Whole cell structural imaging at 20 nanometre resolutions using MeV ions

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Abstract

MeV proton and alpha (helium ion) particle beams can now be focused to 20 nm spot sizes, and ion/matter simulations using the DEEP computer code show that these resolutions are maintained through the top micrometre or so of organic material. In addition, the energy deposition profiles of the transmitted ions are laterally constrained to a few nanometers from the initial ion path. This paves the way for high resolution structural imaging of relatively thick biological material, e.g. biological cells. Examples are shown of high resolution structural imaging of whole biological cells (MRC5) using on-axis scanning transmission ion microscopy (STIM). Nanoparticles have the ability to cross the cell membrane, and may therefore prove useful as drug delivery probes. We show that the combination of on-axis STIM for imaging the cell interior, and off-axis STIM for imaging gold nanoparticles with enhanced contrast within the cell, represents a powerful set of ion beam techniques for tracking gold nanoparticles in biological cells. Whole cell imaging at high spatial resolutions represents a new area for nuclear microprobes.

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1. Introduction

Although nuclear microprobes have been around for over 3 decades, they have still to reach their true potential in terms of technological development and application areas. However, electron technology, e.g. electron beam writing, electron microscopy, and electron spectroscopy are areas that have essentially reached a high level of maturity. In many respects, fast ion (e.g. MeV proton) technology is analogous to electron technology, in that protons can be used for fabricating structures (e.g. proton beam writing), imaging features (e.g. scanning transmission ion microscopy – STIM), and carrying out spectroscopy (e.g. Rutherford Backscattering Spectrometry-RBS). Electron beam technology has achieved great success primarily because it is able to image and fabricate features with higher spatial resolution compared to optical techniques, which in general are limited by the diffraction of light to around 250 nm. It is sensible to suggest therefore that proton technology will have a similar impact in the near future, especially when sub-100 nm spatial resolutions have now been reached.

The prime characteristics of fast ion technology (e.g. MeV protons) can be summed up as follows: (a) because ions have high mass compared to atomic electrons, the trajectory of a focused beam of fast ions into matter is essentially a straight line until the end of range, where beam spreading occurs; (b) as the ions traverse the material, they can knock out many thousands of second-
models, namely Hansen–Koch–Stolterfoht model [4]. Fig. 1 shows the energy density profiles for 3.0, 1.0 and 0.5 MeV protons travelling through 1 micrometre of organic material, in this case a commonly used resist material widely used in lithography (PMMA). The simulations indicate that the protons travel in a straight line, and that the lateral energy deposition caused by secondary electrons is low, reaching minimal levels for the primary particle energies of 0.5 MeV. As a comparison, we have included simulations for electrons (1 MeV, 100 keV, and 25 keV) travelling through the same material. Table 1 shows the relevant stopping powers of 1 MeV protons compared with 1 MeV, 100 keV and 25 keV electrons, showing that the energy deposition for one proton greatly exceeds that of an electron. For a proper comparison, we need to match the total energy deposition of the protons to that of the electrons. For example, a 1 MeV proton (stopping power of 253 (MeV cm^2/g)) as compared to a 100 keV electron (stopping power of 4.01 (MeV cm^2/g)) has a ratio of energy deposition of 253/4.01 = a factor of 63. Therefore it requires 63 times more 100 keV electrons to produce the same energy deposition as one 1 MeV proton.

As can be seen from Fig. 1 the lateral electron energy deposition profiles are significantly worse compared with protons. This is to be expected, since the low mass of the primary electrons transfers significantly more energy to secondary electrons, and suffers much more large angle scattering compared to the higher mass primary protons.

For microscopy, a highly focused MeV ion beam can pass through thin sections e.g. a whole single cell, without significant loss of spatial resolution, and produces only short range lateral electrons. Radial energy deposition is much reduced compared with electron microscopy. Scanning Transmission Ion Microscopy and Proton Induced Fluorescence should be possible at levels of 1–5 nm.

For lithography, practical considerations indicate that proton lithography using 1 MeV proton (p-beam writing) could be 25–60 times faster than the equivalent e-beam writing (per incident particle), depending on the primary particle energy used. P-beam writing also has minimal proximity effects (unwanted lateral energy deposition) and maintains a vertical path through the resist material (straight and smooth sidewalls). Structures down to 1–5 nm should therefore be possible in the top 1 micrometre of resist material.

Table 1

<table>
<thead>
<tr>
<th>Particle</th>
<th>Stopping power: average energy loss in PMMA of the particle per unit path length</th>
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<tbody>
<tr>
<td>1 MeV protons</td>
<td>253 (MeV cm^2/g)</td>
</tr>
<tr>
<td>1 MeV electrons</td>
<td>1.8 (MeV cm^2/g)</td>
</tr>
<tr>
<td>100 keV electrons</td>
<td>4.01 (MeV cm^2/g)</td>
</tr>
<tr>
<td>25 keV electrons</td>
<td>10.8 (MeV cm^2/g)</td>
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The simulations discussed above have been tested from SEM observations of proton beam written structures. In an experiment involving the fabrication of an array of nanopillars in HSQ resist [5] (see Fig. 2a), some of the nanopillars were detached during the development process, and these detached horizontal pillars allowed accurate dimensional imaging using scanning electron microscopy. The detached pillars were measured to be 850 nm long, and the diameters at each end of the pillars were measured to be 85 ± 5 nm. No differences were observed between the ends of each pillar within experimental measuring errors, indicating that the proton beam essentially travelled in a straight path in the resist. In addition, this also indicates that the lateral energy deposition was constant throughout the initial 850 nm path of the proton in the resist. This implies that the simulations outlined above are consistent with our observed experimental results.

3. Current state-of-the-art spatial resolutions for focused MeV ion beams

The state-of-the-art spatial resolutions for MeV proton beams (low current) are 20 × 30 nm, with a best figure of 13 nm for the width of a line [6]. These tests were carried out using the 2nd generation proton beam writing beam line facility in CIBA, a facility based on a spaced triplet of Oxford Microbeams compact OM52 quadrupole lenses. The spatial resolution for MeV alpha particles beams has been measured using the new cell imaging facility at CIBA [7], which has the same beam optical configuration, and the results are shown in Fig. 3. Using the calibration target fabricated using a combination of proton beam writing and nickel electroplating [8], a 1000 × 1000 pixel STIM image was accumulated using a current of approximately 10,000 alpha particles per second. From the image, vertical and horizontal edge profiles were extracted, and resolutions of 20 nm horizontal and 25 nm vertical were measured.

4. Imaging of whole cells using on-axis STIM

The straight path characteristics of fast ion beams focused to sub 100 nm spot sizes can lead to a wide variety of useful applications. One such application is the imaging of whole dried biological cells using Scanning Transmission Ion Microscopy (STIM). The thickness of a dried whole cell is around 1–2 micrometre on average, and so the spatial resolution of the STIM image would be expected to be the same throughout the cell, and be consistent with the original dimensions of the initial focused beam.

5. Imaging of whole cells using off-axis STIM

One of the emerging fields in biomedicine is the use of nanoparticles in drug delivery systems (DDS) [10]. Gold nanoparticles (Au NPs), Carbon NanoTubes (CNTs) and Quantum Dots (QDs) exhibit characteristics that have high potential for successful DDS, since they have dimensions that allow passage through cell membranes without modifying their integrity. The nanoparticles can also undergo surface functionalization so as to target and deliver drugs to the required part of the cell. The success of such procedures require the nanoparticles to be tracked within the cell through advanced microscopies. The problem is that there are very few microscopy technique capable of tracking nanoparticles within whole cells at high resolutions: Optical microscopy is limited by diffraction effects, SEM has high resolutions only at the cell surface, and because electrons scatter from their initial trajectory when they enter the cell, then in general TEM requires multiple thin cellular sections. We have carried out some preliminary experiments in order to test the hypothesis that fast ion STIM has the requisite characteristics to image gold nanoparticles within whole cells at high resolutions.

MRC5 fetal lung fibroblasts were seeded on 100 nm thick silicon nitride windows at 2500 cells/cm² in RPMI medium supplemented with fetal bovine serum (FBS, 10%), penicillin (100 units/ml) and streptomycin (100 μg/ml). After 24 h, cells were washed with phosphate-buffered saline (PBS) followed by incubation in

Fig. 2. (a) SEM image of an array of vertical nanopillars written in 850 nm thick HSQ resist: and (b) a selection of the pillars that have fallen over. The pillars measure 85 nm ± 5 nm at both the top and the bottom of the pillar, indicating no beam widening at a depth of 850 nm.
supplemented medium containing FBS-coated gold nanoparticles (5 pM) for another 24 h. Following several washes with PBS to remove unbound nanoparticles, cells were fixed in formaldehyde (4%) overnight before post-fixation in osmium tetroxide (1%) for 1 h in order to stabilize the cell surface. Cells were then dehydrated through an ethanol gradient followed by critical point drying.

One MRC5 cell was scanned using a 1.6 MeV alpha particle beam focused to around 20 nm, and the results are shown in Fig. 5. Fig. 5a shows a SEM image of the cell, depicting the surface features, and Fig. 5b shows a corresponding on-axis STIM image indicating the nucleus, nucleoli and various structures not observed in the SEM image. Fig. 5c shows a higher magnification SEM image, and Fig. 5d shows the equivalent on axis STIM image. The nucleus is clearly shown in the STIM image, as well as small circular structures of the order of 1 micrometre in diameter or less. Some of these structures are also observed in the SEM image and are therefore present on the surface. It is possible that these structures may be caused by increased localized levels of osmium tetroxide, which was introduced in the cell preparation protocol to stabilize lipids by cross linking at unsaturated double bonds. The osmium tetroxide, which is more dense than organic material, helps stabilize the cell membrane, and accumulates in lipid vesicles within the cell and on the cell surface. The 100 nm gold nanoparticles are not apparent in the on-axis STIM image. In order to increase the contrast for the 100 nm gold nanoparticles, we have utilized off-axis STIM. This technique collects and measures the

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**Fig. 3.** (a) SEM image of a calibration grid fabricated using proton beam writing and nickel electroplating, (b) STIM image of the grid using a focused 1.6 MeV alpha particle beam approximately 10,000 alpha particles per second, (c) STIM image of a portion of the grid, (d) and (e) horizontal and vertical line scans extracted from the regions depicted as shown in 3(c), showing spot size of 20 × 25 nm.
Fig. 4. STIM images at different magnifications of a MRC5 cell using a 1.7 MeV alpha particle beam. Scan sizes 50 \( \mu \text{m} \) and 25 \( \mu \text{m} \) respectively. Median fit using 11 alpha counts per pixel for pixel normalization.

Fig. 5. (a) SEM micrograph of a dried MRC5 cell that has been exposed to 100 nm gold nanoparticles, (b) on-axis STIM image of the same cell indicating the nucleus, nucleoli and various structures not observed in the SEM image, (c) a higher magnification SEM image showing the central region of the MRC5 cell, (d) shows the equivalent on-axis STIM image, and (e) shows the equivalent off-axis STIM image, indicating high contrast for the gold nanoparticles.
energy of the transmitted ions scattered at a slight angle (around 10 degrees) from the beam axis. This technique is therefore governed by the well-known Rutherford scattering formula:

\[
\sigma = \frac{Z_1 Z_2 e^4}{4E_c \sin^2 \left( \frac{\theta_e}{2} \right)}
\]

where \(Z_1\) and \(Z_2\) are the atomic numbers of the incoming ion, and the target ion respectively, \(E_c\) is the incoming ion energy, and \(\theta_e\) is the scattering angle from the original ion path.

Because gold has an atomic number of 79, then the cross section for scattering is greatly enhanced compared with normal organic elements such as carbon (atomic number 6). Fig. 5e shows the greatly increased contrast for the gold nanoparticles as compared with both the cell constituents and also the osmium tetroxide stained vesicles. Thus a combination of on-axis STIM to highlight the internal cell features, and off-axis STIM to image the gold nanoparticles has proved highly successful in imaging AuNPs within a whole cell.

6. Conclusions

The field of nuclear microprobes has been developing for over 30 years, and has still to reach its full potential. The point at which MeV ion beam spot sizes reached sub 100 nm represented a significant step, since this represents the approximate limit of optical resolutions. Our latest results in CIBA show that we can focus MeV ion beams (both protons and alpha particles) to 20 nm, and theoretical simulations indicate that if we can attain spot sizes below 10 nm (which should be possible using high brightness ion sources), then this spatial resolution can be maintained through at least a micrometer below the surface of a sample. Coupled with a greatly reduced lateral deposition of energy through induced secondary electrons, the fast ion has a significant role to play in both direct write lithography and microscopy.

One interesting new application area is the use of nanoparticles for drug delivery, since the nanoparticle can both cross the cell membrane and be functionalized to include targeting and drug delivery molecules. There are currently very few advanced microscopical techniques that can image nanoparticles inside a whole cell at high resolutions, and this lack of imaging technology is limiting advances in this field. We have shown however that by using on-axis STIM, the interior structure of a whole cell can be imaged at high spatial resolution, and that by using off-axis STIM we can significantly enhance the contrast for internalized gold nanoparticles. This in our opinion represents a new area for high spatial resolution nuclear microprobes.

References