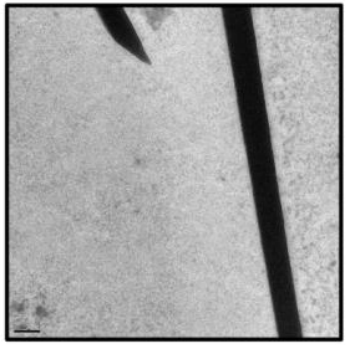


Signature of defined size aggregates forming

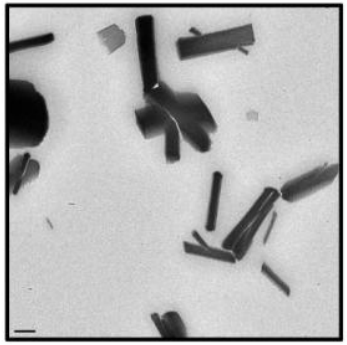
Other techniques (Electron microscopy)



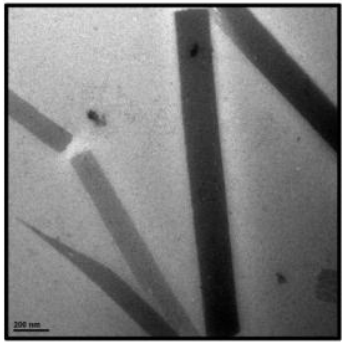
CD3Delta



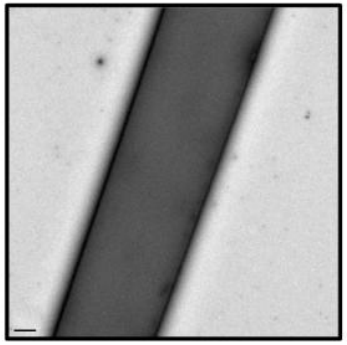
tPTHR



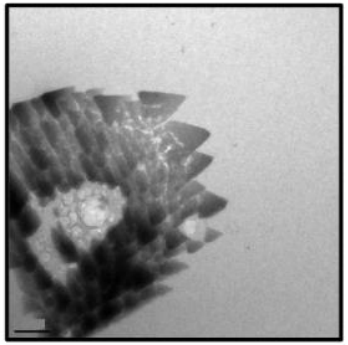
H5N1



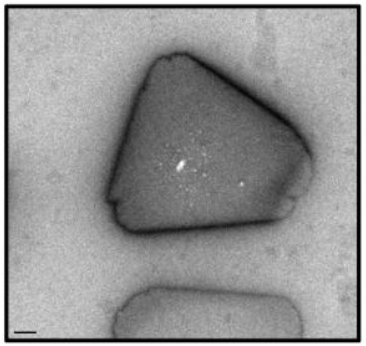
TFIIH



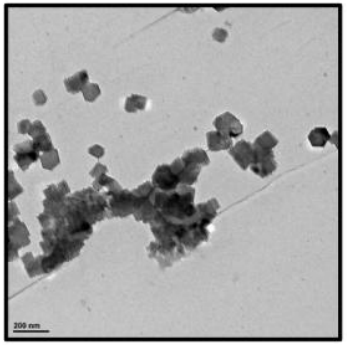
DSZS AT



Spt4/5



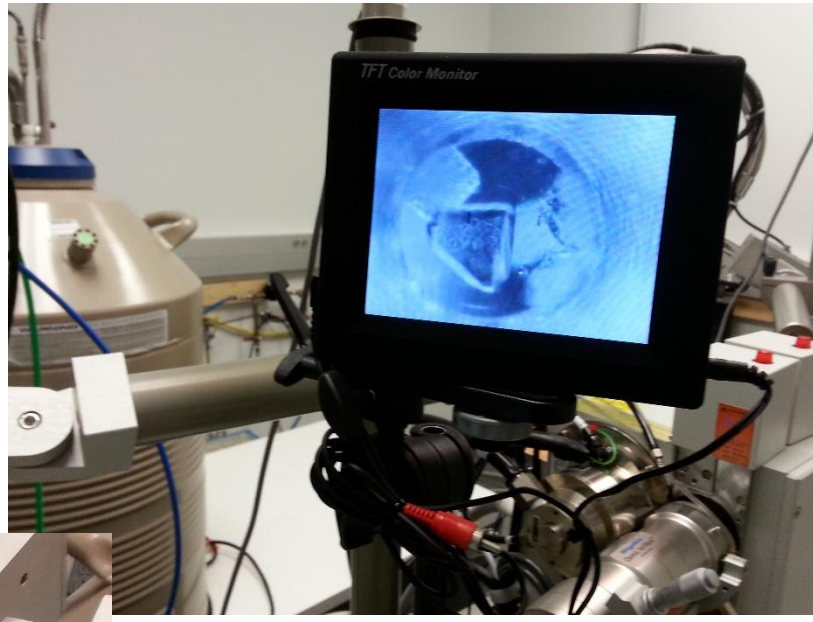
RNA-Pol II

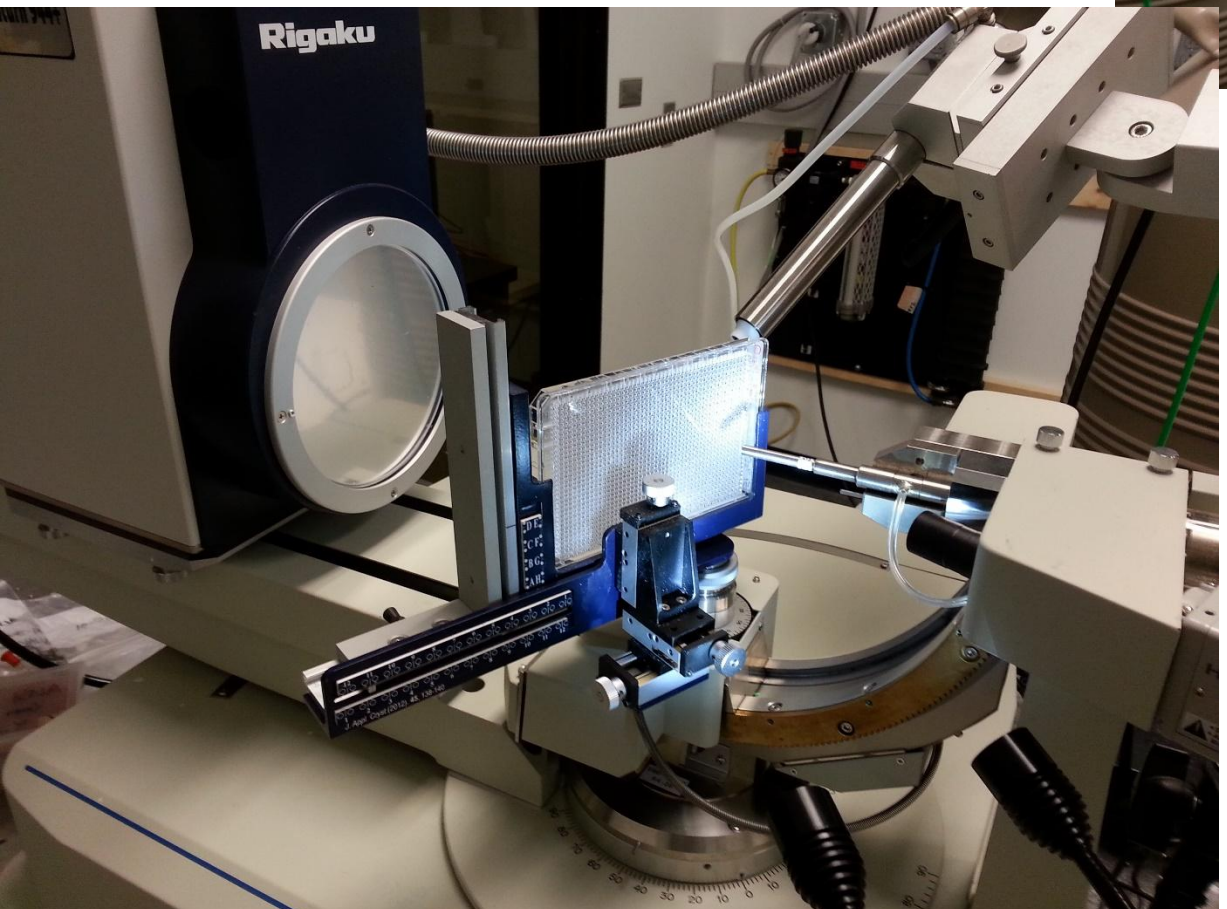


RNA-Pol II-
TFIIB-Spt4/5

X-ray analysis in the laboratory

Crystallization plate considerations





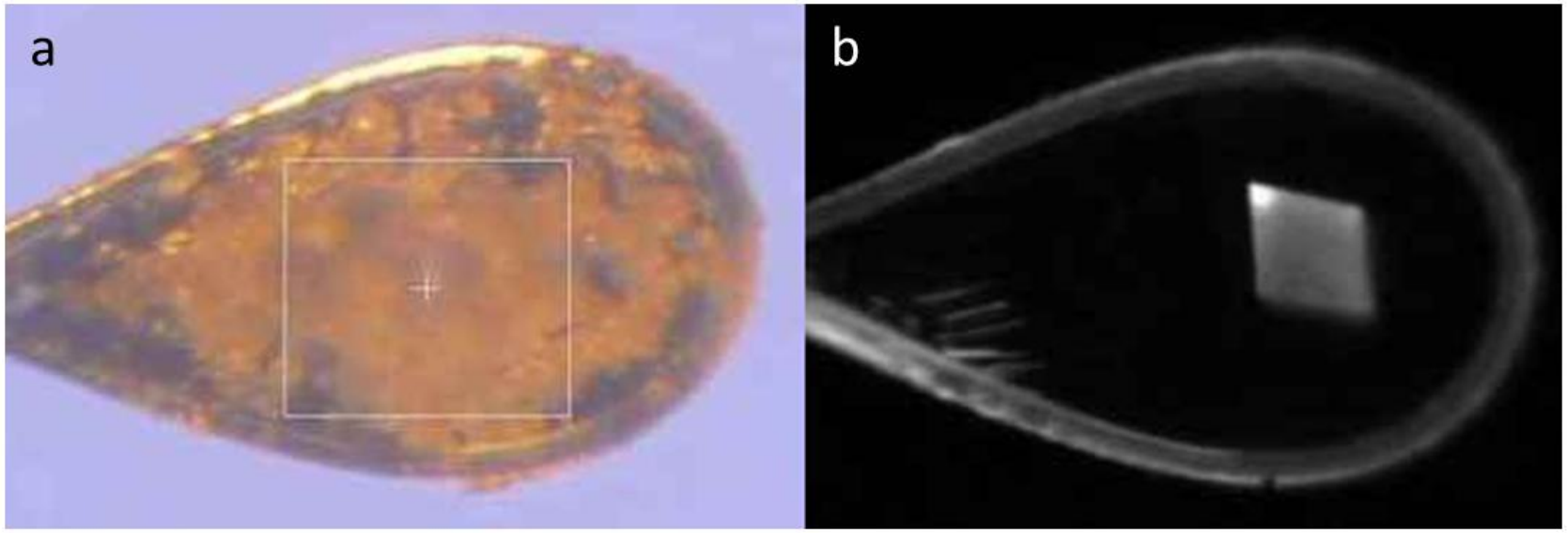
Can even be done as a powder experiment in house.

Summary

Technique	Spatial resolution achieved	Time	Expense	Outcome
Eye	~500 µm	Rapid	None	Identification of presence and position of large crystals. Measurement of number and size.
Visual microscopy	~2 µm	Rapid	Medium.	Identification of presence and position of large and small crystals. Measurement of number and size
UV microscopy	~2 µm	Medium	Medium	Identification of presence and position of large and small crystals. Measurement of number and size. Characterization between biological and salt in many cases.
Light scattering	few nm	Medium	Medium	Characterization of size distribution. No number or positional information.
SONICC	~0.2 µm	Medium	High	Identification of presence and position of large to nano crystals. Measurement of number and size. With TP-UV, Characterization between biological and salt.
Electron microscopy	50-100 Å (Individual proteins)-5-10 µm	Long	Very high	Identification of presence and position of large to nano crystals. Measurement of number and size. With TP-UV, Characterization between biological and salt. Characterization of diffraction quality.

Observation for data collection

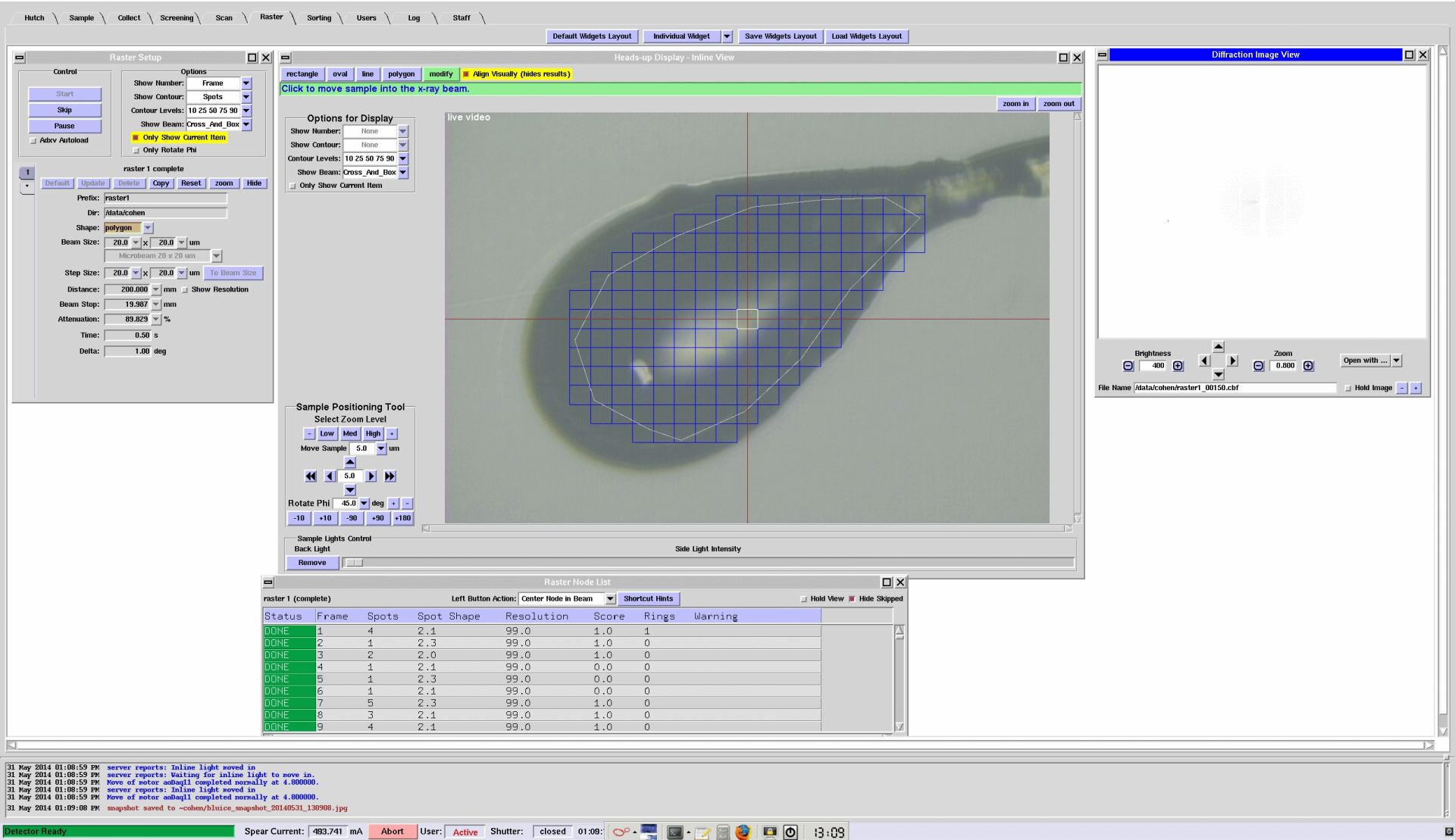
Optical methods



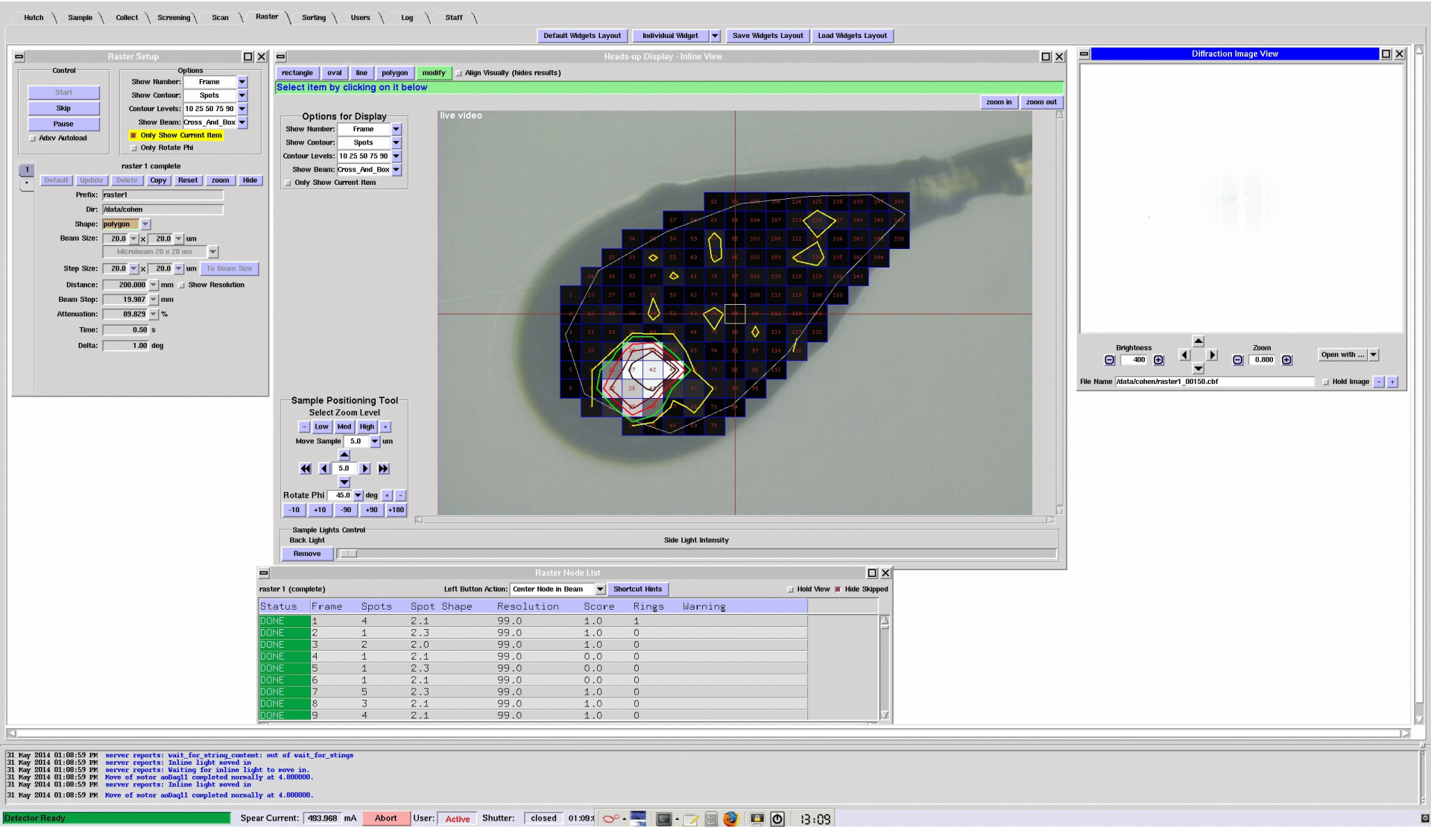
A cryogenically cooled crystal within a nylon loop is obscured by surrounding material when imaged using visible light microscopy (a). The same protein crystal may be clearly observed using visible light emission (b).

Single photon excited visible light emission has been in use at SSRL BL7-1 since 2012 to aid in visualizing colorless crystals inside loops or on meshes, and has been incorporated into the ‘click-to-center’ video display of the beam line control software. Protein crystal excitation is in the near UV and emission at wavelengths longer than 430 nm is collected.

Optical based techniques



X-ray based techniques



Summary

Technique	Spatial resolution achieved	Time	Expense	Outcome
Optical microscopy	~2 µm	Rapid	Medium	For visible crystals the crystal can be centred accurately.
UV fluorescence	~2 µm	Rapid	Medium.	If the crystal fluoresces it can be centered accurately.
Visible two photon flourescence	~2 µm			Similar to UV
SONICC	~2 µm	Medium	Very High	Similar to UV
X-ray rastering	Sensitive to the diffraction properties despite the size	Short	High	Not only can the crystal be centered but the spatial diffraction properties can be characterized.

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Identifying, studying and making good use of macromolecular crystals

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Structural biology has contributed tremendous knowledge to the understanding of life on the molecular scale. The Protein Data Bank, a depository of this structural knowledge, currently contains over 100 000 protein structures, with the majority stemming from X-ray crystallography. As the name might suggest, crystallography requires crystals. As detectors become more sensitive and X-ray sources more intense, the notion of a crystal is gradually changing from one large enough to embellish expensive jewellery to objects that have external dimensions of the order of the wavelength of visible light. Identifying these crystals is a prerequisite to their study. This paper discusses developments in identifying these crystals during crystallization screening and distinguishing them from other potential outcomes. The practical aspects of ensuring that once a crystal is identified it can then be positioned in the X-ray beam for data collection are also addressed.

1. Introduction

The relationship between the intensity of the X-ray data produced and the volume of the crystal was originally captured by Darwin's formula (Darwin, 1922),

$$I(hkl) = I_0 r_e^2 \frac{V_{\rm crystal}}{V_{\rm cell}} \frac{\lambda^3 L}{\omega V_{\rm cell}} PA|F(hkl)|^2, \qquad (1)$$

where $I(hkl)$ is the intensity of a fully recorded reflection, I_0 is the intensity of the incident beam, r_e is the classical electron radius, $V_{\rm crystal}$ is the illuminated crystal volume, $V_{\rm cell}$ is the unit-cell volume,