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Crystallization Screening using Trace Fluorescent Labeling

Or

How Using Fluorescence can be a Brilliant Answer to YOUR Crystallization Problem.

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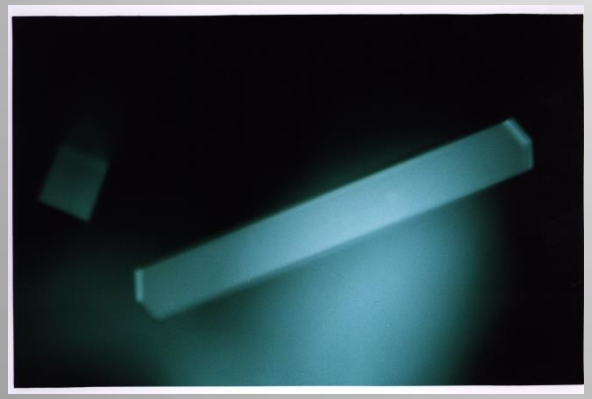
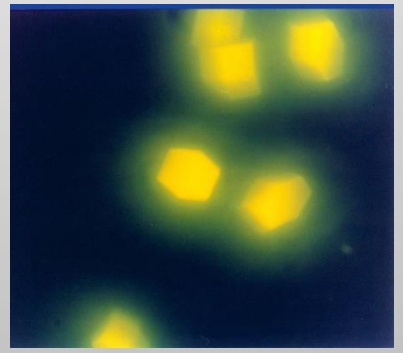
Even Heavily Labeled Proteins Crystallize

Original impetus was to produce FRET pairs for use in studying the lysozyme crystal nucleation process. Lysozyme was site-specifically labeled on asp-101, then Cation-exchange purified to remove labeled from unlabeled protein. Crystallization experiments were carried out to determine if the labeled proteins would indeed crystallize.



Cascade blue-lys
Ex = 396 nm, Em = 410 nm



Lucifer yellow – lys
Ex = 428 nm, Em = 536 nm

Texas red - UCAN

The proteins in the above images were > 50% (cascade blue and lucifer yellow-lys to > 90%) labeled with the indicated fluorescent probe. This gave an early indication that protein could be labeled and would still crystallize. The fluorescent microscope was set up for green fluorescent protein.

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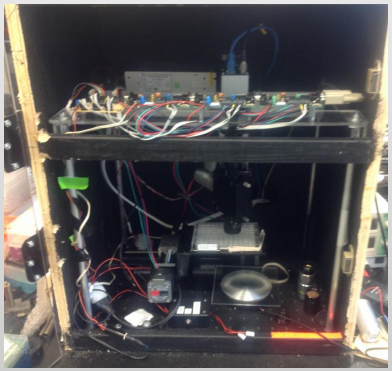
Evolution of the Trace Fluorescence Idea - Implementation

Original work used an Olympus Stereo Fluorescence microscope. Had to manually grab and save each image. Supported by NIH R21 grant while I was at NASA.



Frustration led to development of an in-house assembled scanning microscope, that imaged every well and saved them to disk. Supported by NIH Phase I STTR.

The in-house design has been developed by a local design engineering company to a more presentable product. Currently supports 2 fluorescent and white light imaging, or 3 fluorescent and no white light imaging. ~15 minutes to scan a 96 well plate, 3 drops/well, single wavelength.

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Fluorescence Methods

Different Approaches

Native tryptophan fluorescence

Positives – protein is 'untouched'

Negatives – requires UV optics, illumination, therefore much higher cost

- cannot use a 'hands (eyes) on' approach.
- not all proteins have a tryptophan.

Adding free probe to crystallizing solution

Positives – Easy to implement

- no covalent modification needed

Negatives – possible uncertainty about where probe is partitioning

- cannot be used with IMPs
- less sensitive

Covalent modification of protein with probe

Positives – Certainty about following the protein

Negatives – Most work to use

- Worries about effects on nucleation, crystal quality, inhibit use.

Other benefits found using the covalent modification approach
That may or may not be found with the other two.

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Trace Fluorescent Labeling

The methodology to be described is TRACE fluorescent labeling, where on average **ONLY ~ 0.1 – 0.2 %** of the protein molecules have a fluorescent probe covalently attached. This is 1 or 2 molecules per thousand being modified. IF your protein was 100% pure (it almost certainly isn't) then this would drop the "purity" to 99.9 or 99.8 %.

The basis of the technique is:

Fluorescence intensity is proportional to fluor concentration.
The highest solute concentration is in the crystalline state.
Therefore crystals are brighter objects.
This gives the guiding rule ➔ **INTENSITY = STRUCTURE**

Note the emphasis on TRACE, meaning < 0.5% (and preferably Lower. NOT 1%, 5%, 10%, 50%, or 100%, but < 0.5%!!

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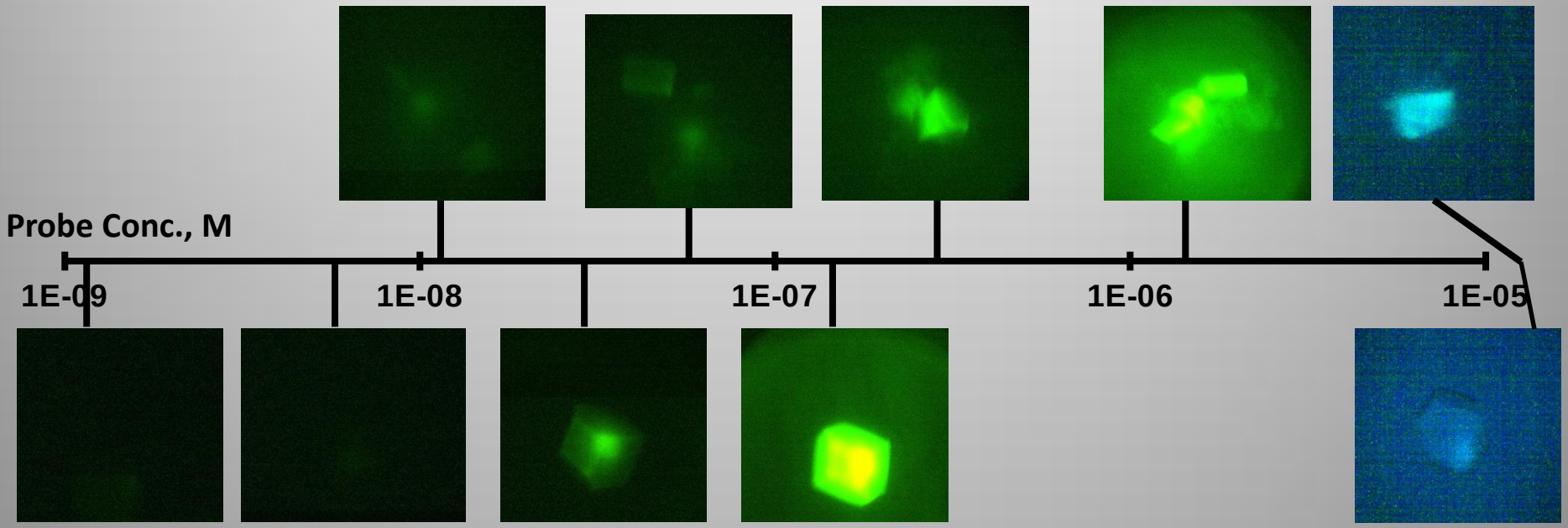
Sensitivity – How Low?

Glucose Isomerase and Xylanase were derivatized with CR, then dilutions made in 1/5x steps and crystallization plates set up. For a 2nd level of comparison, Crystals were also set up using $2x10^{-5}$M ANS (8-Anilino-1-napthalenesulfonic acid) using the IZIT approach.



The intensity of the excitation source also affects the lower detection limit. The above images were collected using a single LED for the source illumination. By now using a higher powered LED source I estimate the sensitivity can be adjusted ~ an order of magnitude lower. A more sensitive camera would lower this still more.

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Trace Labeling is Simple and Quick

A procedure to quickly prepare trace fluorescently labeled protein solution.

Typically takes 5 to 30 minutes and Is found to yield ~0.2 % derivatized protein.

Start with -
0.8 mL of protein @ 125% desired final conc.

Remove aliquot of 0.08 mL, buffer exchange if necessary ~2 min.

Add 0.5-2.0 µL of fluorescent probe solution (more with higher protein MWs)

Incubate while reequilibraing the desalting column (if using a single desalting column) ~10 min.

Buffer exchange (to stock solution buffer) ~2 min.

Add back to the Starting solution, Bring volume to 1.0 ml

DONE!!

This procedure can be scaled up or down as needed. To reduce the amount of protein the reactive probe solution should be diluted. Smaller centrifugal desalting columns are available.

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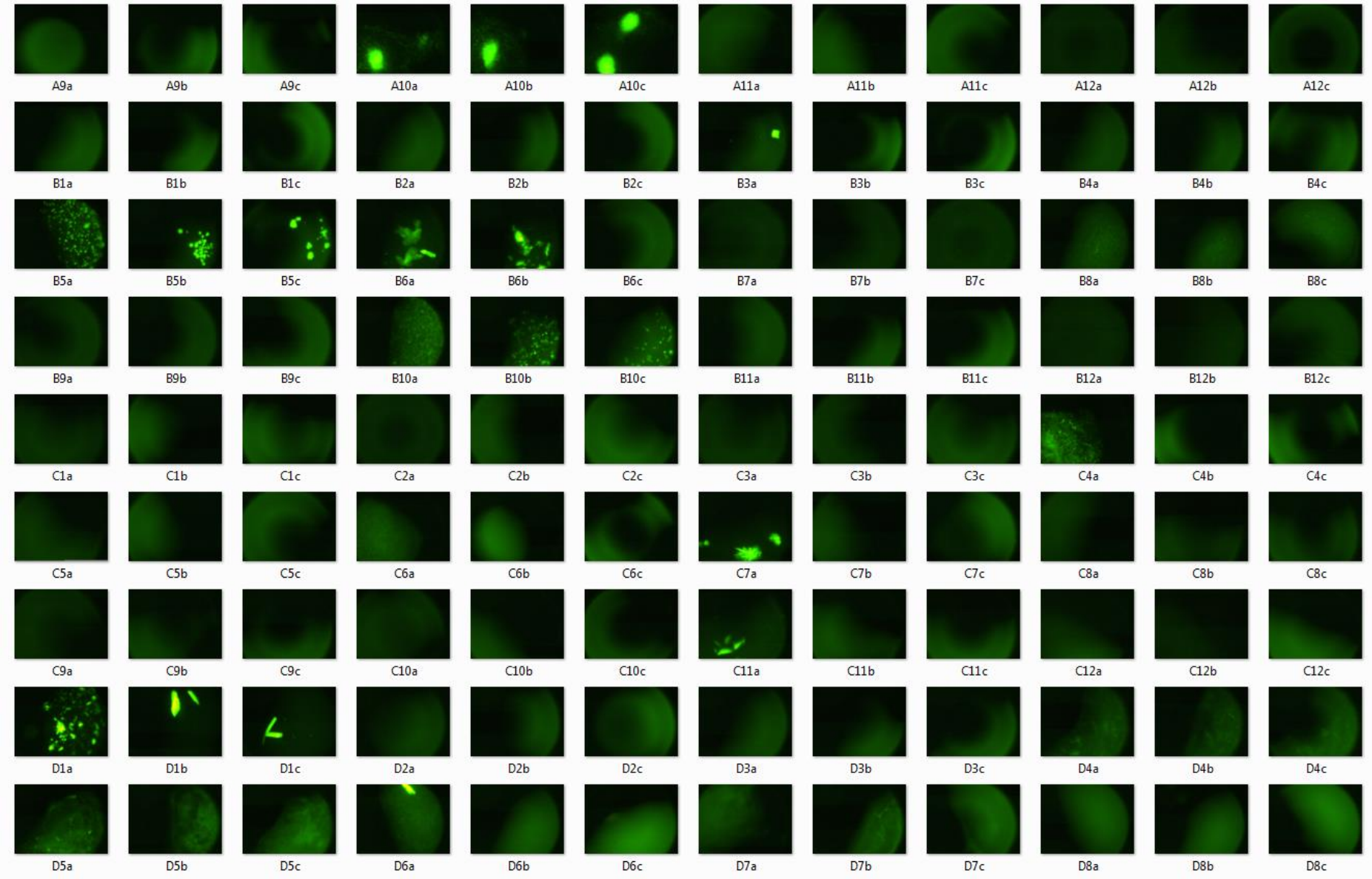
WHY DO IT?

If intensity is proportional to structure – find the crystals & conditions of interest below.



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The BIG QUESTION

Does the presence of the fluorescent probe affect the crystallization?

The short answer is – <u>NO!</u>

(but that won't stop me from talking about it in detail for the next couple of minutes)

Experiments to again[1] determine the answer (now in progress)

Using a series of test proteins, some previously worked on, some not:

- **Using a single protein preparation –**
  - **Prepare labeled and unlabeled protein at same concentrations.**
  - **Set up all plates at the same time.**
- **Track results over time**
- **Compare outcomes, with respect to:**
  - **Same hits found?**
  - **Improvement (or not) in hits, labeled vs unlabeled?**
  - **Additional leads found (for labeled protein)?**

1. Previously shown – Forsythe, Achari, & Pusey, Acta Cryst. D62, 339-346.

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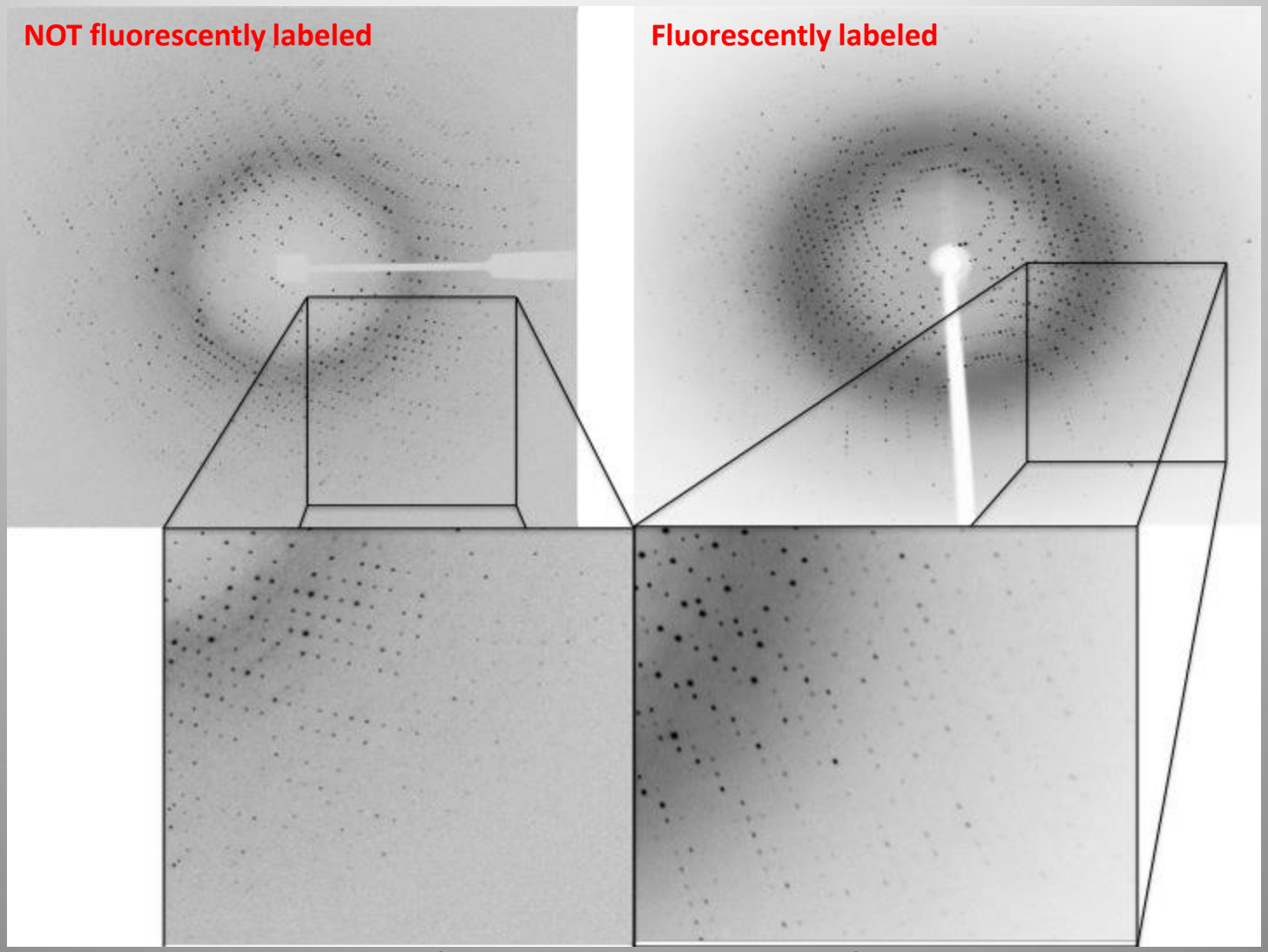
Diffraction Images – For Starters

IPPase, room temperature, home source, 10 min. exposure.

We have not found any effects on diffraction data from trace fluorescent labeling.



Crystals grown in and filled capillaries, mosaicity for both was equivalent

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Ongoing Experiment Design/Plan

Test at least 30 different proteins – more if possible.

- Soluble proteins – as available
- Complexes – individual proteins and mixed
- IMP's

Set up 6 plates for each protein – from the same stock solution/protein preparation.

- 3 labeled as per protocol
- 3 not labeled.
- both at same final concentration.
- use Nanodrop robot to set up plates

Use ONE screen – Hampton Research High Throughput 96 condition screen.

Follow plates for at least 8 weeks, scoring as described below.

Carry out optimization on all lead conditions (score = 4, to be described below).

Compare outcomes – labeled vs not.

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Consistency Within Sets of Plates

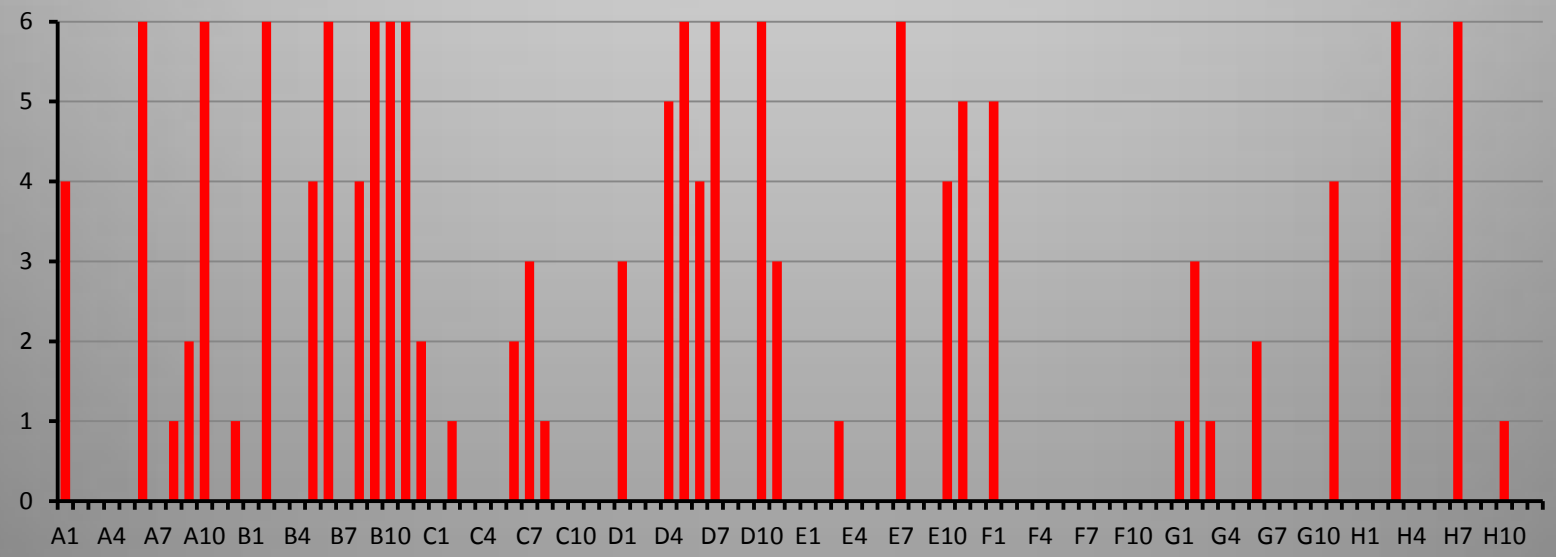
A starting impetus for these experiments came from a review of six plates of one of the proteins (Tt141). A total of 38 hits were found (spheroids ➔ 3D xtals), but not in all of the plates. These results represented at least two different protein preparations, with the plates set up at various times for each preparation.

Of the 38 total crystallization conditions found: (with still more testing, now up to ~50 conditions)

- **13 (34%) were found in all 6 plates – 1/3 of all conditions found in all 6 plates.**
- **16 (42%) were found in 5 of the plates.**
- **22 (58%) were found in 4 of the plates – 2/3 of the plates had just over ½ the conditions.**
- **26 (68%) were found in 3 of the plates – ½ of the plates had just over 2/3 of the conditions.**
- **30 (79%) were found in 2 of the plates.**
- **<u>NO ONE PLATE HAD ALL OF THE HITS FOUND!</u> Two plates had 27 hits (71%), but <u>did not have the same hits</u>.**

More Questions

Would I have found more conditions if I set up more plates using the same screen? What if you have 0 hits in a single plate w/a single screen? Is setting up more of the same plate approaching a definition for insanity?



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Data Analysis

Scoring is on a (slightly) modified 9 point scale:
1 = clear solution
2 = phase separation
3 = precipitate
4 = precipitate with 'bright spots' – no discrete structure observed.
5 = non faceted structures (urchins, dendrites, spheroids) w/bright fluorescence – critical distinction vs. score of 4 is association with an observable structure.
6 = needles (pointed ends – flat ends = rods = 8 or 9)
7 = plate
8 = 3D crystals < 0.2 mm in size
9 = 3D crystals > 0.2 mm in size

Both labeled and unlabeled plates examined under white light for first scoring pass.

Labeled plates then examined under fluorescent illumination (primary source of score of 4) to adjust scores on basis of fluorescence image.

The well score is from the highest scoring structure in that well. Thus, if the well is mostly spheroids (score of 5), but one has developed facets, then the score is for a 3D crystal.

Unlabeled plate scores were 'edited' by reference to labeled plates. If similar structures appeared in both, but were not fluorescent, then the score was adjusted accordingly.

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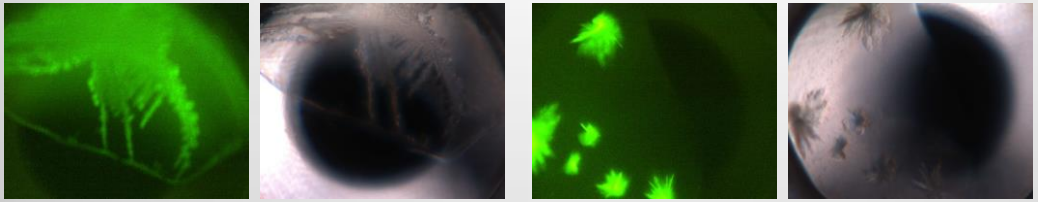
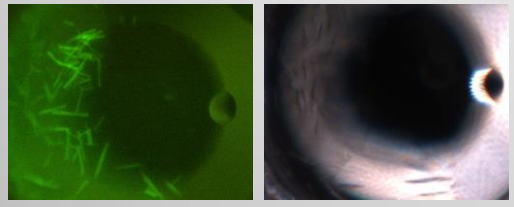
Scoring Examples

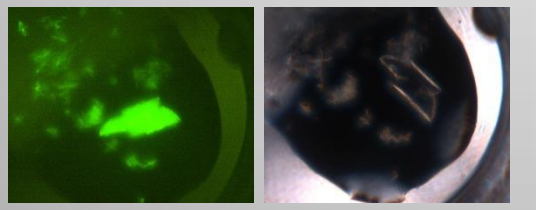
Score = 5



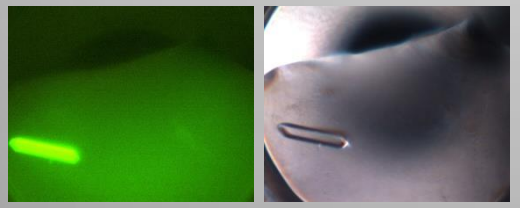
Score = 6



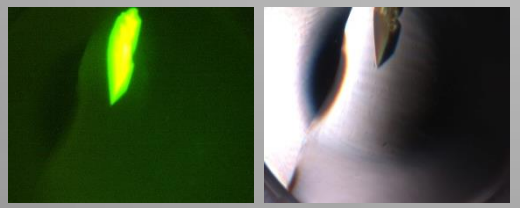
Score = 7



Score = 8



Score = 9



Score = 4

Examples to be shown…

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Preliminary Results

By Conditions

17 proteins tested so far

Protein - activity	|----------SCORES OF----------| Labeled / Unlabeled					
	5	6	7	8,9	4 (leads) ➔ Crystals (Total/unique)	(Tested/found)
Tt55 – Translation Initiation Factor	26/21	12/14	3/1	3/3	31/14	14/4
Tt71 – Intracellular Protease	5/5	23/26	0/0	7/8	13/5	NT
Tt82 – HAD Superfamily Hydrolase	5/8	1/0	5/4	0/0	23/22	12/6
Tt81 – Haloacid Dehalogenase	42/54	9/5	0/0	18/16	11/0	NT
Tt80 – Sugar HAD Phosphatase	17/12	1/0	0/0	9/6	31/24	NT
Tt94 – RNA 3'-Terminal Phosphate Cycl.	9/8	11/11	0/0	8/8	21/14	NT
Tt102 – Endonuclease Methyltransferase	9/6	2/4	1/0	1/1	20/15	15/-IP
Tt106 – Nucleotide Kinase	5/3	0/0	0/0	0/0	16/15	14/4-IP[1]
Tt189 – Nucleoside Diphosphate Kinase	10/10	1/1	1/1	7/3	21/11	5/1
Tt186 – Alcohol Dehydrogenase	18/21	10/8	4/5	15/12	10/3	NT
Tt75 – Prolyl Endopeptidase	18/13	3/0	5/4	1/5	29/15	4/1
Tt46 – SS DNA-specif. Exonuclease	13/6	1/1	1/0	0/0	31/22	22/7
Tt141 – Inorganic Pyrophosphatase	21/23	13/14	4/6	20/12	11/3	1/1
Tt97 – Aspartate Racemase	24/2	3/2	0/0	6/5	14/8	NT
PCP – Pyrrolidone Carboxylate Peptidase	39/21	36/9	5/2	5/2	49/16	15/10-IP[1]
PCNA – Proliferating Cell Nuclear Antigen	22/22	10/6	3/0	14/11	21/8	NT
Phas – Kidney Bean Phaseolin	17/19	0/0	0/0	15/17	13/5	NT
			Totals	129/109		Ave. of TESTED 39 % (34/87)

CRYSTALS FROM FLUORESCENCE

1) Capillary counter diffusion and IL-based optimization used.

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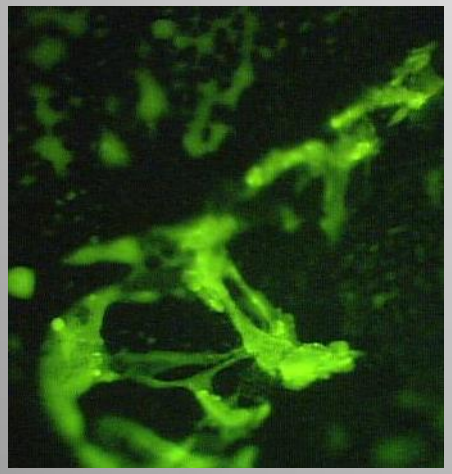
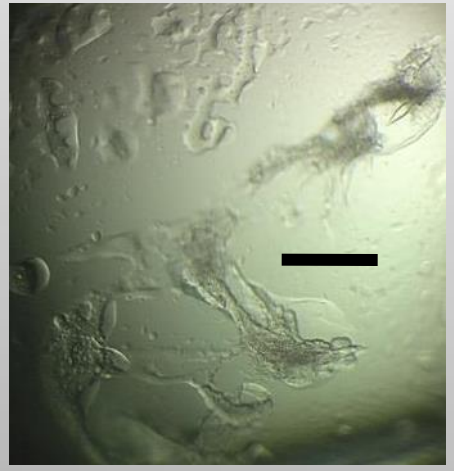
Bright Spots in Precipitant For Lead Conditions

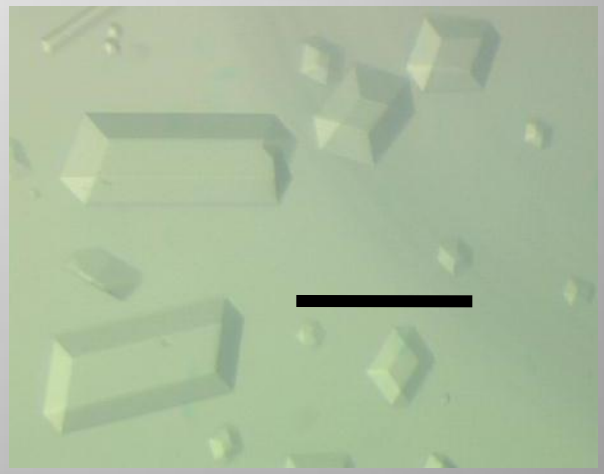
(Those outcomes with a score of 4)

Bright spots are often observed, which do not correspond to any identifiable discreet structures.

If intensity = structure, could they indicate proximity to crystallization conditions?







Canavalin – HSHT condition F1 (30%PEGMME 2000, 0.2M ammonium sulfate, 0.1M NaAcetate, pH 4.6).

Screening was first with respect to pH, then PEG MME & ammonium sulfate concentrations, and resulted in crystals as shown on the right (5% PEG MME 2000, 0.15M ammonium sulfate, 0.1M NaCacodylate, pH 6.5, 5 mg/ml protein).

Scale bars are 200um.

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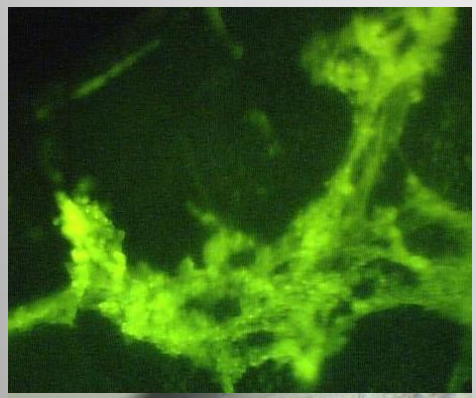
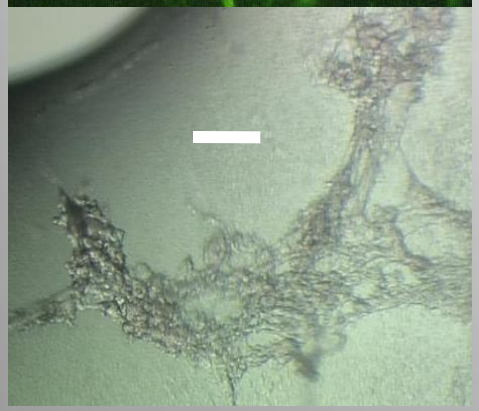
Bright Spots in Precipitant For Lead Conditions

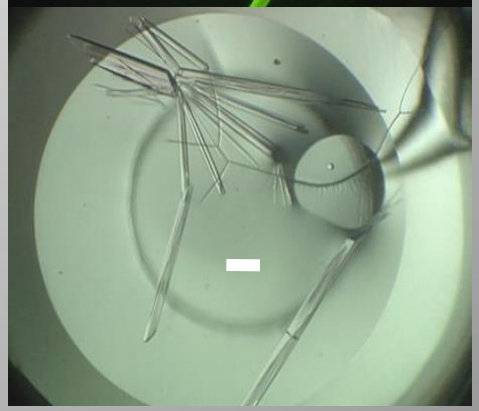




18% PEG 8K, 0.2M ZnAcetate, 0.1 M NaCacodylate, pH 6.5

Optimized to

10% PEG 8K, 0.01M ZnAcetate, 0.1M NaCacodylate, pH 6.5



β-Lactoglobulin B, left side shows results of HSHT screen condition D9.
This was optimized as shown on the right.
The Zn was necessary – crystals were not obtained without. Scale bars = 200 um.

Conclusion – Some precipitation conditions hid crystallization conditions.

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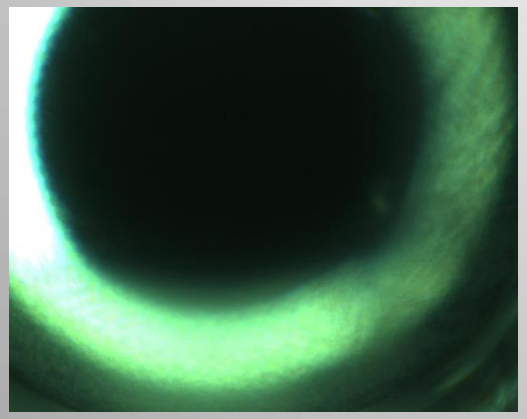
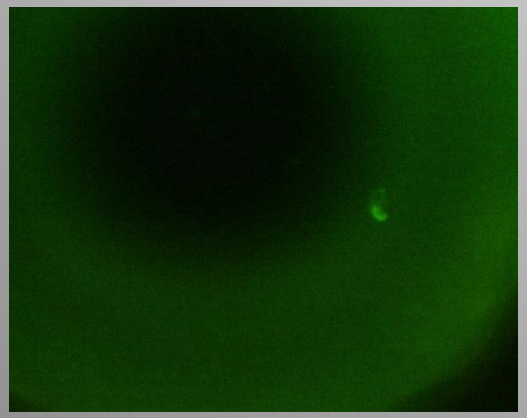
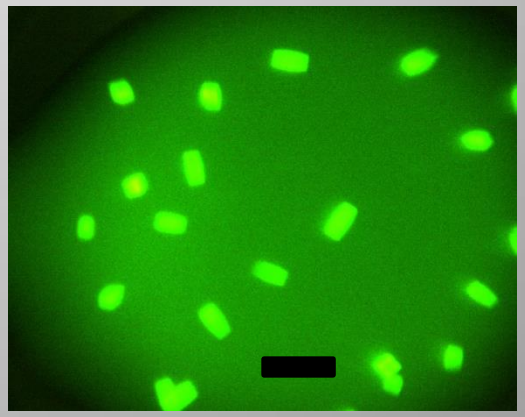
More 'Bright Spot' Optimizations

Sometimes the spots are not all that bright





PCP, Hampton Research HT condition C9. Optimization is condition C9 at 0.1 M C2[mim]-Cl, mixed 1:4 (v:v, screen:protein)



Scale bar = 200 um

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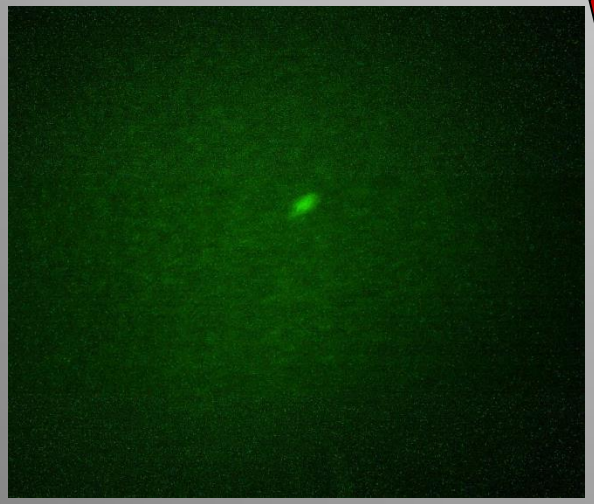
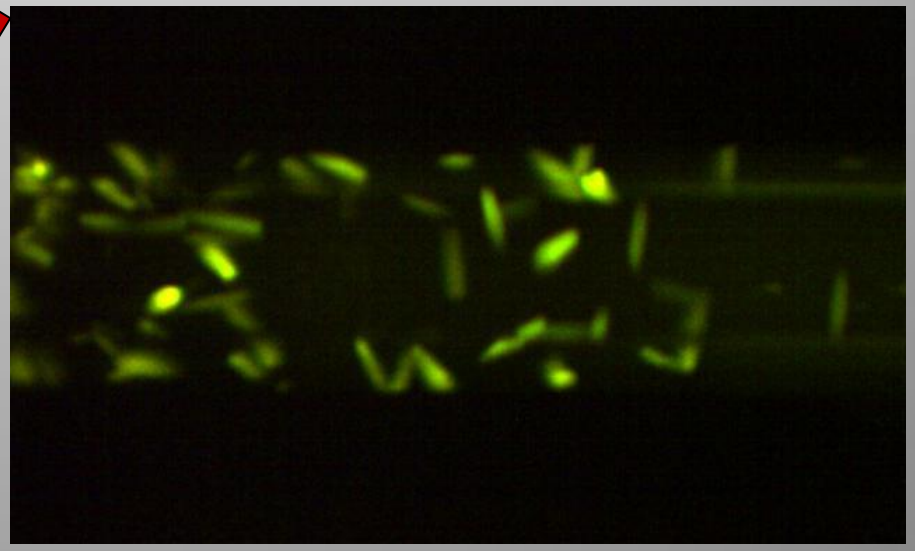
Bright Spots & Low Volume Imaging

Protein is Tt169, annotated as an exoribonuclease. The crystallizations conditions are an optimization of HSHT D6, the result of a 'bright spot' (below). The set-up is in a Microlytic counterdiffusion plate, with the imaged volume estimated as 15 nL.





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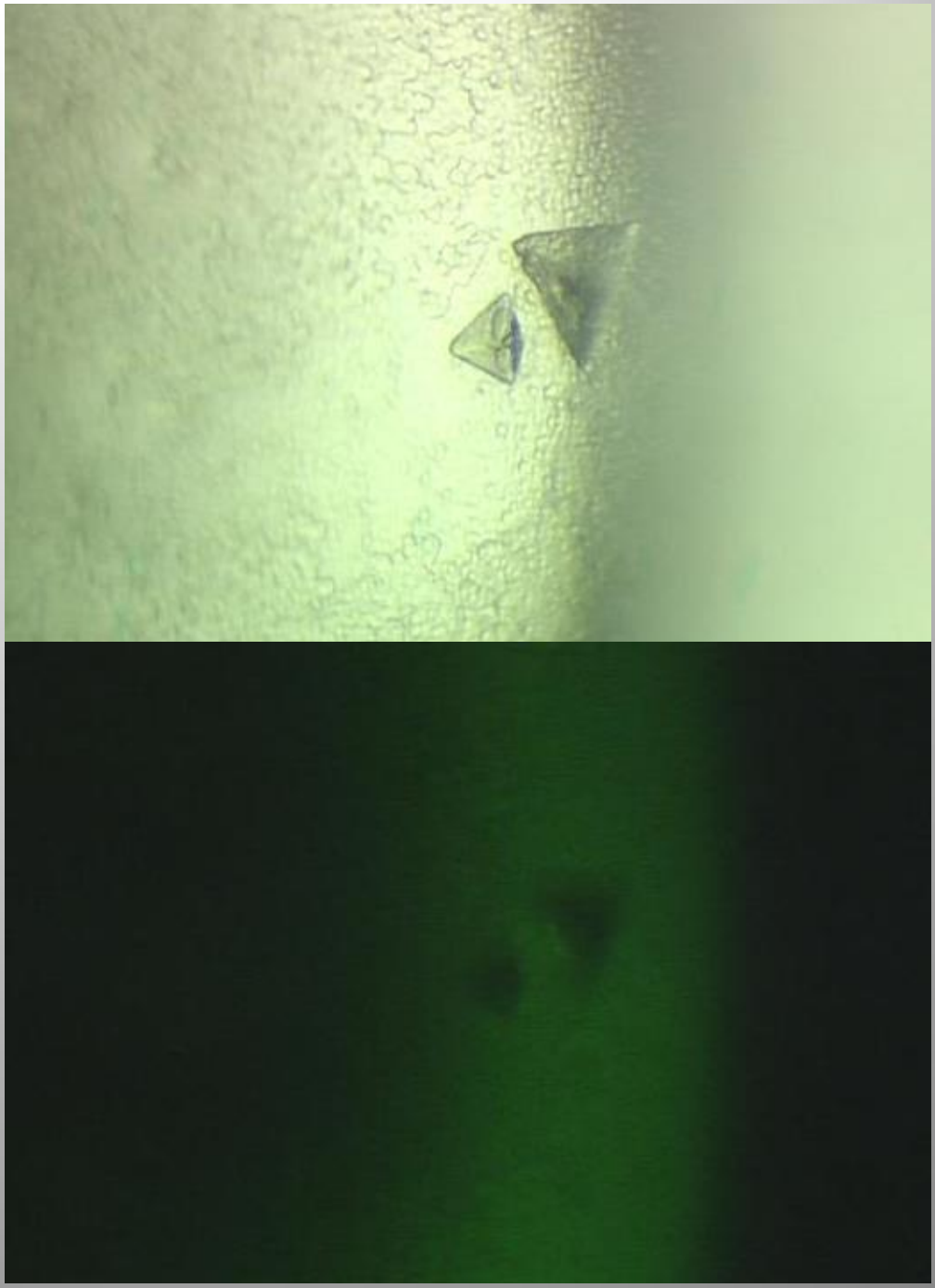
Benefits –
Disappearing salt crystals the original reason for using fluorescence

Native Canavalin, labeled with CR

HS II, #13



Last night I saw upon the stair
A little man who wasn't there
He wasn't there again today
Oh, how I wish he'd go away.

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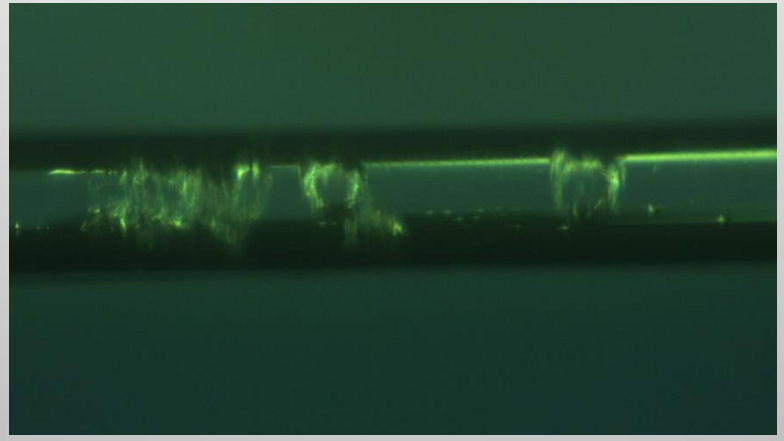
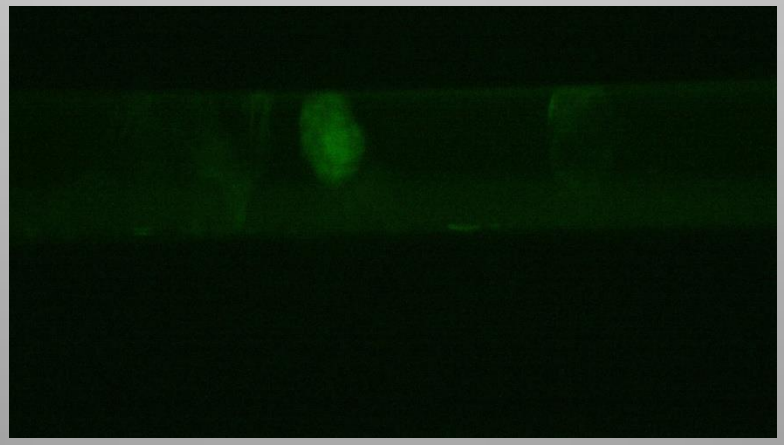
Another Example

(Joe thought he had me with this one!)

When the first diffraction attempt was made they centered the beam on the salt.





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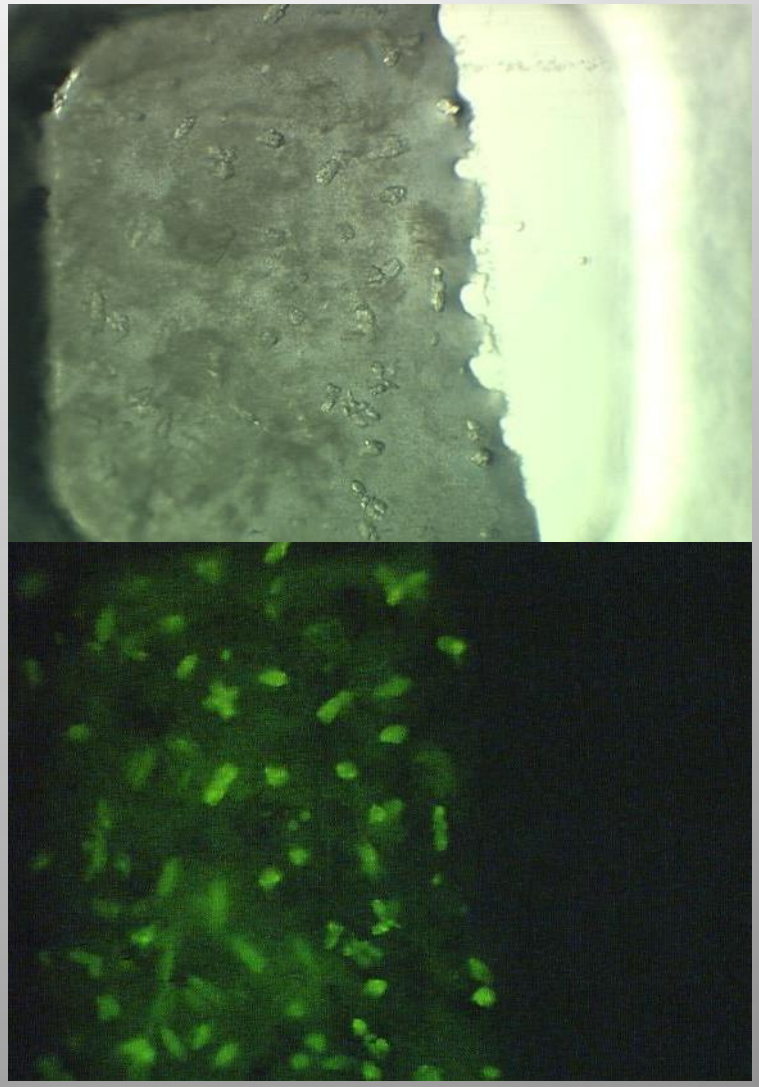
Finding Hidden Crystals

Native Canavalin, N-terminal amine labeled w/ the fluorescent probe PyMPO

HS 1, #14

PyMPO was used in early experiments as it had Ex and Em characteristics similar to GFP, for which the microscope was set up.



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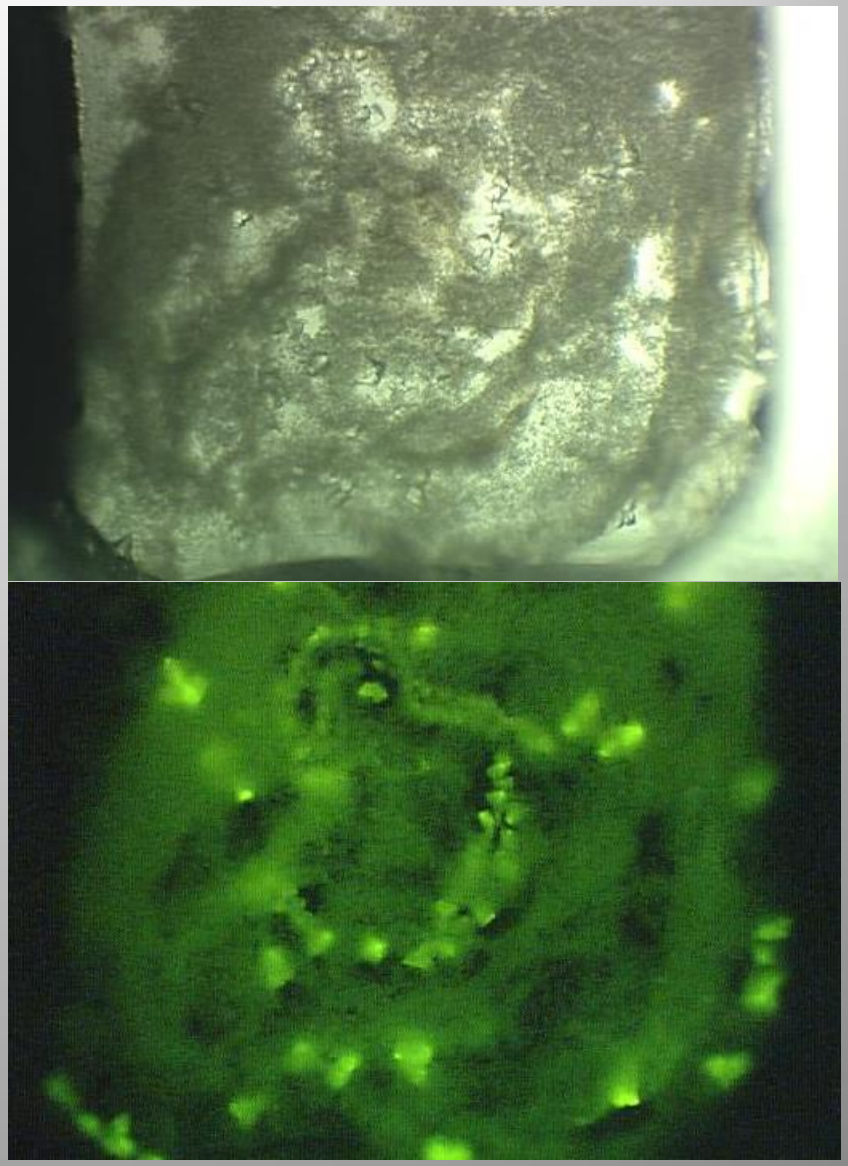
Finding Hidden Crystals

Native Canavalin, N-terminal amine labeled w/ PyMPO

HS 1, #23



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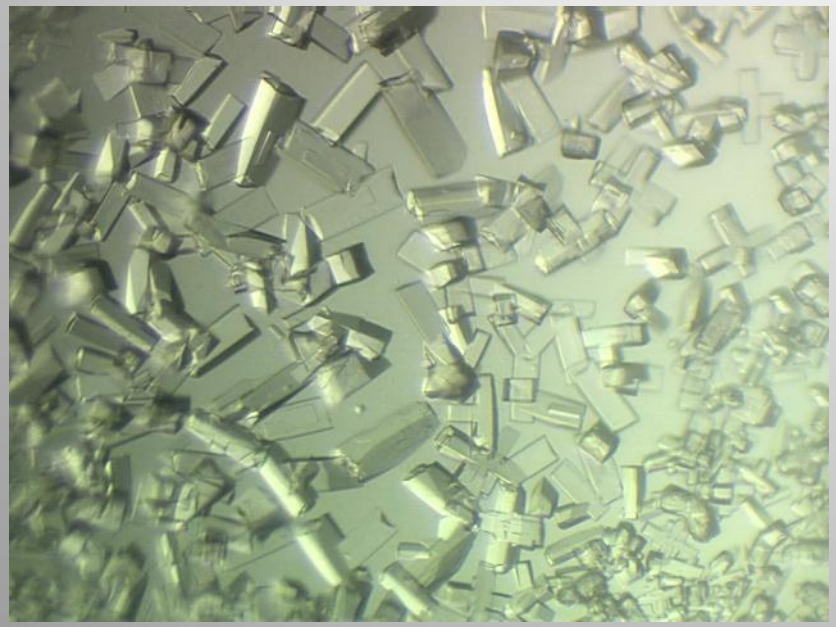
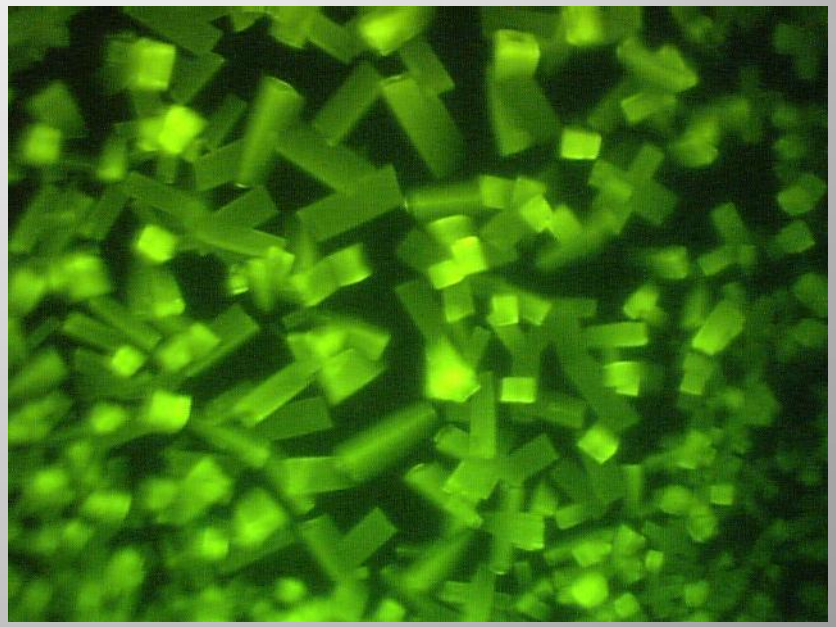
A Light Pipe Effect from Rod-Shaped Crystals





I have learned to closely examine precipitate showing pinpoints of light, which often indicate the presence of needles due to this light-pipe effect.

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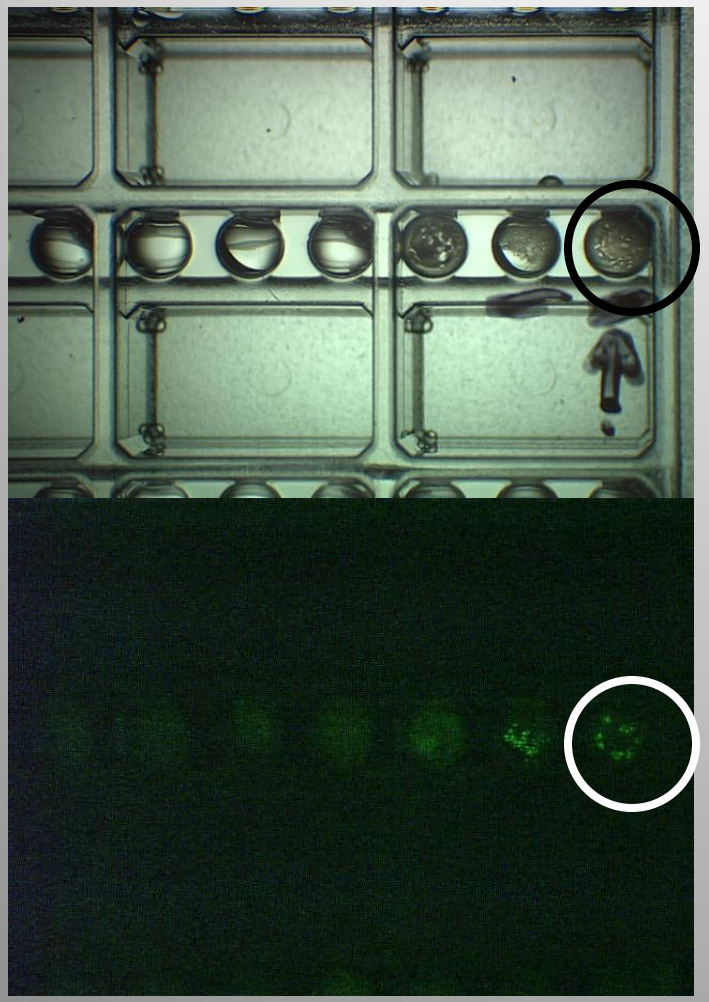
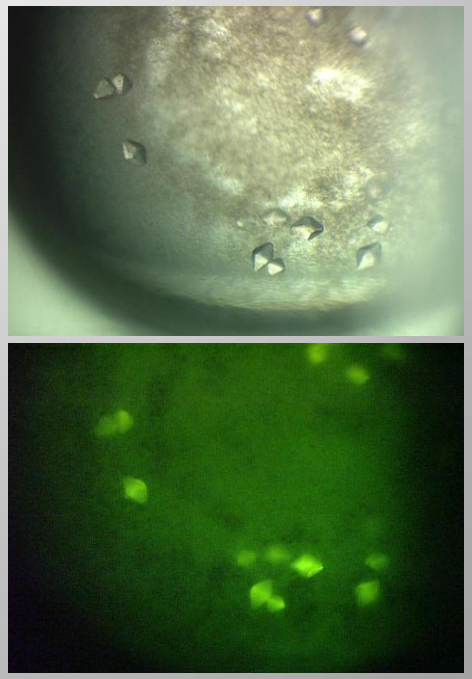
Quick Visual Scans are Facilitated Using Fluorescence

Even at low resolution





Finding crystals can be a pain in the ass
Fluorescence is a method with real class
You really feel fine
Seeing them shine
And you can review the plates very fast!

Not recommended with excitation using UV light!

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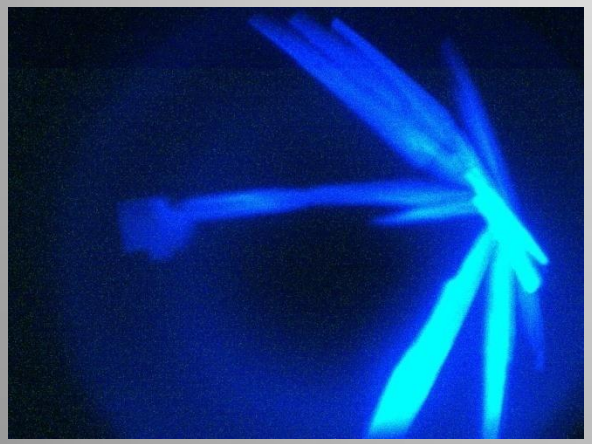
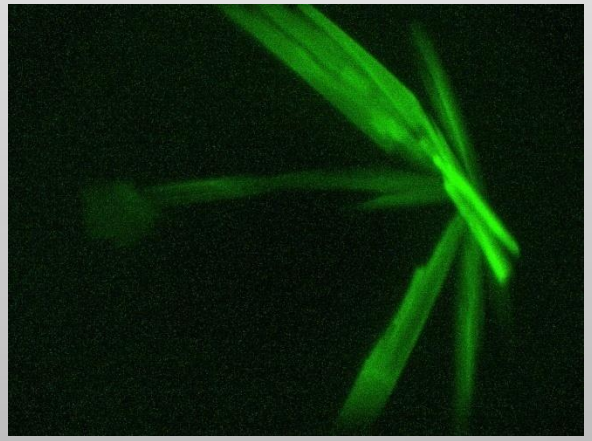
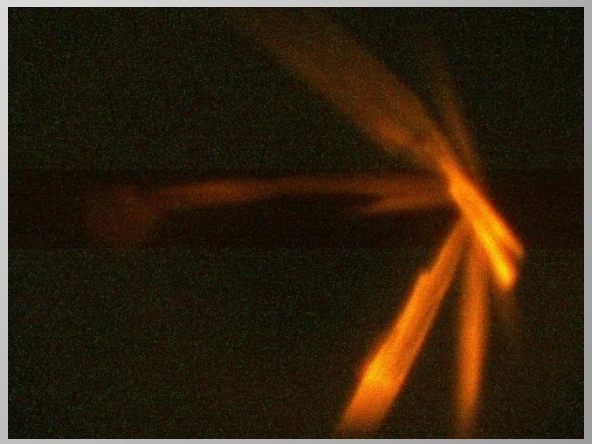
Future (now current) Directions

Multicolor Fluorescence for Complexes

Three different IPPase solutions individually labeled, each with a unique dye. The solutions were mixed, then a screening plate set up.







From right to left-
Cascade Yellow, Ex @ 405 nm, Em 550-570 nm
Carboxyrhodamine, Ex @ 525 nm, Em 540-560 nm
Pacific Blue, Ex @ 405 nm, Em 450-460 nm.

Images acquired using original prototype system. This required changing the (single 5 mm) LED and the filter cube for each imaging run. The new system can do two colours and white light, or three colors without white light.

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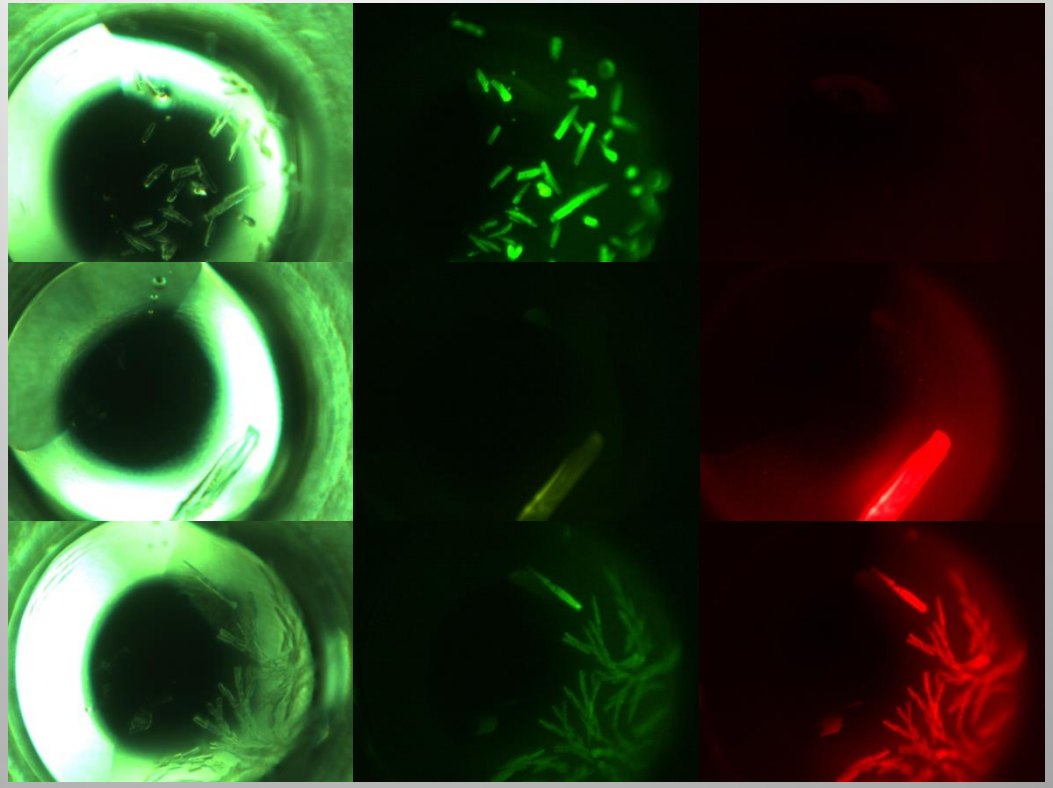
Multicolor Imaging

Protein is Tt141, HSHT condition E10

	White Light	CR Source & Filters	TR Source & Filters
CR labeled protein only			
TR labeled protein only			
CR & TR labled protein			



Probe selection is important! A higher wavelength (Ex and Em) red probe should be used instead of the TR. Probes chosen should be relatively immune from effects due to pH, solution components, etc.

Images acquired using X2 system.

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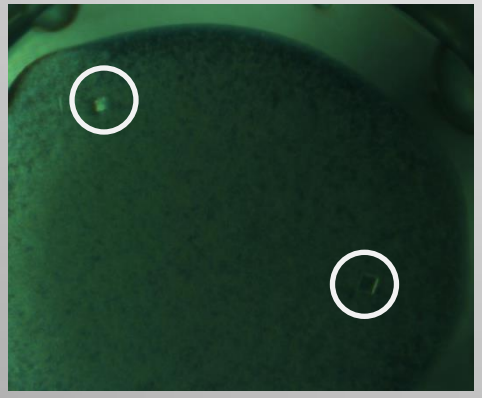
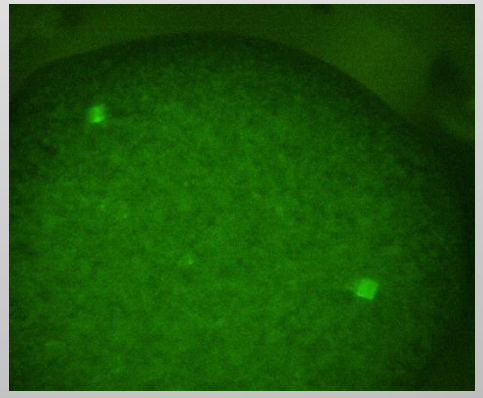
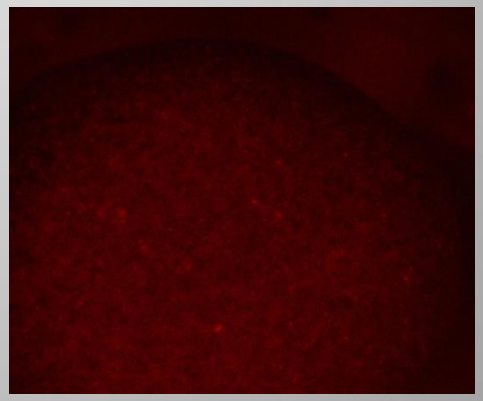
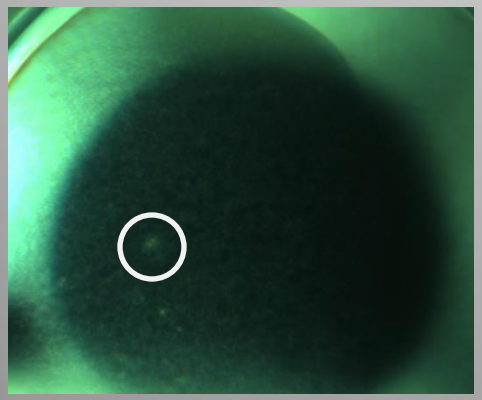
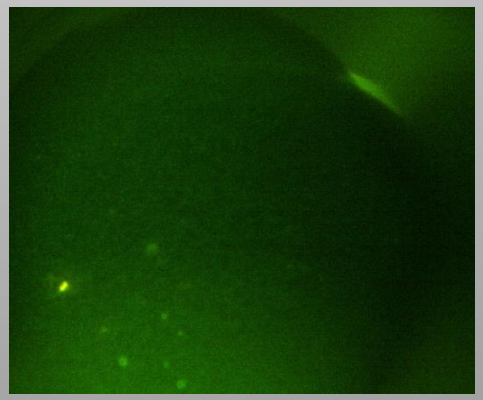
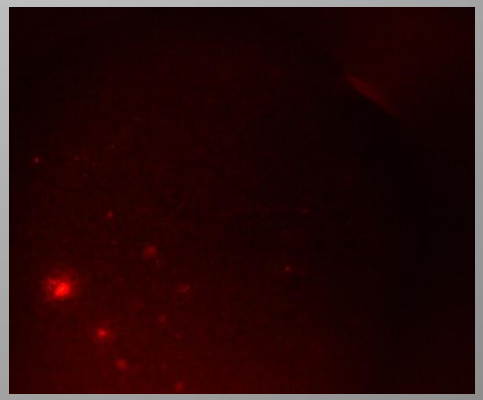
Multicolor Fluorescence For Complexes – 1'st Results

PCNA + Flap Endonuclease: The PCNA is labeled with Carboxyrhodamine (green) and the FEN with Texas Red (red). They were purified separately, mixed together at a 1:1 ratio, and a HSHT screening plate set up.

	White Light	Carboxyrhodamine	Texas Red
E5c			
G9c			

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CAN IT BE USED WITH LCP?

Different labeling considerations when working with IMPs

<u>Negatives</u>

Fluorescent probes will usually (almost always) partition into the hydrophobic phase.

If not removed, detergent micelles can become a source of false positives.

<u>Positives</u>

Fluorescence yield is typically better in hydrophobic environments.

So – Fluorescence should be better for trace fluorescently labeled protein, but only if the free probe can be removed.

Solution So Far – Label the protein just after extraction and just prior to the first chromatography step. Use this step to do a detergent exchange, to remove unbound fluorescent probe. First test did not work (very low yield of probe-protein product) and we are now rethinking this approach.

Same considerations about pH and the presence of 1° and 2° amines applies (if labeling amines) when labeling.

Initial testing (and learning curve climbing) now underway with *E. coli* OmpF.

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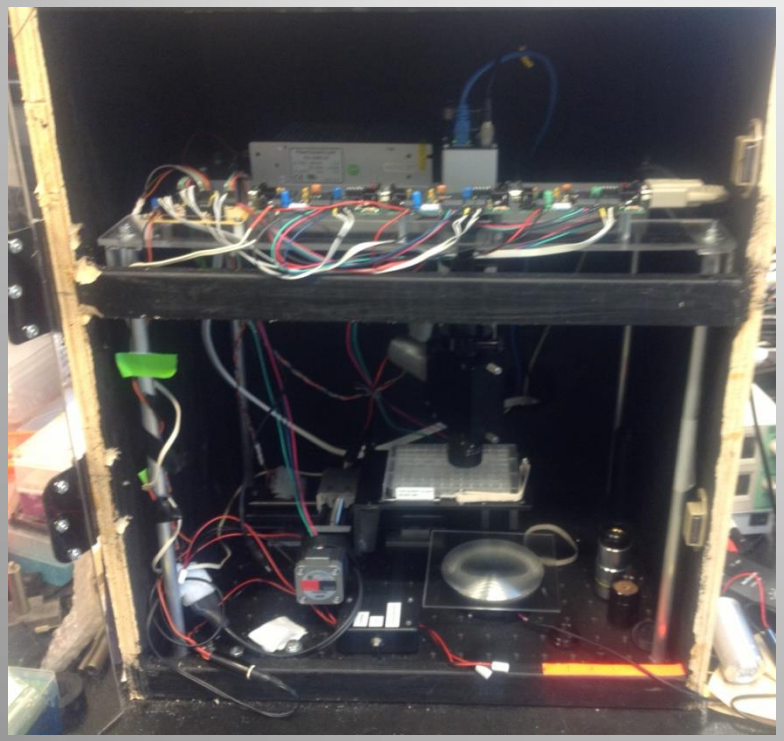
Future (Now Current) Directions
An Automated Plate Imager

My Laboratory Prototype

~6 months assembly & programming



Has Evolved To This

(Thanks to Fitz-Thors Engineering!)

This prototype is still useful for testing out of new ideas, light sources, etc. However, It is limited in only being able to use a single wavelength. Routine data collection is now carried out using this system, 2 fluorescent and 1 white light colours (also possible to have 3 fluorescent colours, no white light). Eventual goal is 4 fluorescent colors & white light.

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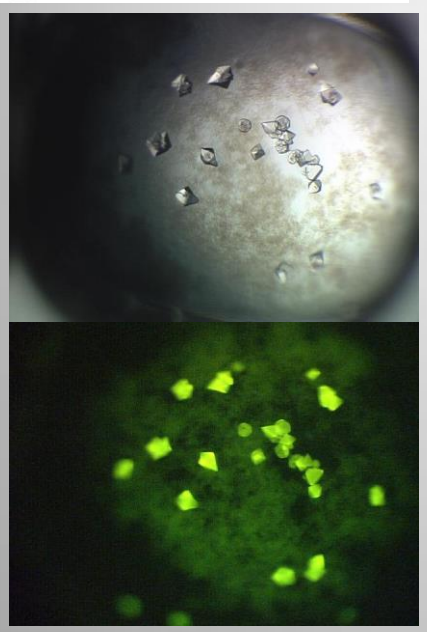
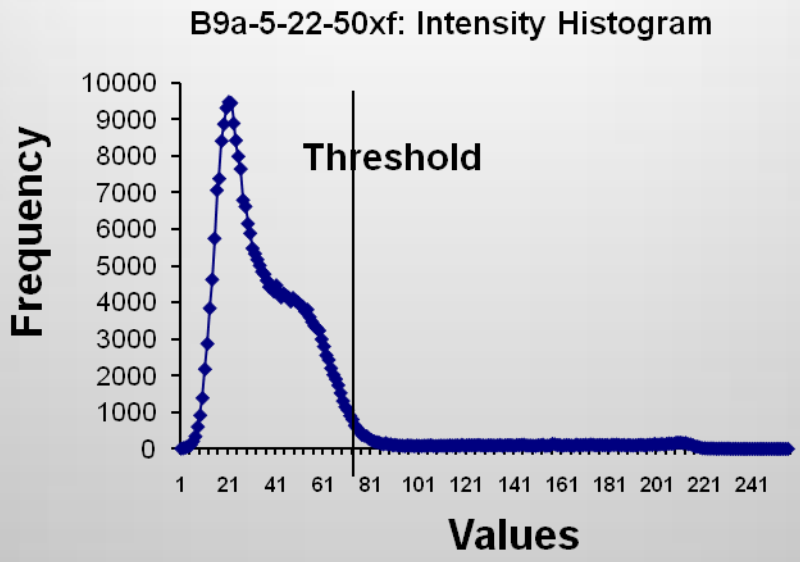
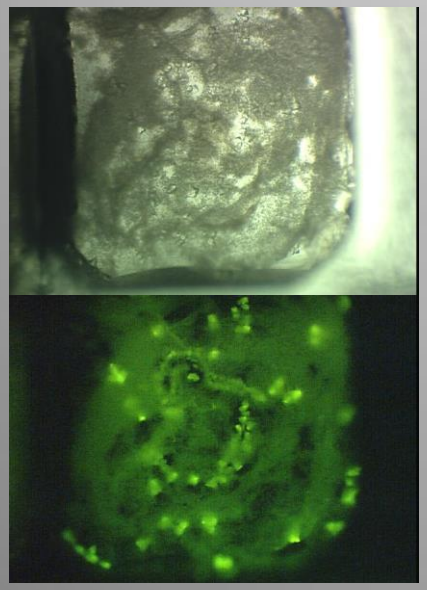
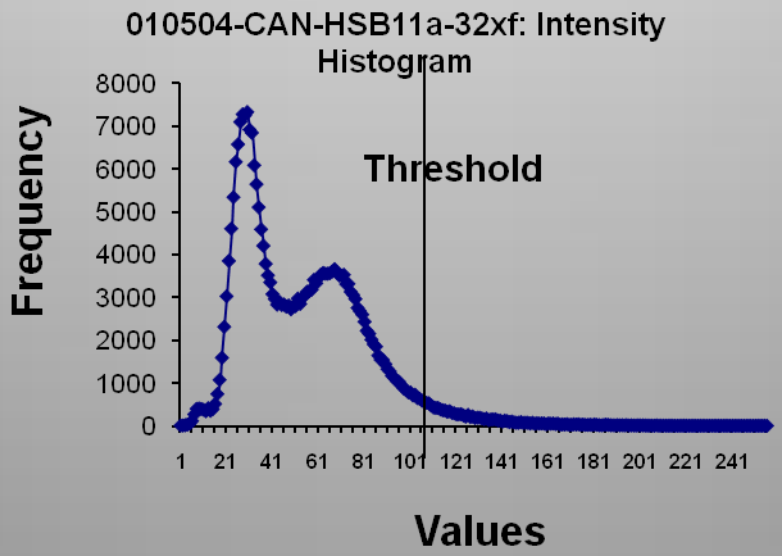
Future Directions – Automated Scoring

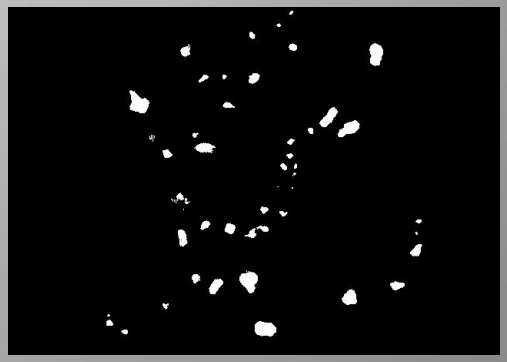
Intensity makes it very easy to identify objects of interest











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Future Directions – Automated Scoring

➔ Focus is important, although fluorescence is apparently more tolerant than white light images. Now Looking to apply autofocusing on images having sufficient contrast.

➔ False positives vs. false negatives – preferable to have 100% correct false negatives and 80% correct false positives than the reverse.

➔ Preliminary test goals were to divide the images into crystals, potential leads (bright spots that are not obvious crystals), precipitate, and clear.

➔ Crystals were successfully identified ~85% of the time, with missed crystals being primarily due to images out of focus, resulting in 'bright spot' lead conditions being classified as crystals and visa-versa.

➔ Future goals for image analysis software are to include morphological analysis, to facilitate differentiation of crystals from 'bright spots' and Include crystal morphology in the scoring.

➔ 'Primitive' scoring/outcome prioritization routine now built in to the Crystal X2 software.

Work in progress in collaboration with Dr. Ramazan Aygun, Dept. of Computer Sciences, UAH, as part of a Phase II STTR.

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Trace Labeling Conclusions

➔ Fluorescence can be a powerful tool in support of macromolecule crystallization, having many potential applications.

➔ It can be used to find lead crystallization conditions that would not be apparent using 'standard' plate-based methods.

➔ On a more 'routine' basis it can be used to rapidly find crystals or even potential lead conditions in plate screens.

➔ Intensity is a easier search parameter than straight lines, simplifying automated and/or visual plate-based screening analysis.

➔ Multi-color fluorescence can be used to verify the presence of multiple components in the crystallization of complexes .

➔ Probe derivatization levels of ≤ 0.5% do not affect nucleation rates or crystal quality. – <u>do not have to use modified protein for growth of crystals for analysis!!</u> After all – you now have the Information you needed – the crystallization conditions.

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Acknowledgements

iXpressGenes:

Dr. Joe Ng – crystallography
Michelle Morris – screening, crystal growth
Dr. Qunying Yuan – molecular biology
Jorge Barcena – protein purification

Univ. of Alabama, Huntsville

Dr. Ramazan Aygun – lead for image analysis software
Madhav Sigdel – student who does the work

Fitz-Thors Engineering, (recently moved to Birmingham, Alabama)
-Conversion of prototype system to the Crystal X2

Funding – NIH – Phase I & II STTRs (GM090453)

Any Questions?

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