Automated Fluorescence tomography of frozen-hydrated cells at the Bionanoprobe

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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.

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[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 Fe₃O₄/TiO₂ nanocomposites (NCs):

TiO₂ 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

- **Stepper-motor stages (X, Y, Z)**
  - X: ~4 mm
  - Y: ~5.5 mm

- **Piezo stages (X, Y)**
  - X: 100 µm
  - Y: 30 µm

- **Rotation stage ± 90 deg**

- **Sample**

- **Liquid nitrogen temperature**

- **Room temperature**

- 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 $\theta_i$, $\vec{b}_i - \vec{a} = \vec{r}$;

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

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

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

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 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.

\[ \vec{a} \]

\[ \vec{c} [X_0 \ Y_0 \ Z_0] \]

\[ \vec{r} \]

translate by \[ [\Delta x \ \Delta y \ \Delta z] \]
sample (s) coordinate system

base coordinate system
(“zero” position)

sample stage (ss) coordinate system
(“non-zero” position)

θ coordinate system

coordinate transformation matrix:

\[
b_s^T = b_{ss}^T \cdot b_{ss}^{\theta T} = \begin{bmatrix}
1 & 0 & 0 & \Delta X + X_0 \\
0 & 1 & 0 & \Delta Y + Y_0 \\
0 & 0 & 1 & \Delta Z + Z_0 \\
0 & 0 & 0 & 1
\end{bmatrix}
\]

general case: \( \theta \neq 0 \) when set the POI

\[
b_s^T g = b_{ss}^T \cdot b_{ss}^{\theta T} \cdot b_{ss}^{\theta T} = \begin{bmatrix}
\cos \theta & 0 & \sin \theta & X_0 \cos \theta + Z_0 \sin \theta + \Delta X \\
0 & 1 & 0 & Y_0 + \Delta Y \\
-\sin \theta & 0 & \cos \theta & -X_0 \sin \theta + Z_0 \cos \theta + \Delta Z \\
0 & 0 & 0 & 1
\end{bmatrix}
\]
Calculation of the offset vector \([X_0 Y_0 Z_0]\)

When set the POI, the known parameters: \((\theta \Delta X \Delta Y \Delta Z) (X_a Y_a Z_a)\)

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:

\[
\begin{aligned}
X_0 &= X_a \cos \theta - Z_a \sin \theta - \Delta X \cos \theta + \Delta Z \sin \theta \\
Y_0 &= Y_a - \Delta Y \\
Z_0 &= X_a \sin \theta + Z_a \cos \theta - \Delta X \sin \theta - \Delta Z \cos \theta
\end{aligned}
\]
For tomographic data collection: Motor positions are needed!

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:

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

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

Then, the motor positions for each \(\theta_i\) are:

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

For initial alignment:
- Record $\theta_i$, $X_i$, $Y_i$, $Z_i$ in a spreadsheet calculator;
- Obtain the calculated alignment vector, $\hat{a}$;
- Enter to an MEDM GUI window.

<table>
<thead>
<tr>
<th>Number</th>
<th>theta (deg)</th>
<th>stagex (um)</th>
<th>stagey (um)</th>
<th>stagez (um)</th>
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<tbody>
<tr>
<td>1</td>
<td>-90.00</td>
<td>954.00</td>
<td>-1360.00</td>
<td>204.40</td>
</tr>
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<td>90.00</td>
<td>605.00</td>
<td>-1360.00</td>
<td>192.30</td>
</tr>
</tbody>
</table>

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

Set the vector from sample stage CR to the focus position
(when sample stage motors are at 0 position)

- SampleX Align 776.339 um
- SampleY Align 0.000 um
- SampleZ Align 199.099 um

(data from center of rotation spreadsheet calculator)
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 $\theta$, determine $\Delta x$ using again X-rays;
Correction along the beam direction

- Determine X position using X-rays;
- Rotate by $\theta$, determine $\Delta x$ using again X-rays;
- Calculate $\Delta z$;

![Diagram of visible light microscope](image)

$\Delta z$
Correction along the beam direction

- Determine X position using X-rays;
- Rotate by $\theta$, determine $\Delta x$ using again X-rays;
- Calculate $\Delta z$;
- Translate the stage in Z by $\Delta z$. 
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

[Yuan et al., submitted]
Image registration

Alignment by cross correlation;
Trimming off the signals from adjacent cells
Reconstruction and visualization

**Trial and error:** try various methods to get better results

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