Automated Fluorescence tomography of frozen-hydrated cells at the Bionanoprobe

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TWG Meeting

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A year ago…

the Bionanoprobe at LS-CAT:

sample-scanning, hard X-ray fluorescence nanoprobe

- 30 nm spatial resolution for fluorescence imaging
- Vacuum, cryogenic sample environment

10^{-7}-10^{-8} torr, < 110 K

- Robotic sample transfer in cryogenic conditions
- Fast “fly scan” mode
- Automated fluorescence tomography

Outline

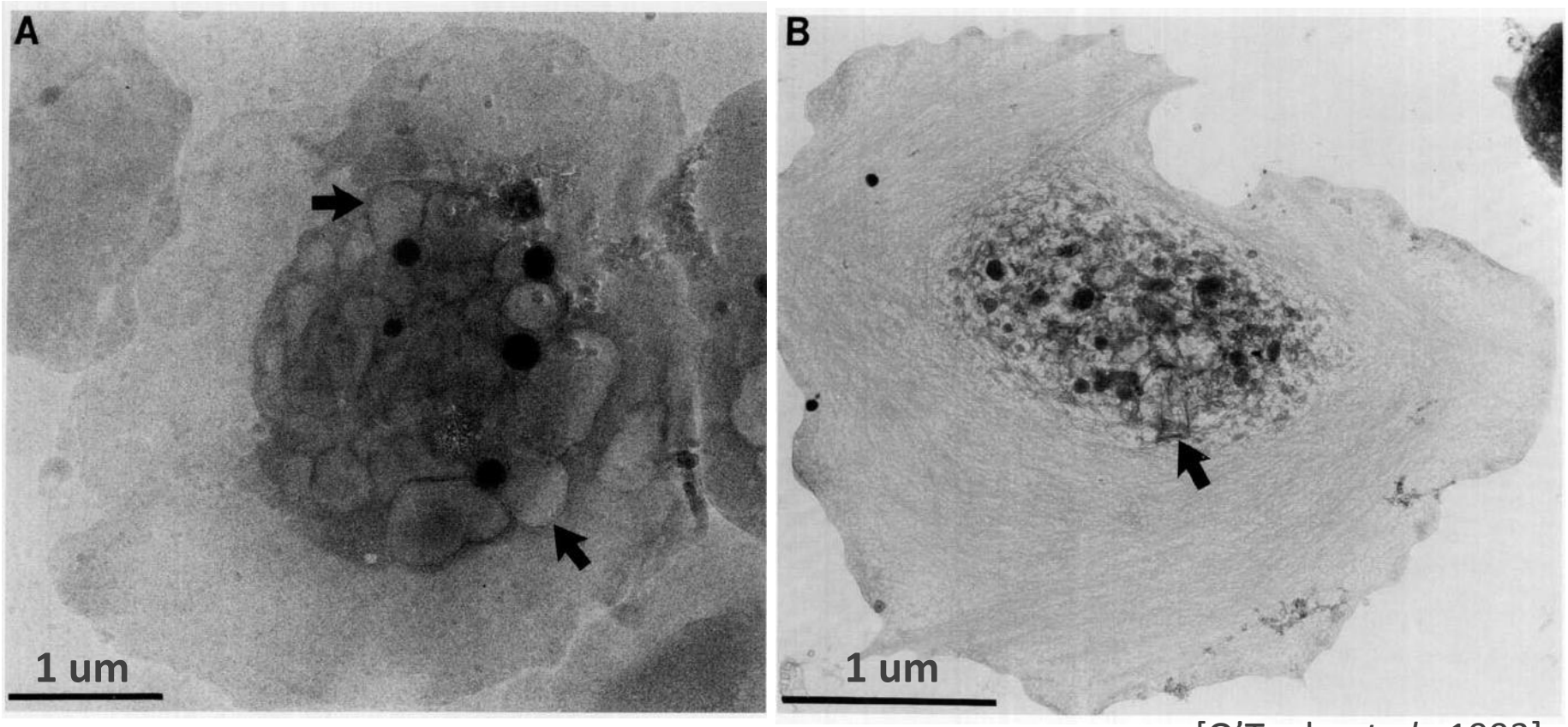
- Why “frozen-hydrated” cells?
- Why tomography?
- Tomography

- Projection acquisition at different angles
- Image alignment: cross-correlation
- Cross section reconstruction
- 3D volume visualization

- Conclusion and future work

Why “frozen-hydrated” cells?

- Imaging of frozen-hydrated whole cells under cryogenic conditions is the only reliable way to fully preserve the three-dimensional architecture of the cell while minimizing radiation damage and rearrangement or loss of diffusible ions.



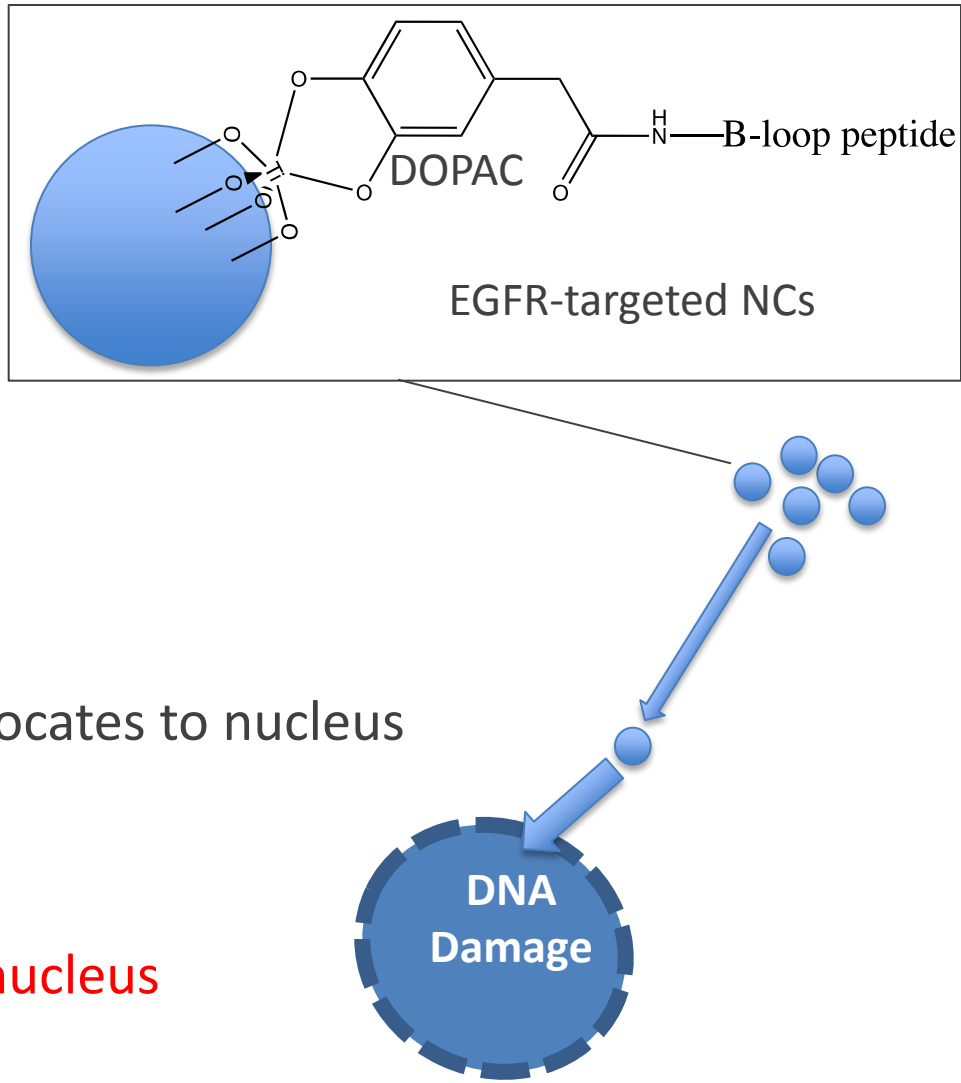
[O’Toole *et al*., 1993]

frozen-hydrate platelet, imaged at -160˚C

chemically-fixed, dehydrated platelet, imaged at room temperature

Why tomography?

Intracellular distributions of Fe_3O_4/TiO_2 nanocomposites (NCs):



TiO_2 nanoparticles are a promising vehicle for delivering therapeutic and diagnostic agents. XRF has been used to study how EGFR (epidermal growth factor receptor) positive HeLa cells internalize or take up EGFR-targeted NCs.

- EGFR is overexpressed, and translocates to nucleus
- EGFR-targeted NCs bind EGFR

- Do EGFR-targeted NCs reach the nucleus after internalization?

Why tomography?

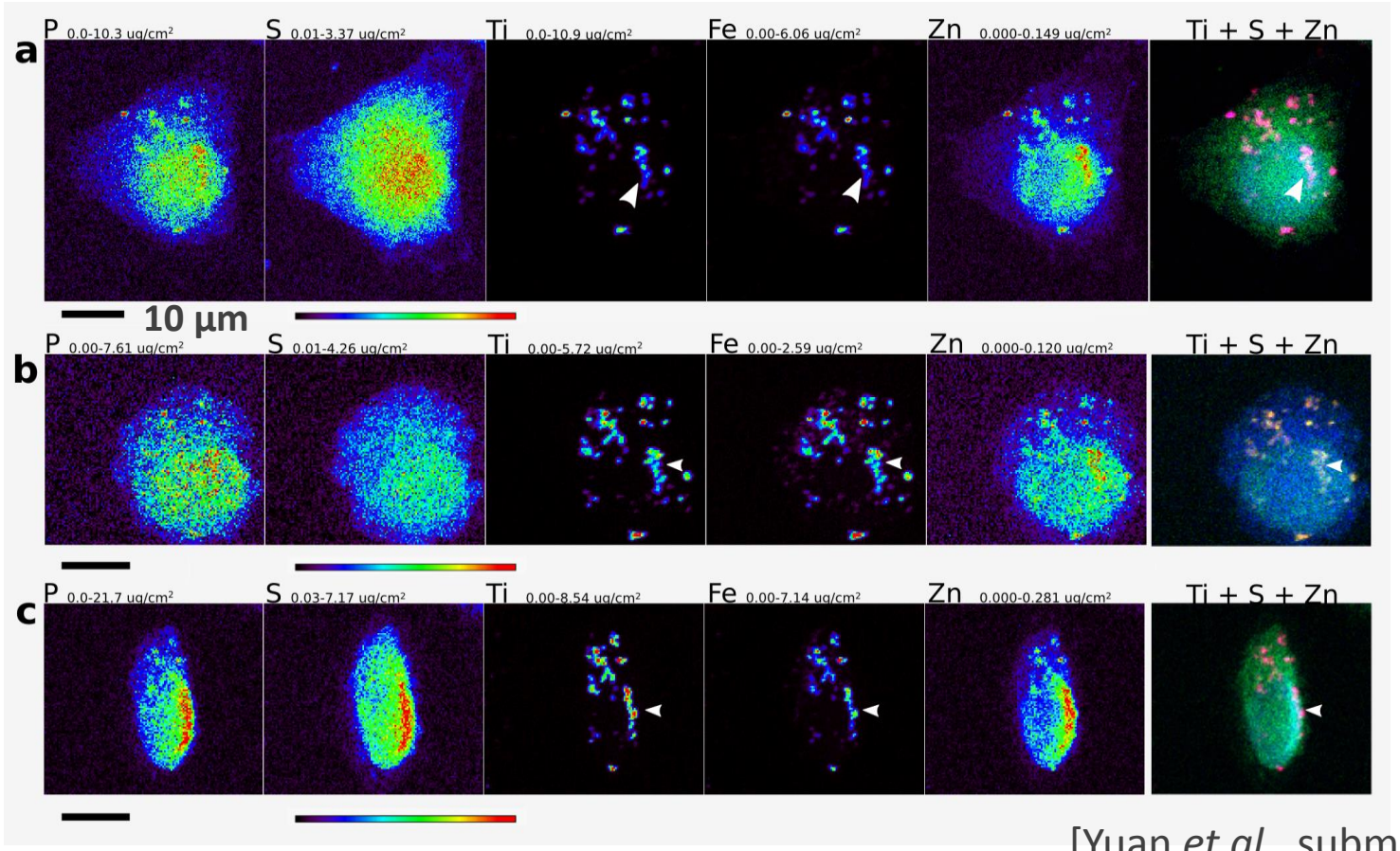
- A typical scanning XRF experiment results in a 2D projection of the elemental distributions within the examined sample with very limited depth information.
- Tomography enables visualization of internal structures and composition in a nondestructive manner.

HeLa Cell

@ 0 deg

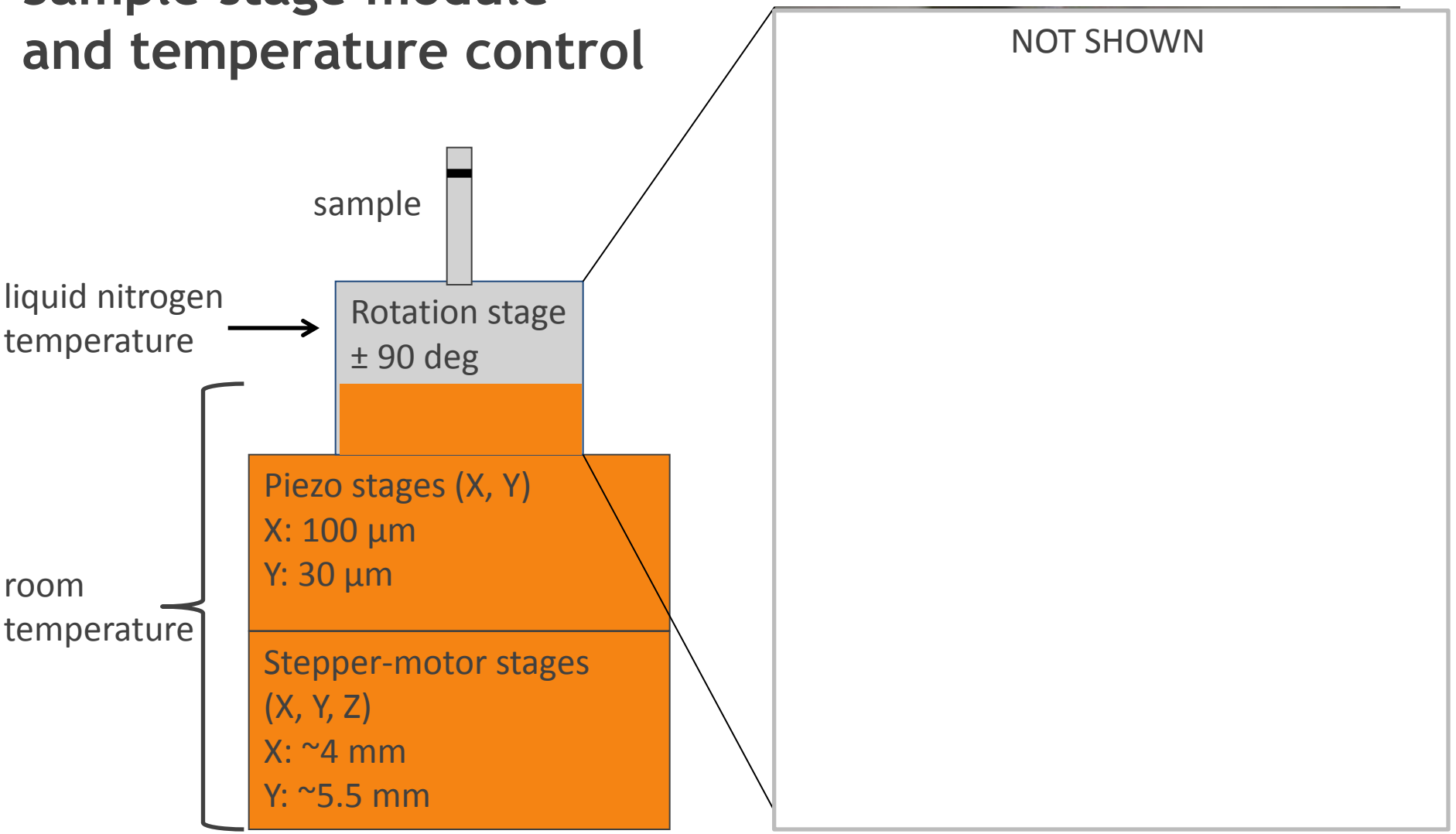
@ 6 deg

@ 60 deg

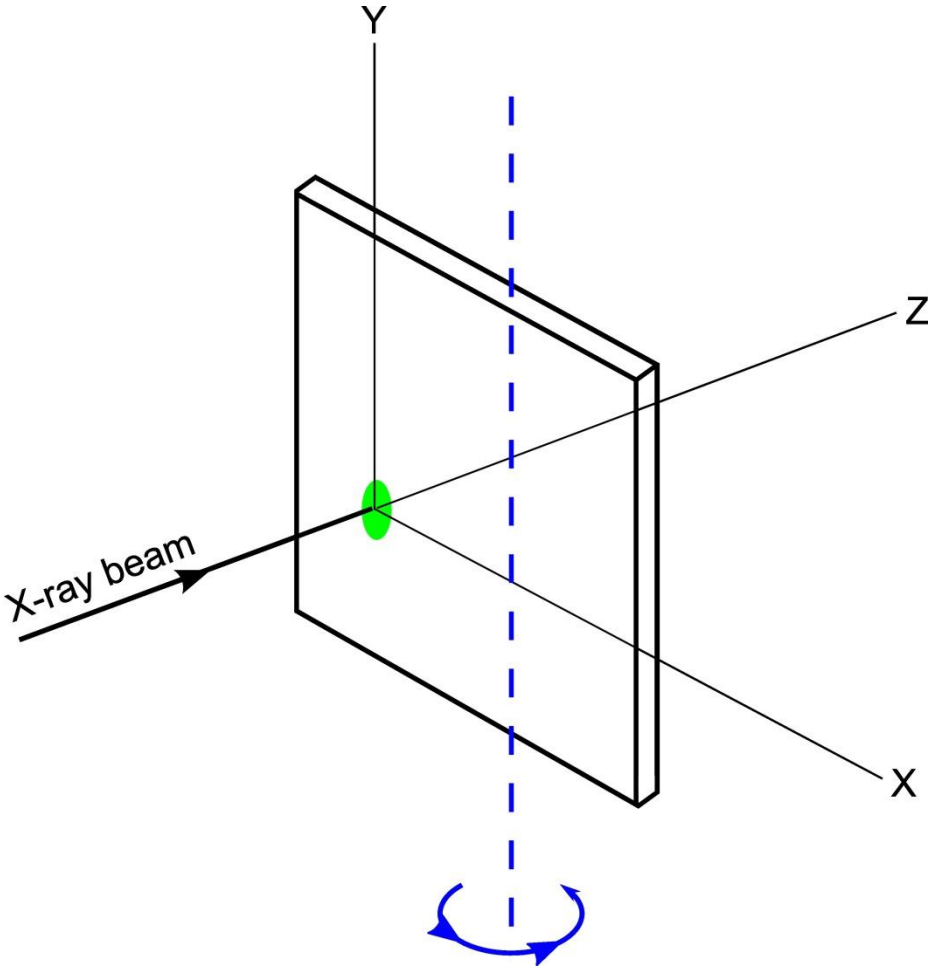


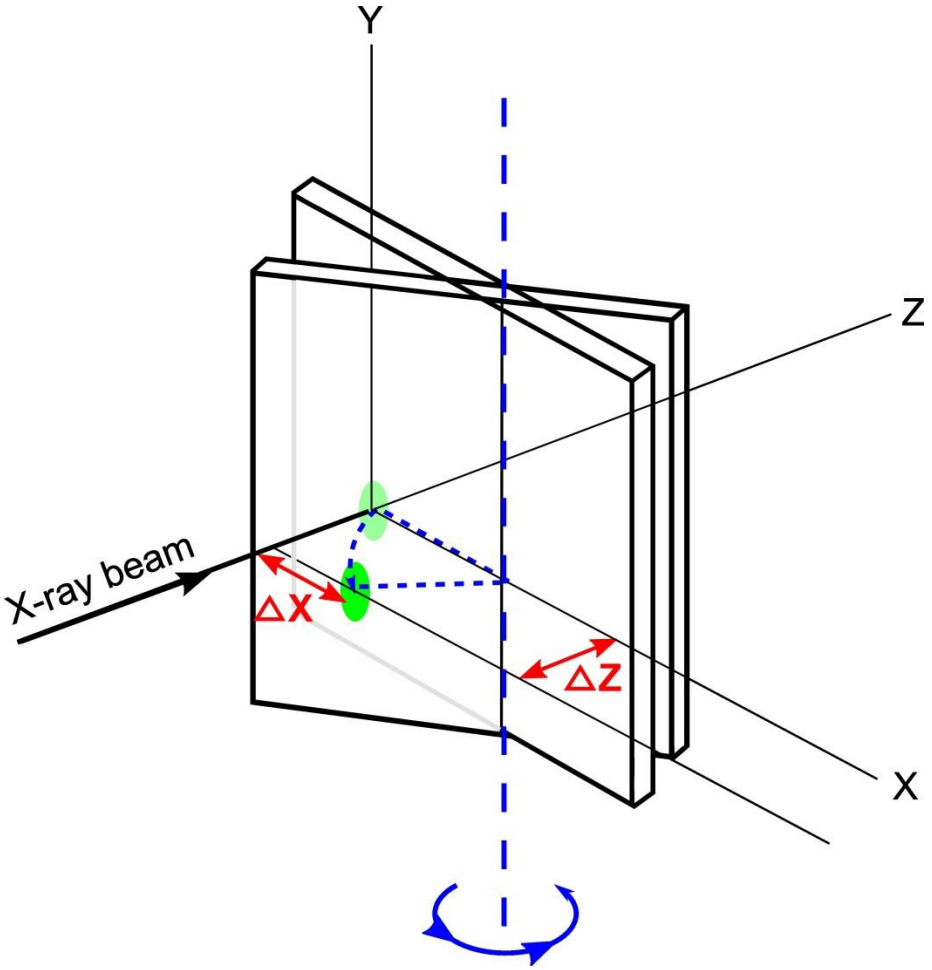
[Yuan *et al.*, submitted]

Sample stage module and temperature control



- Reduce the weight on the rotation stage to improve stability;
- POI cannot be easily moved to the center of the rotation.





Translate X/Y stages to bring the POI to the focused X-ray beam.

However, after a simple rotation , the POI will likely be out of the beam.

Corrections in both X and Z directions are needed after each rotation!

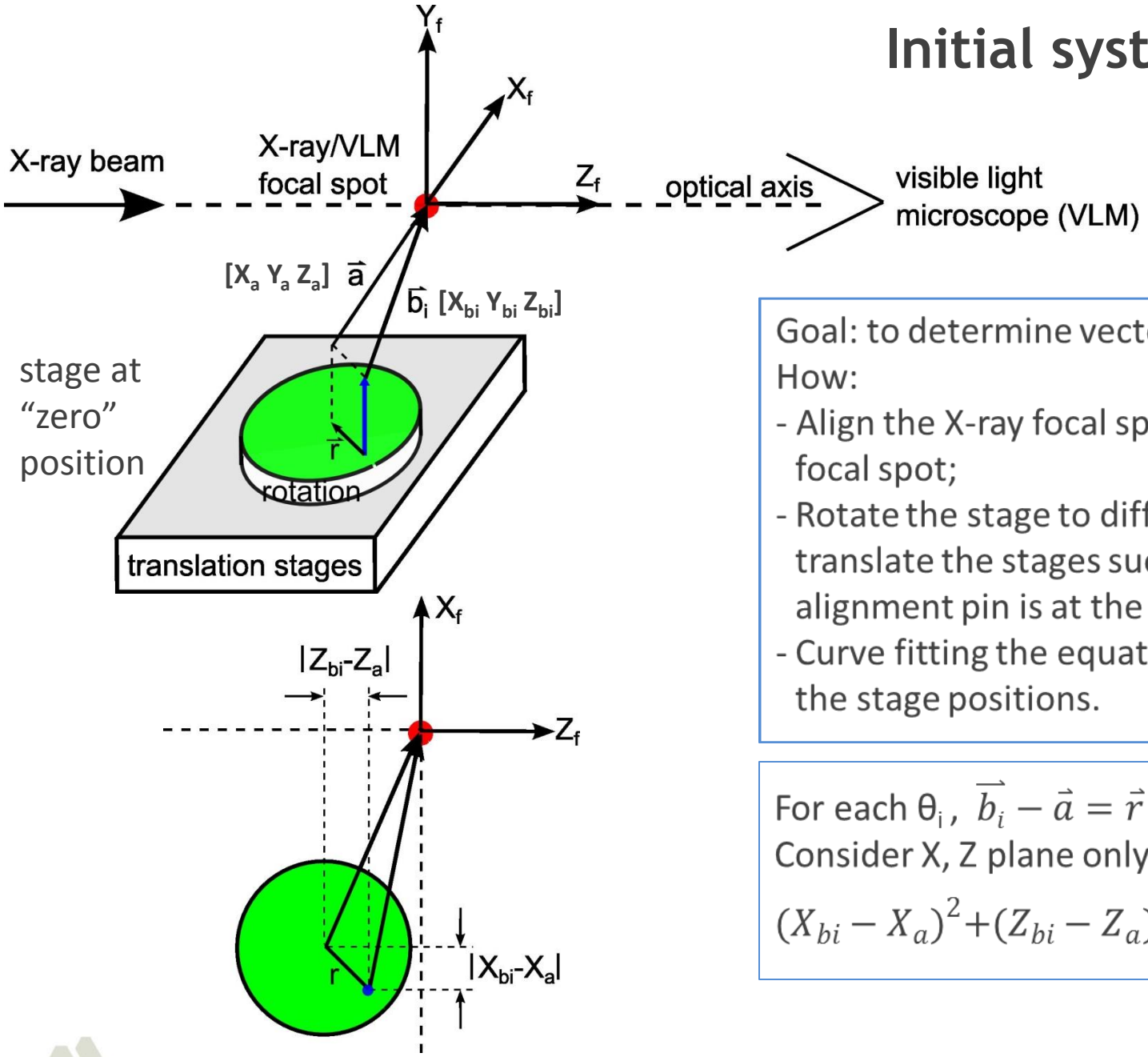
- Automated correction method:

-- Initial system alignment: alignment vector

-- Calculation of the offset between the desired scanning area and the rotation axis: offset vector

-- Calculation of the motor positions for each rotation angle

Initial system alignment



Goal: to determine vector $\vec{a}$

How:

- Align the X-ray focal spot with the optical focal spot;
- Rotate the stage to different angles, translate the stages such that the alignment pin is at the focal spot;
- Curve fitting the equation of a circle using the stage positions.

For each θ_i, $\overrightarrow{b_i} - \vec{a} = \vec{r}$;

Consider X, Z plane only (Y_a=Y_{bi}=constant):

$$(X_{bi} - X_a)^2 + (Z_{bi} - Z_a)^2 = r^2,\ i = 1,2,\cdots,n$$

Then…

$$(X_{bi} - X_a)^2 + (Z_{bi} - Z_a)^2 = r^2,\ i = 1,2,\cdots,n$$

Expansion:

$$X_{bi}{}^2 - 2X_{bi}X_a + X_a{}^2 + Z_{bi}{}^2 - 2Z_{bi}Z_a + Z_a{}^2 - r^2 = 0$$

Substitution:

$$P = X_a^2 + Z_a^2 - r^2$$

$$2X_{bi}X_a + 2Z_{bi}Z_a - P = X_{bi}{}^2 + Z_{bi}{}^2$$

Matrix calculation:

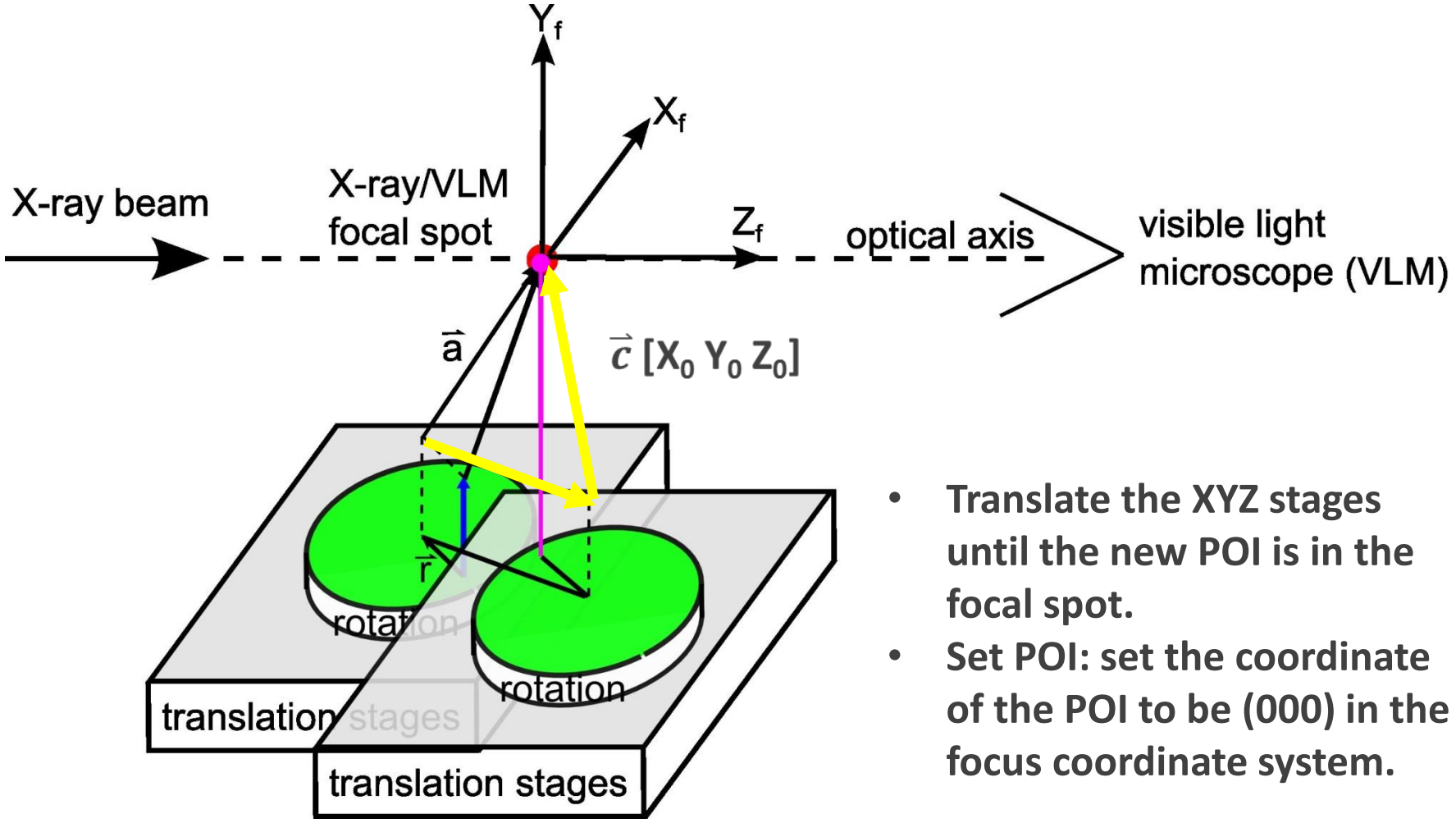
$$\begin{bmatrix} 2X_{b1} & 2Z_{b1} & -1 \\ 2X_{b2} & 2Z_{b2} & -1 \\ \vdots & \vdots & \vdots \\ 2X_{bn} & 2Z_{bn} & -1 \end{bmatrix} \begin{bmatrix} X_a \\ Z_a \\ P \end{bmatrix} = \begin{bmatrix} X_{b1}{}^2 + Z_{b1}{}^2 \\ X_{b2}{}^2 + Z_{b2}{}^2 \\ \vdots \\ X_{bn}{}^2 + Z_{bn}{}^2 \end{bmatrix}$$

End up with:

$$\begin{bmatrix} X_a \\ Z_a \\ P \end{bmatrix} = \begin{bmatrix} \sum_{i=1}^{n} 4X_{bi}{}^2 & \sum_{i=1}^{n} 4X_{bi}Z_{bi} & \sum_{i=1}^{n} -2X_{bi} \\ \sum_{i=1}^{n} 4X_{bi}Z_{bi} & \sum_{i=1}^{n} 4Z_{bi}{}^2 & \sum_{i=1}^{n} -2Z_{bi} \\ \sum_{i=1}^{n} -2X_{bi} & \sum_{i=1}^{n} -2Z_{bi} & n \end{bmatrix}^{-1} \begin{bmatrix} \sum_{i=1}^{n} 2X_{bi}(X_{bi}{}^2 + Z_{bi}{}^2) \\ \sum_{i=1}^{n} 2Z_{bi}(X_{bi}{}^2 + Z_{bi}{}^2) \\ \sum_{i=1}^{n} -(X_{bi}{}^2 + Z_{bi}{}^2) \end{bmatrix}$$

alignment vector

Offset vector between a POI and the rotation axis

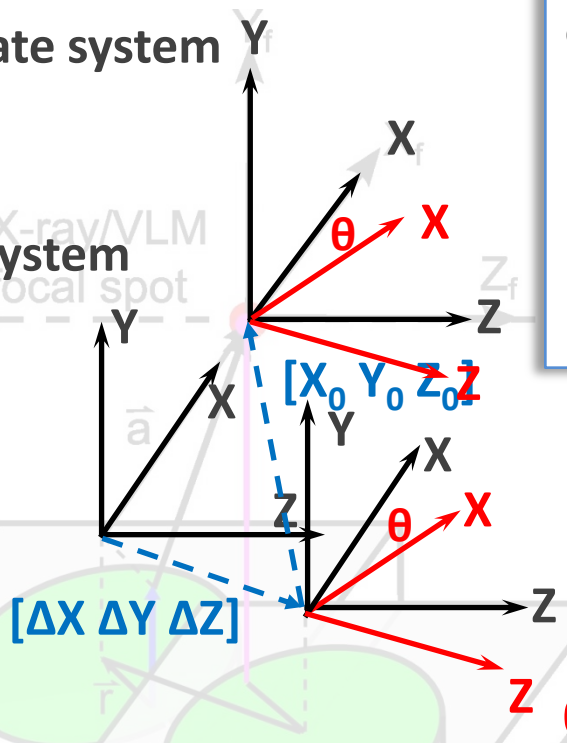


translate by [Δx Δy Δz]

- **Translate the XYZ stages until the new POI is in the focal spot.**
- **Set POI: set the coordinate of the POI to be (000) in the focus coordinate system.**

sample (s) coordinate system

base coordinate system
("zero" position)



sample stage (ss) coordinate system
("non-zero" position)

θ coordinate system

coordinate transformation matrix:

$$ {}^{b}_{s}T = {}^{b}_{ss}T * {}^{ss}_{s}T = \begin{bmatrix} 1 & 0 & 0 & \Delta X + X_0 \\ 0 & 1 & 0 & \Delta Y + Y_0 \\ 0 & 0 & 1 & \Delta Z + Z_0 \\ 0 & 0 & 0 & 1 \end{bmatrix} $$

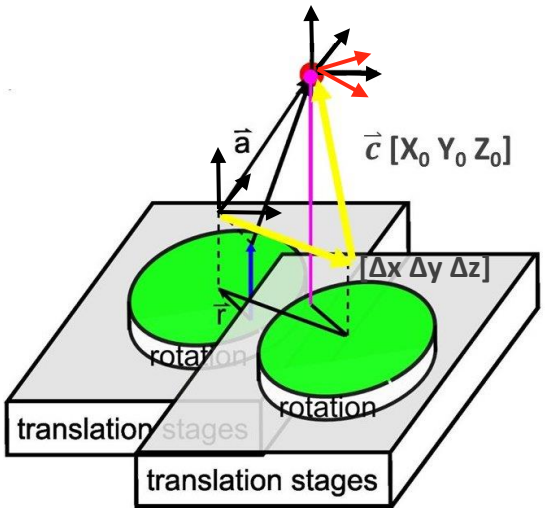
general case: $\theta \neq 0$ when set the POI

$$ {}^{b}_{s}T_g = {}^{b}_{ss}T * {}^{ss}_{\theta}T * {}^{\theta}_{s}T = \begin{bmatrix} \cos\theta & 0 & \sin\theta & X_0\cos\theta + Z_0\sin\theta + \Delta X \\ 0 & 1 & 0 & Y_0 + \Delta Y \\ -\sin\theta & 0 & \cos\theta & -X_0\sin\theta + Z_0\cos\theta + \Delta Z \\ 0 & 0 & 0 & 1 \end{bmatrix} $$

Calculation of the offset vector $[X_0 Y_0 Z_0]$

When set the POI, the known parameters: (θ ΔX ΔY ΔZ) (X_a Y_a Z_a)

After setting the POI, the coordinate of POI is (0 0 0), in the sample coordinate system, (X_a Y_a Z_a), in the base coordinate system.



They satisfy the following relation:

$$\begin{bmatrix} \cos\theta & 0 & \sin\theta & X_0\cos\theta + Z_0\sin\theta + \Delta X \\ 0 & 1 & 0 & Y_0 + \Delta Y \\ -\sin\theta & 0 & \cos\theta & -X_0\sin\theta + Z_0\cos\theta + \Delta Z \\ 0 & 0 & 0 & 1 \end{bmatrix} * \begin{bmatrix} 0 \\ 0 \\ 0 \\ 1 \end{bmatrix} = \begin{bmatrix} X_a \\ Y_a \\ Z_a \\ 1 \end{bmatrix}$$

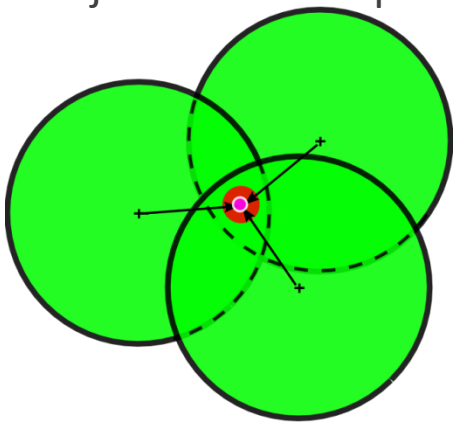
Thus, the vector $[X_0 Y_0 Z_0]$ is obtained as:

$$\begin{cases} X_0 = X_a\cos\theta - Z_a\sin\theta - \Delta X\cos\theta + \Delta Z\sin\theta \\ Y_0 = Y_a - \Delta Y \\ Z_0 = X_a\sin\theta + Z_a\cos\theta - \Delta X\sin\theta - \Delta Z\cos\theta \end{cases}$$

offset vector

For tomographic data collection: Motor positions are needed!

Projections in X-Z plane



Requirement: the POI stays in the focal spot for all rotation angles;
which means: the coordinate of POI is:
constant
(0 0 0) (generally: (x' y' z')) , in the sample coordinate system;
(X_a Y_a Z_a), in the base coordinate system.

They satisfy the following relation:

$$\begin{bmatrix} \cos\theta_i & 0 & \sin\theta_i & X_0\cos\theta_i + Z_0\sin\theta_i + \Delta X_i \\ 0 & 1 & 0 & Y_0 + \Delta Y_i \\ -\sin\theta_i & 0 & \cos\theta_i & -X_0\sin\theta_i + Z_0\cos\theta_i + \Delta Z_i \\ 0 & 0 & 0 & 1 \end{bmatrix} * \begin{bmatrix} x' \\ y' \\ z' \\ 1 \end{bmatrix} = \begin{bmatrix} X_a \\ Y_a \\ Z_a \\ 1 \end{bmatrix}$$

What we know: (X_a Y_a Z_a) (X_0 Y_0 Z_0) θ_i (x' y' z')

Then, the motor positions for each θ_i are:

$$\begin{cases} \Delta X_i = X_a - \cos\theta_i(X_0 + x') - \sin\theta_i\,(Z_0 + z') \\ \Delta Y_i = Y_a - (Y_0 + y') \\ \Delta Z_i = Z_a + \sin\theta_i(X_0 + x') - \cos\theta_i(Z_0 + z') \end{cases}$$

motor positions

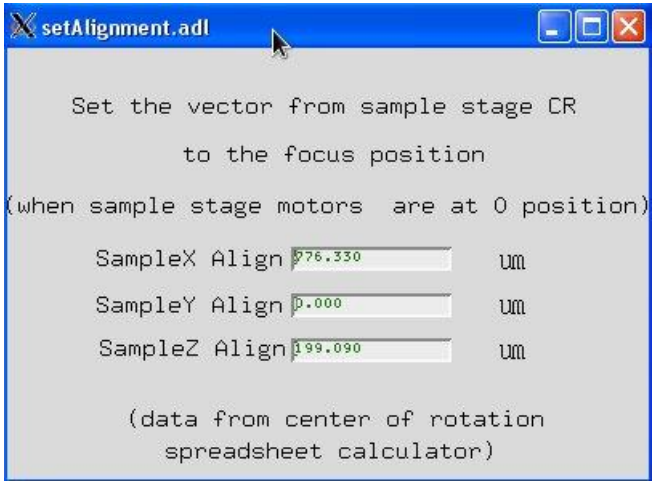
Operation interface

Data Entry				
Number	theta (deg)	stagex (um)	stagey (um)	stagez (um)
1	-90.00	954.00	-1360.00	204.40
1	-70.00	941.00	-1360.00	135.40
1	-50.00	912.00	-1360.00	84.30
1	-30.00	871.00	-1360.00	49.40
1	-10.00	812.00	-1360.00	26.30
1	0.00	781.00	-1360.00	22.50
1	10.00	750.00	-1360.00	27.70
1	30.00	695.00	-1360.00	46.40
1	50.00	644.00	-1360.00	72.80
1	70.00	611.00	-1360.00	124.80
1	90.00	605.00	-1360.00	192.30
0				
0				
0				
0				
	Number of points			
	11			

Results:	
Xalign [um]	776.33
Yalign [um]	0.00
Zalign [um]	199.09

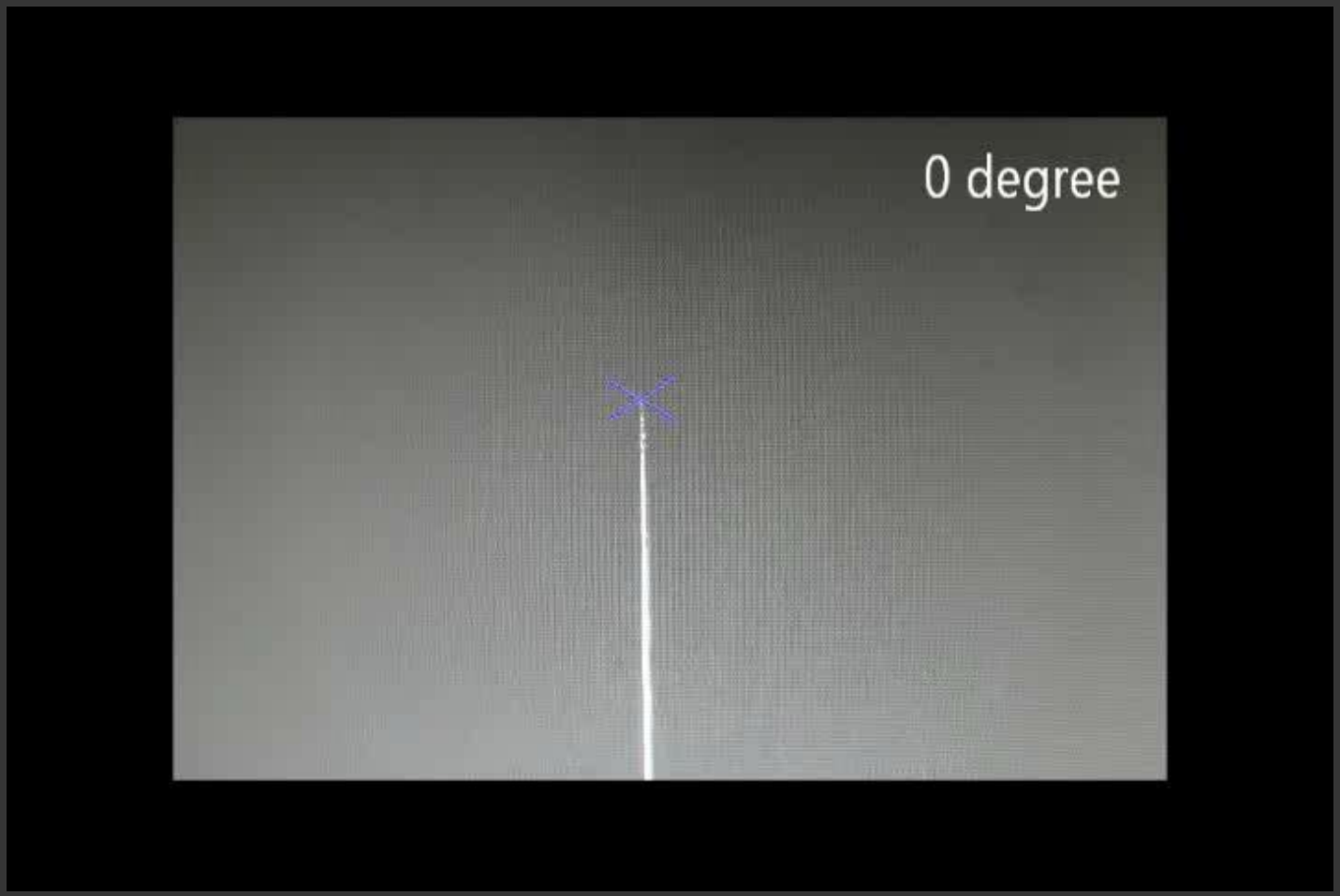
For initial alignment:

- Record θ_i, X_i, Y_i, Z_i in a spreadsheet calculator;
- Obtain the calculated alignment vector, $\vec{a}$;
- Enter to an MEDM GUI window.



XRF Tomographic Dataset Collection

W-tip



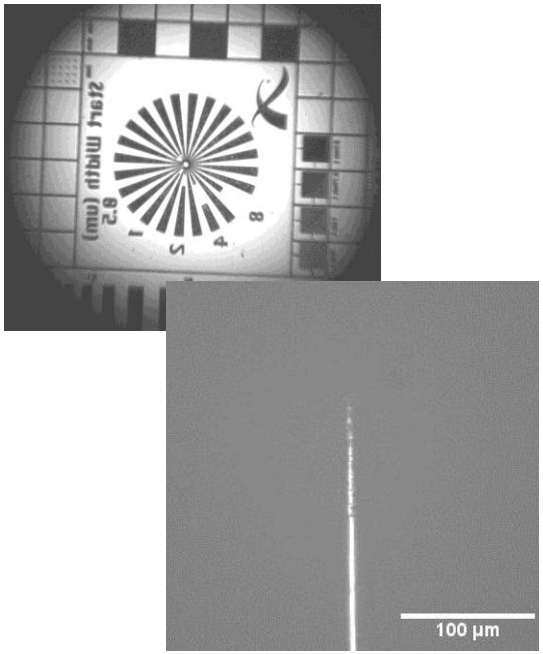
rotation
-20˚ -- +20˚
with a 10˚ step

2D projection
X: 20 µm
Y: 30 µm

rotation, center, focus -- 2D scan -- rotation, center, focus -- 2D scan …

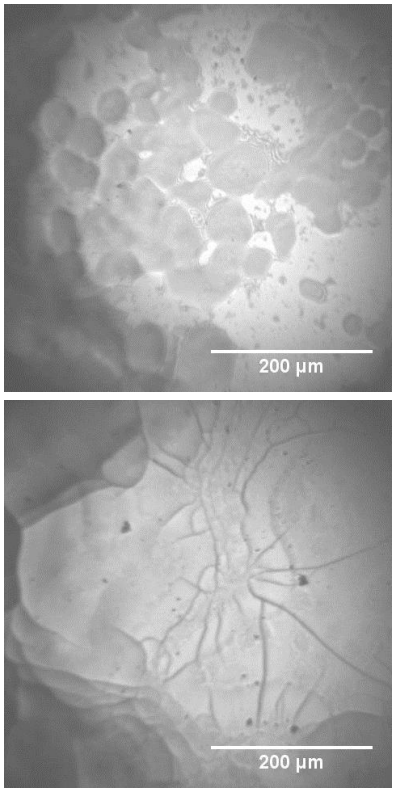
However, it did not work well for frozen samples…

- Issue: we rely on the optical microscope downstream of the sample to determine whether the POI is in the beam focus.



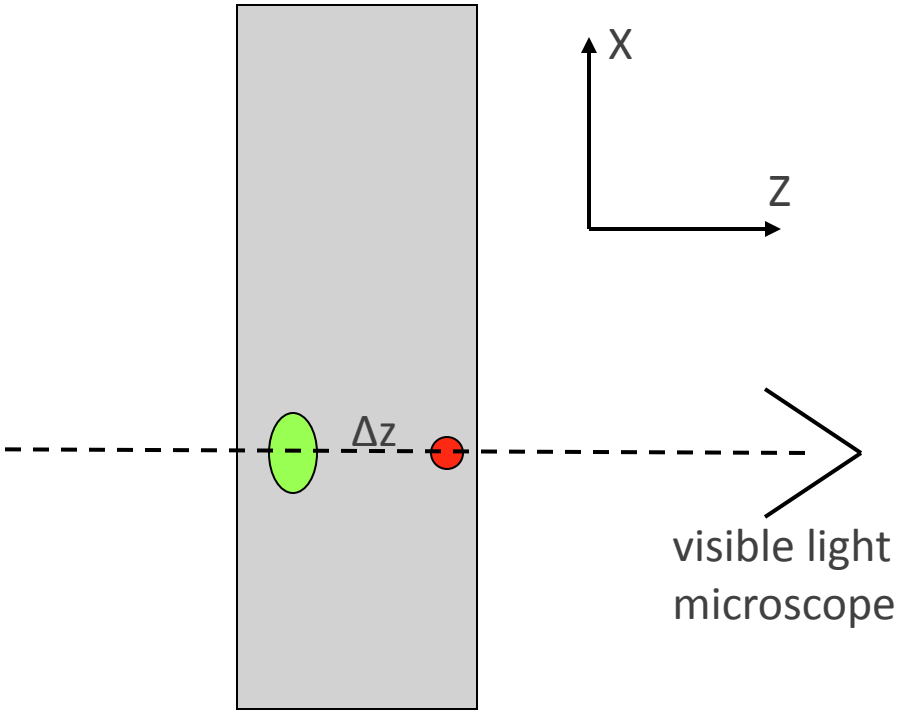
objects with sharp features on the surfaces,
e.g. test pattern, alignment pin

thin biological samples,
e.g. 400-nm section of resin-embedded mouse egg



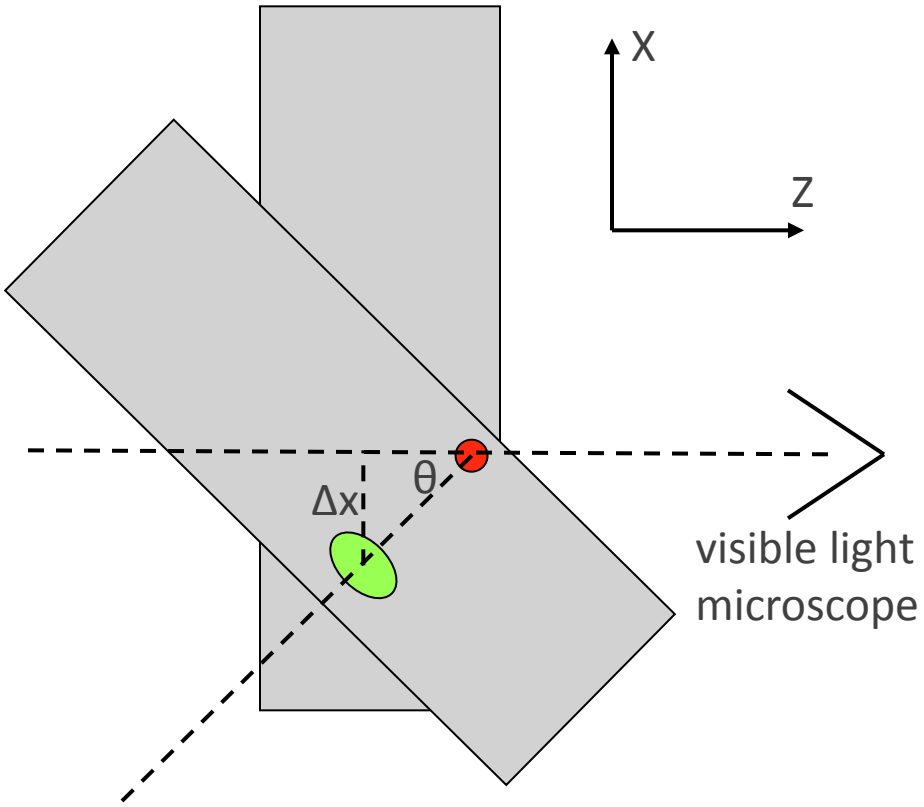
frozen-hydrated samples,
e.g. frozen whole cells (~20 µm)

Correction along the beam direction



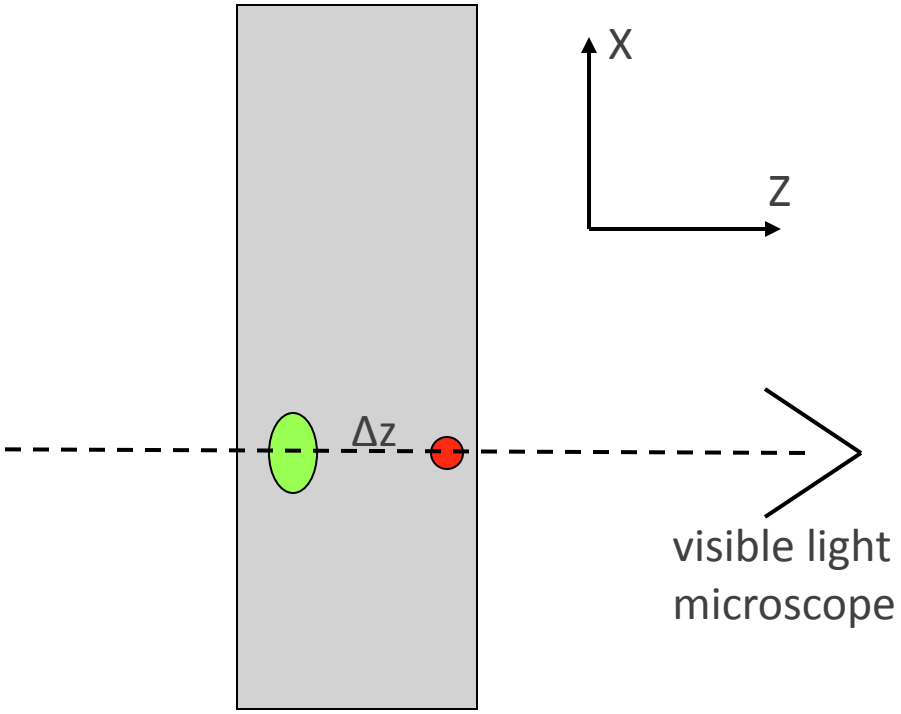
- Determine X position using X-rays;

Correction along the beam direction



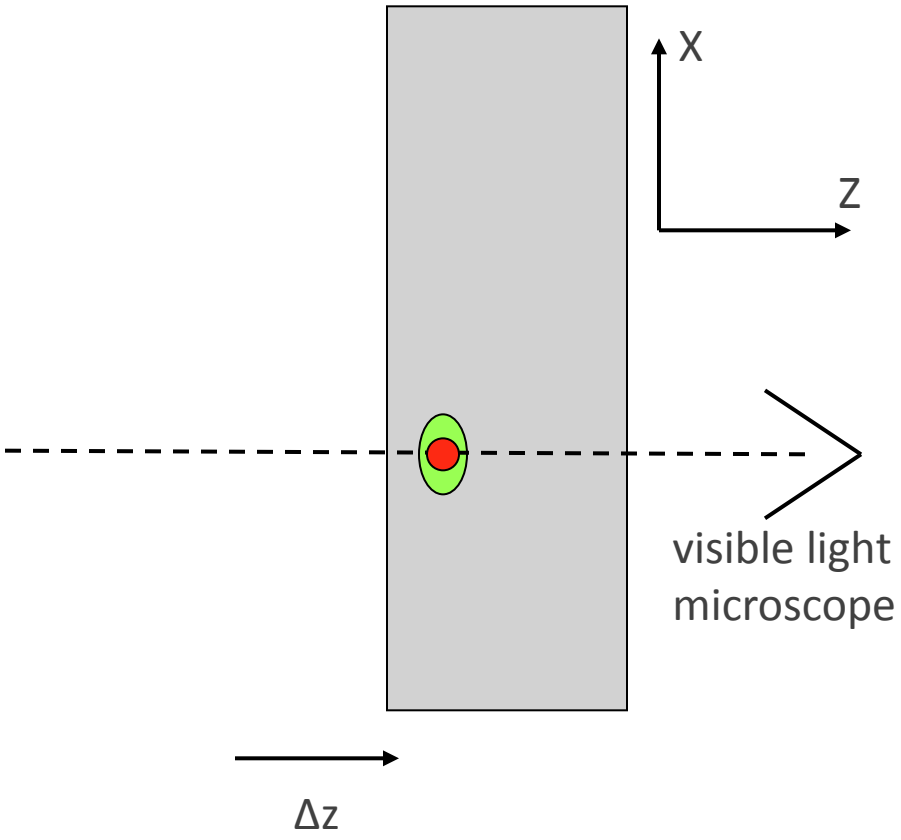
- Determine X position using X-rays;
- Rotate by θ, determine Δx using again X-rays;

Correction along the beam direction



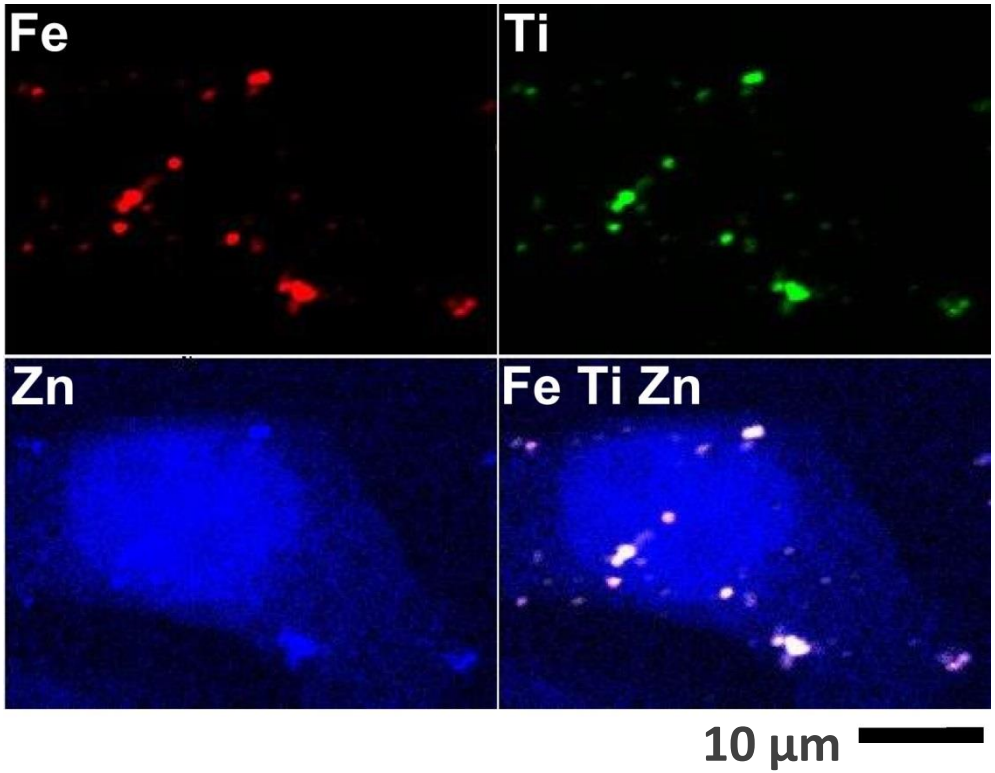
- Determine X position using X-rays;
- Rotate by θ, determine Δx using again X-rays;
- Calculate Δz;

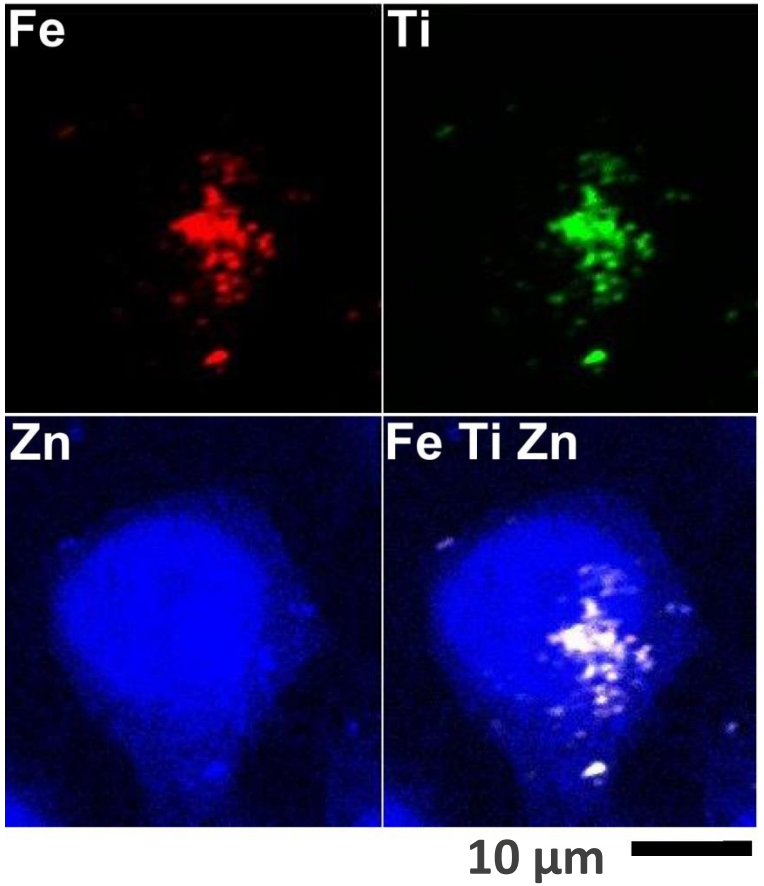
Correction along the beam direction



- Determine X position using X-rays;
- Rotate by θ, determine Δx using again X-rays;
- Calculate Δz;
- Translate the stage in Z by Δz.

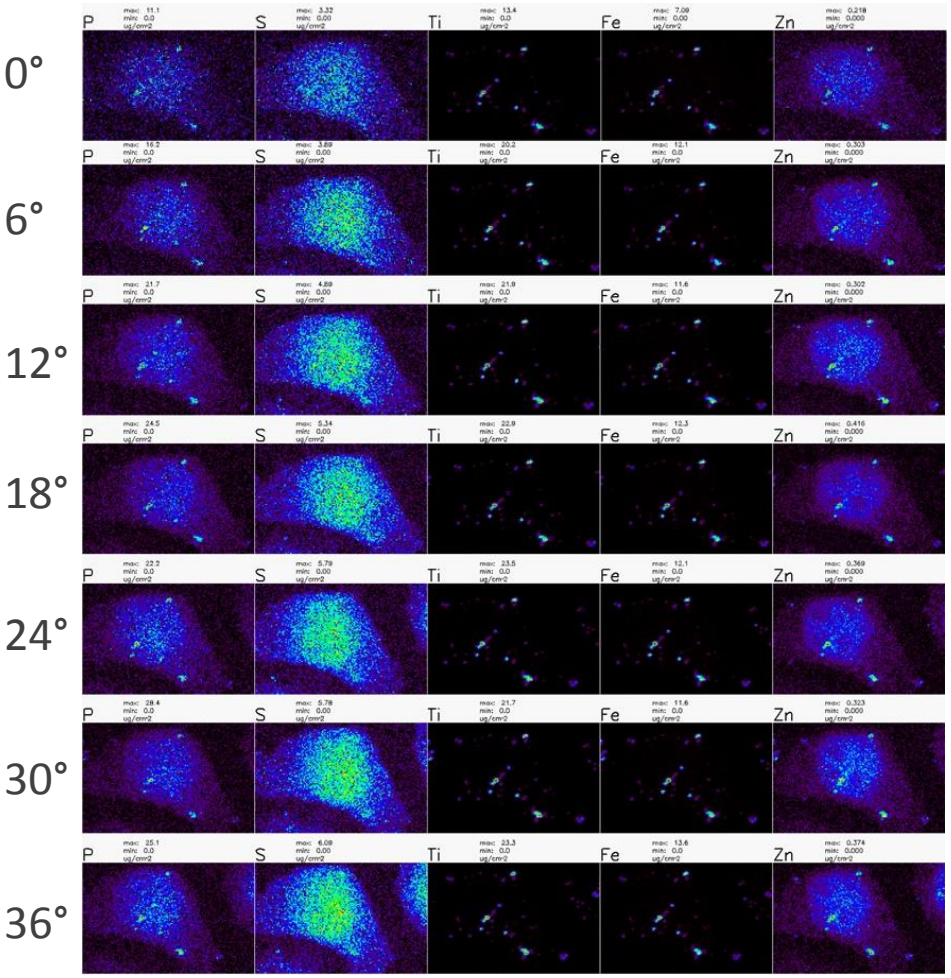
2D elemental mapping of HeLa cells treated with B-loop NCs, incubated for 30 min

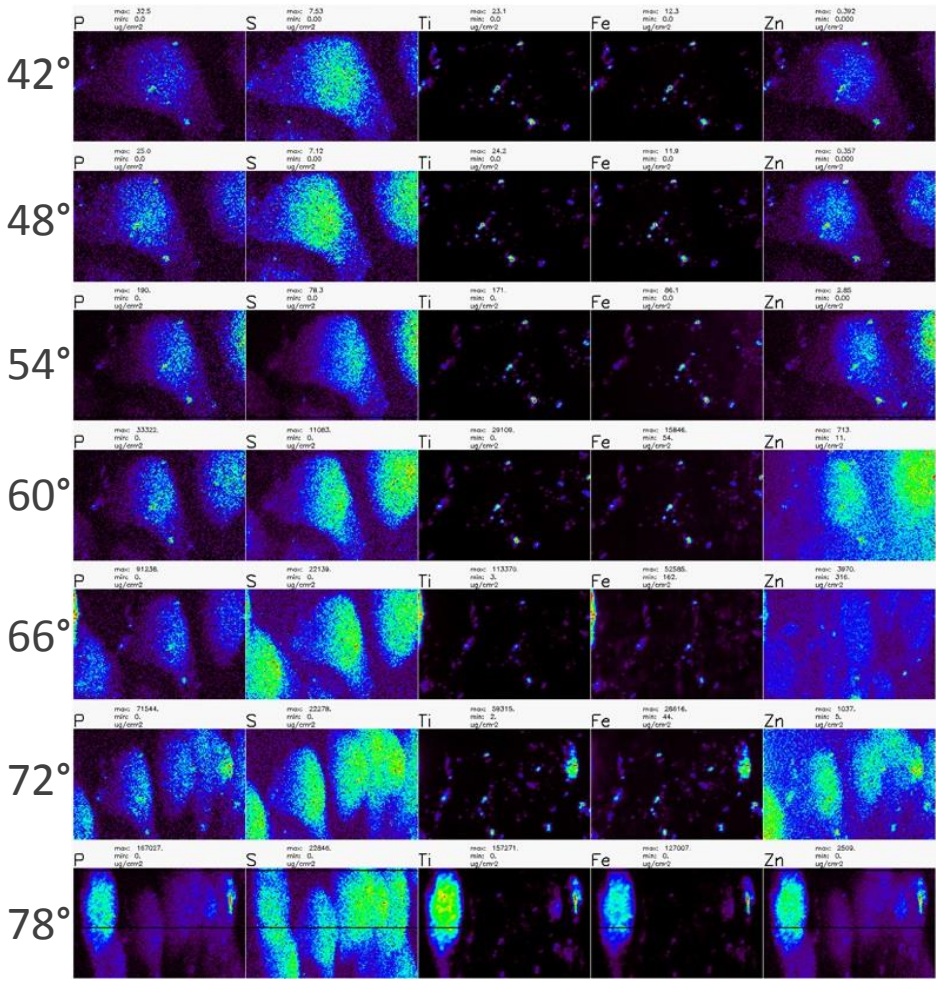




- 150 nm step size, 500 ms dwell time per pixel

Tomographic dataset





10 µm

[Yuan *et al.*, submitted]

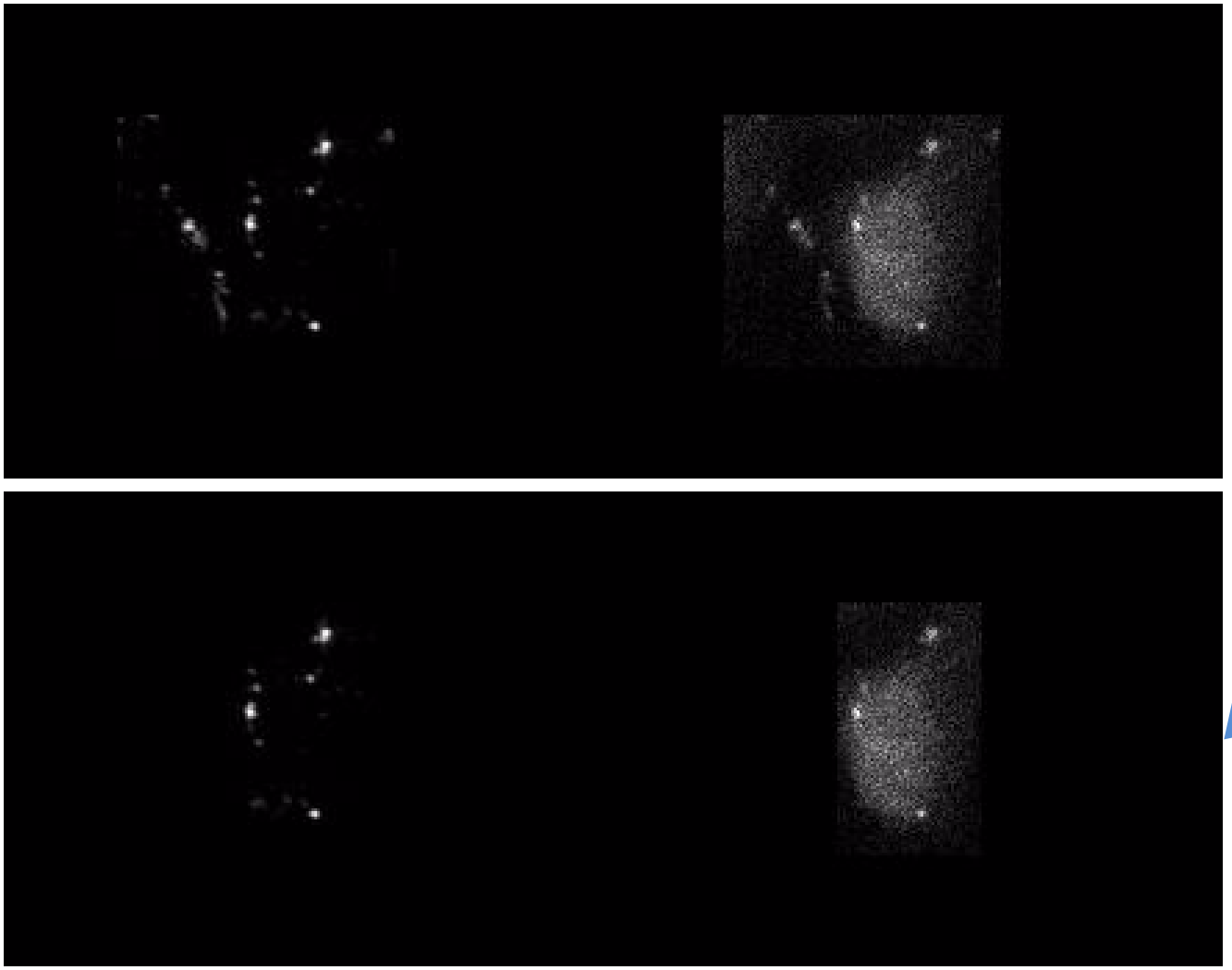
Image registration

Ti

Zn

original series

registered series

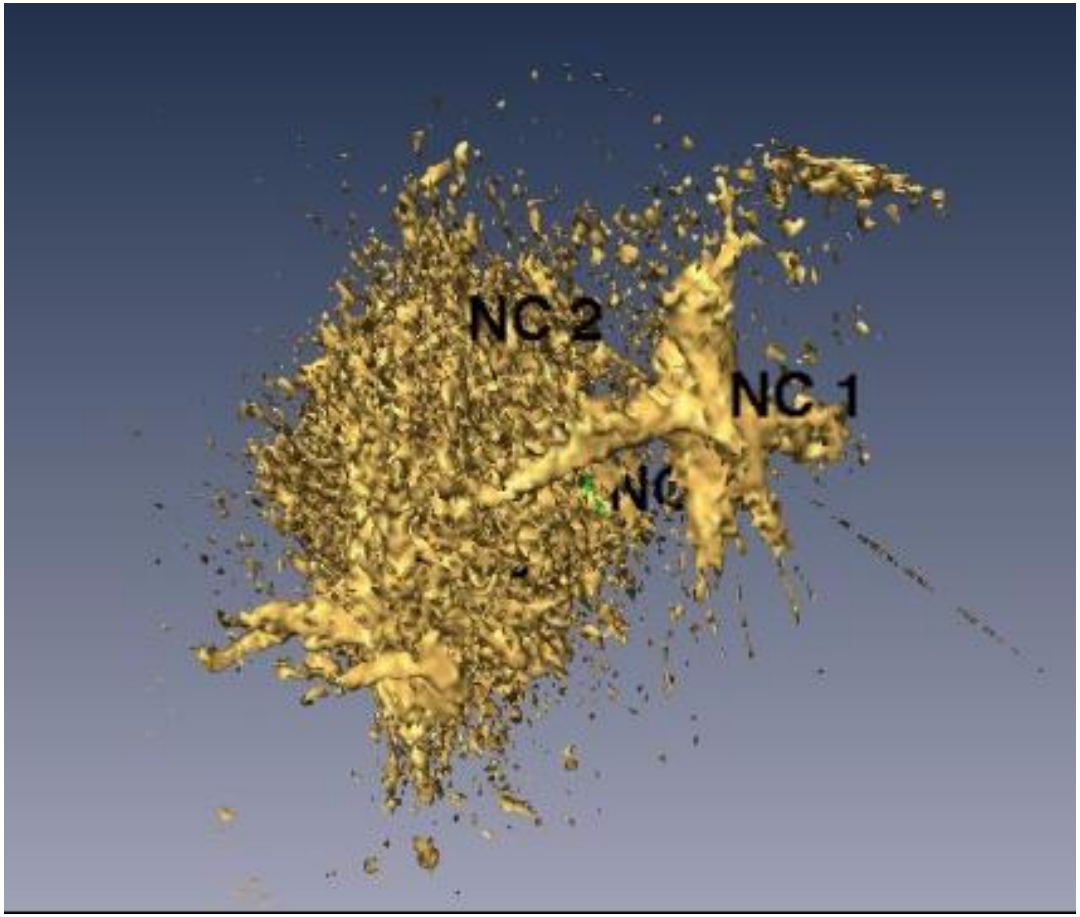


Alignment by cross correlation;

Trimming off the signals from adjacent cells

Reconstruction and visualization

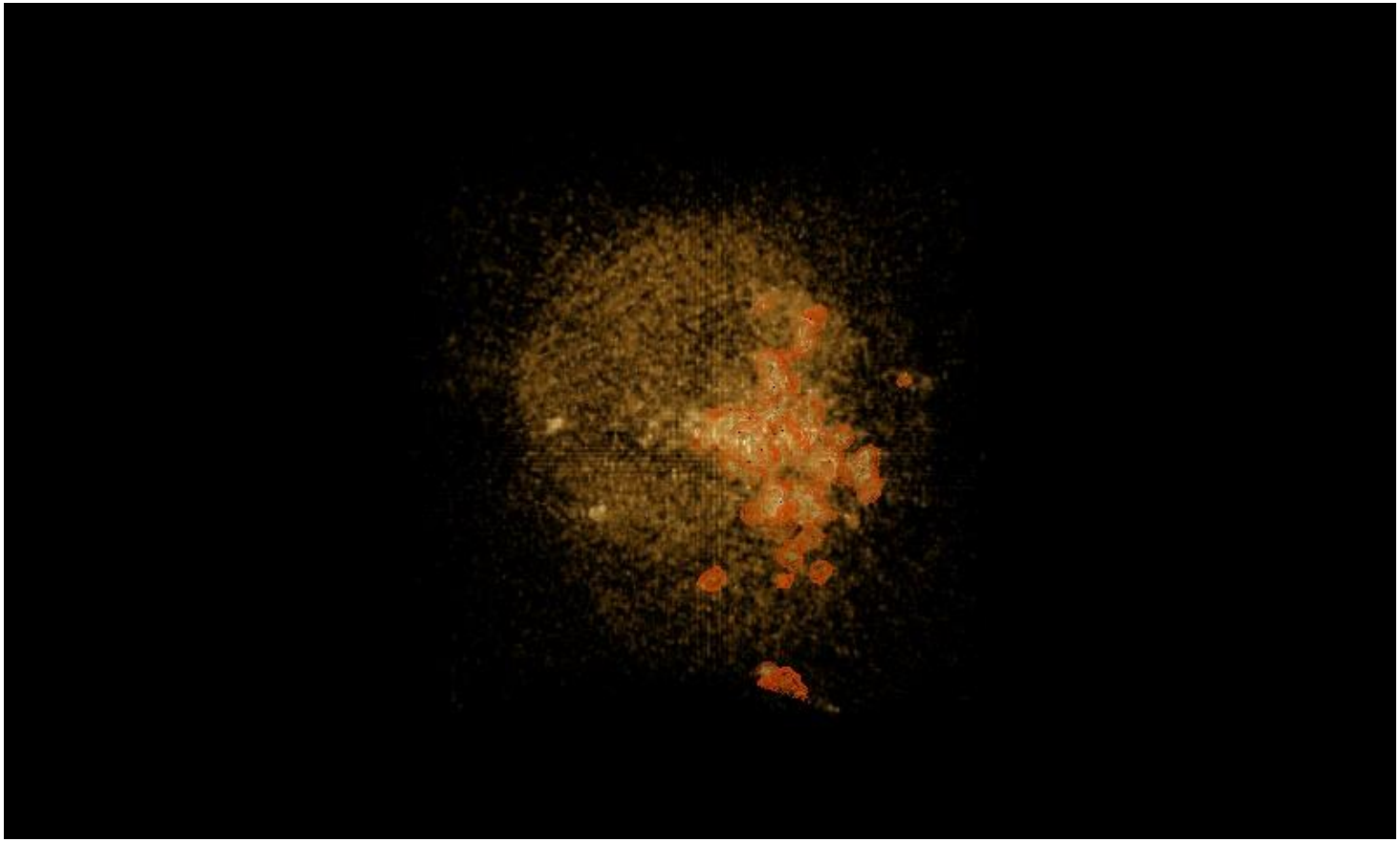
Trial and error: try various methods to get better results



Ti

Zn

reconstruction: TomoJ, simultaneous iterative reconstruction technique; visualization: Amira



Ti

Zn

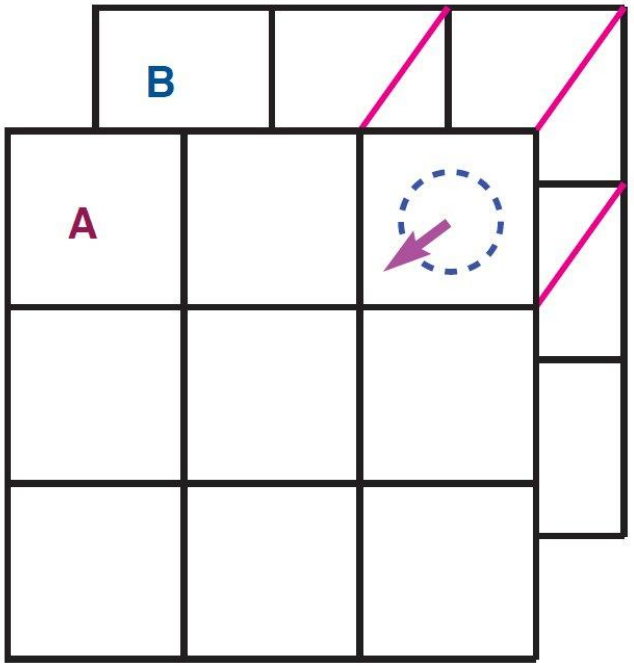
reconstruction: filtered backprojection; visualization: Drishti

Evaluation of image field distortion

- Distortions will complicate fluorescence tomography due to incorrect registration between pixels in projections and the reconstruction volume

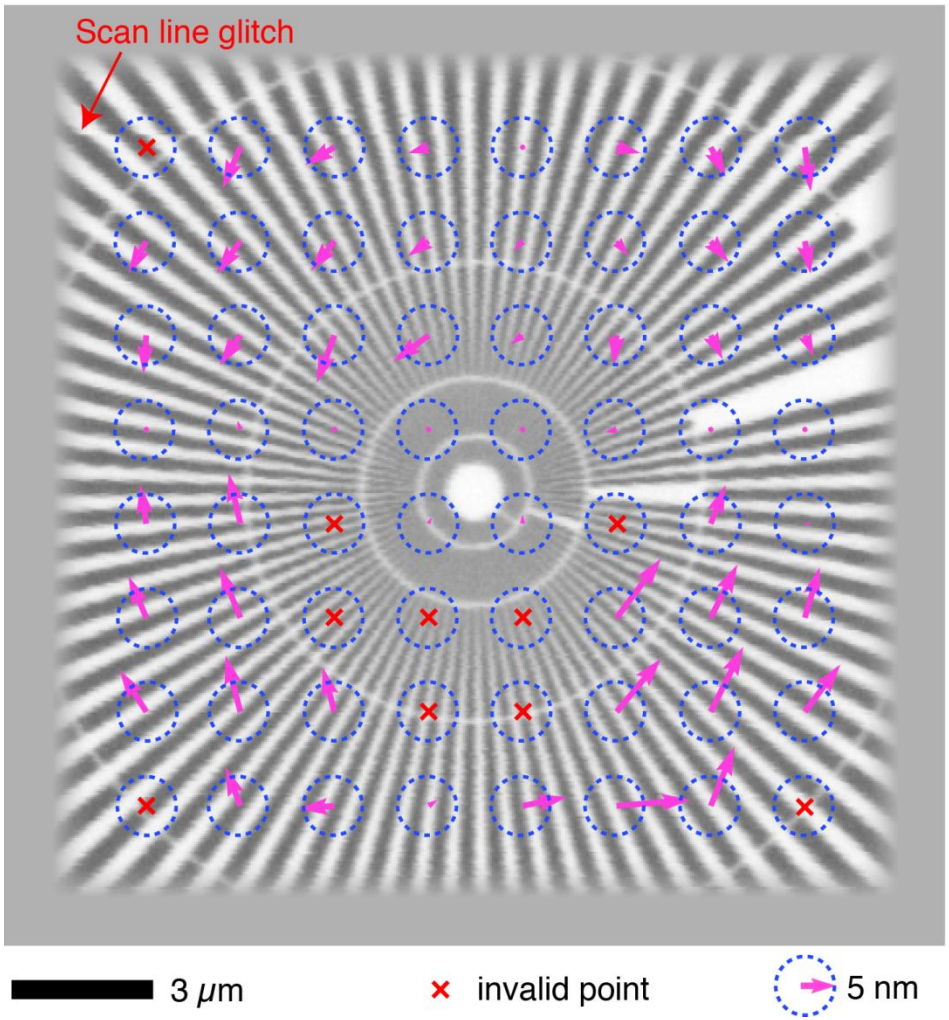
- Image field distortion measurement method:

- Take two images of the same object (**A** and **B**)
- Cross-correlation of center region to correct overall shift
- Divide image into subregions (*e.g.*, 10x10)
- Find sub-pixel registration of subregions, and plot shift of that subregion in image **B** relative to image **A** as a vector with length and direction (arrow)



[Deng *et al.*, in preparation]

Image field distortion map



- 110 K (LN_2-cooled)
- Ni fluorescence images
- 10 keV, 50 nm step, 50 ms/pixel, 360x360 pixels, 70 nm zone plate

- Overall shift: 2.8 pixels (140 nm)
- Subfield shift: <15 nm (85%)

[Chen *et al.*, submitted]

Conclusion and future improvements

- Automated tomographic data collection implementation

  Automated correction for X, Z offset for all the rotation angles;

  Correction in Z for frozen-hydrated samples using X-rays;

  Python scripts to drive rotation/scanning
- Tomography allows visualizing the internal structure in a nondestructive manner
- Future improvements:

  Sample preparation: isolate the object of interest

  Data collection:

  sufficient projections

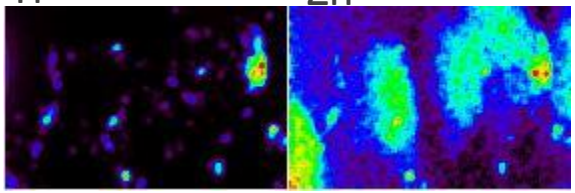
  Registration:

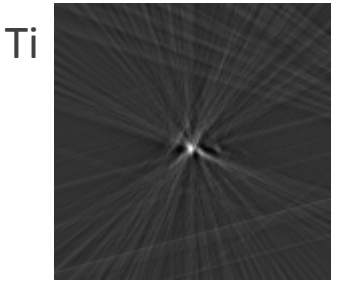
  implementation of distortion correction

  Reconstruction:

  algorithms for missing wedge

  upgrade the downstream optical microscope





cross section

Acknowledgement

APS:

Stefan Vogt, Barry Lai, Chris Jacobsen, Jörg Maser, Christian Roehrig, Deming Shu, Lydia Finney, Sophie Gleber, Qiaoling Jin, Tim Mooney

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