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A correlative optical microscopy and scanning electron microscopy approach to locating nanoparticles in brain tumors

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ABSTRACT

The growing use of nanoparticles in biomedical applications, including cancer diagnosis and treatment, demands the capability to exactly locate them within complex biological systems. In this work a correlative optical and scanning electron microscopy technique was developed to locate and observe multi-modal gold core nanoparticle accumulation in brain tumor models. Entire brain sections from mice containing orthotopic brain tumors injected intravenously with nanoparticles were imaged using both optical microscopy to identify the brain tumor, and scanning electron microscopy to identify the individual nanoparticles. Gold-based nanoparticles were readily identified in the scanning electron microscope using backscattered electron imaging as bright spots against a darker background. This information was then correlated to determine the exact location of the nanoparticles within the brain tissue. The nanoparticles were located only in areas that contained tumor cells, and not in the surrounding healthy brain tissue. This correlative technique provides a powerful method to relate the macro- and micro-scale features visible in light microscopy with the nanoscale features resolvable in scanning electron microscopy.

1. Introduction

The increasing use of nanoparticles in biomedical applications, such as cancer diagnosis and treatment (Devaraj et al., 2009; Ptak et al., 2010; Thakor et al., 2011), joint repair (Ma et al., 2003) and drug delivery (Farokhzad and Langer, 2009) has necessitated the ability to accurately and precisely locate them within complex biological systems. Scanning electron microscopy (SEM) is a powerful tool for characterizing the size, shape and structure of nanoparticles owing to its ability to resolve nanometer scale features (Roussel et al., 2009).

SEM has been successfully utilized to locate nanoparticles in cells and tissue through the use of backscattered electron imaging (BSE) to distinguish between the inorganic nanoparticles and the surrounding organic structure (Koh et al., 2008; Shachaf et al., 2009; Papis et al., 2009). Unfortunately, there is no mechanism to readily distinguish between tissue types, e.g. healthy and cancerous tissue, in the SEM due to a lack of contrast either in BSE or secondary electron (SE) modes.

Through the use of staining protocols (Presnell and Schreibman, 1997) it is possible to discern biologically relevant differences in the structure of tissue sections, including the presence of a brain tumor,

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through optical microscopy (OM) (Kircher et al., 2012). Owing to the relatively low magnification utilized it is possible to image large areas in the microscope, including macroscopic features such as blood vessels. OM however has a spatial resolution of approximately 250 nm due to the wavelength of visible light (Svitkina and Borisy, 1998). Furthermore, OM has a limited depth of field at high magnifications. These limitations restrict conventional OM to the microscopic regime and make it impossible to locate and image nanoparticles.

By utilizing these two techniques in tandem and correlating the results it is possible to exploit the benefits of both while minimizing their respective disadvantages (Sartori et al., 2007; Svitkina and Borisy, 1998). SEM offers evidence of nanoparticle accumulation and localization in the tissue, while OM provides biologically relevant information, such as the presence of tumor or healthy tissue. In the present work, we developed a correlative optical and scanning electron microscopy approach to locate triple modality nanoparticles (magnetic resonance imaging-photoacoustic imaging-Raman imaging (MPRs)) within brain tissue and to determine if they are located in healthy or tumor tissue.

2. Methods and materials

2.1. Mouse and tissue preparation

Male severe combined immunodeficiency (SCID) mice implanted with an orthotopic primary human xenograft glioblastoma, TS543, were injected with MPRs via their tail vein (Kircher et al., 2012). All mice were treated following animal protocol (#06-07-011) approved by the Institutional Animal Care and Use Committee of Memorial Sloan Kettering Cancer Center on October 20th 2006. The MPRs consist of a 60 nm diameter gold core surrounded by a monolayer of the Raman tag *trans*-1,2-bis(4-pyridyl)-ethylene and encapsulated in a 30 nm silica shell. This was then coated with maleimide-DOTA-Gd^{3+} resulting in a gold–silica core–shell surface enhanced Raman scattering (SERS) nanoparticle covered with Gd^{3+} ions (Kircher et al., 2012). It was anticipated that the MPRs would enter the extravascular space of the tumor via the enhanced permeability and retention (EPR) effect and as a result no targeting motif was needed for MPR uptake. 24 h post injection the mice ($n=2$) were sacrificed and the brain excised (Kircher et al., 2012). The brain tissue was then embedded in Tissue-Tek® optimal cutting temperature (O.C.T.) compound (Sakura), and snap-frozen in liquid nitrogen (Kircher et al., 2012). Ten-micron thick sections of frozen tissue were obtained using a Leica cryotome and adjacent tissue sections, one each, were stained following the hematoxylin and eosin (H&E) histochemical and Olig2-specific (OLIG) immunohistochemical staining protocols to reveal the general structure of the tissue section and the exact location and extent of the tumor respectively (Kircher et al., 2012; Ligon et al., 2004; Presnell and Schreibman, 1997). The OLIG stain was selected because the TS543 tumor model overexpresses the Olig2 transcription factor. H&E stained tissue sections were prepared without a cover slip contrary to standard protocols because SEM is a surface sensitive technique and the cover slip would inhibit the ability to image the nanoparticles.

2.2. Optical microscopy imaging of tissue samples

The OLIG stained sections were imaged using a Leica TCS SP2 AOBS Confocal Laser Scanning Microscope or a Leica DMI6000 inverted fluorescence microscope.

H&E stained tissue sections were imaged using a DM Leica 2000 light microscope at 100× magnification. Between 50 and 100 images were acquired from each tissue section and stitched together by hand using Adobe Photoshop to create a large composite image of the entire brain section. Images were taken with partial overlap to provide regions to align each image with its respective neighbor images.

2.3. Scanning electron microscopy imaging of tissue samples

After imaging the H&E stained tissue sections in the optical microscope, the sections were then sputter coated with a thin layer of AuPd using a Cressington 108 Sputter Coater for 30 s with a current of 20 mA to provide a conductive surface layer.

The samples were then imaged using a Magellan XHR SEM operated at 15 kV with a probe current of 50 pA and spatial resolution of 1 nm in secondary electron (SE) mode. In BSE mode, the resolution is slightly worse at approximately 5 nm due to decreased signal intensity and in-sample electron scattering. A low magnification montage image was created of the entire brain section using the automated montage stitching program embedded in the SEM software. Two-hundred and twenty-five (225) low magnification (i.e. 200 times magnification) images each with a 1 mm field of view were taken and stitched together creating the montage image with a 14.9 mm field of view. The tissue was then examined at 5000× magnification using the BSE detector to locate the higher atomic number MPRs in the tissue. The MPRs appear as bright spots in the image due to their gold core. This magnification was selected to maximize the analysis efficiency by covering the largest possible area while still being able to resolve individual MPRs.

The beam conditions were optimized to maximize the BSE signal while minimizing charging and false positives. An accelerating voltage of 15 kV was selected to provide enough energy for the primary beam to penetrate through the silica shell and interact directly with the gold core while a low beam current of 50 pA was chosen to minimize the risk of charging. The dwell time was set to 10 μs per pixel while the contrast and brightness settings on the BSE detector were set so that the background tissue appeared black in the image while the individual MPRs oversaturated the detector, making them easy to identify. Through this method any stray salt crystals or surface features would not appear bright and the chance of a false positive was diminished.

When MPRs were located using the BSE detector, the exact location of the nanoparticles was recorded on the montage image created previously. The magnification was then increased until the MPRs in question filled the screen and secondary electron, backscattered electron, and 50:50 mixed signal images were acquired. Through this process the entire brain section was imaged for the presence of MPRs.

2.4. Optical microscopy/scanning electron microscope correlation

The montage image created in the SEM with the MPR locations embedded in it was overlaid manually, utilizing macroscopic tissue features, on the large composite OM image created from the same tissue section. This was accomplished by locating three distinct points on each image and matching them together. Utilizing this overlaid image the exact locations of the MPRs in the tissue were determined. This information was then correlated with the adjacent OLIG stained tissue section to identify the exact location of the MPRs with respect to the tumor and its peripheries. Because the tissue sections were only 10 μm thick, less than the diameter of most cells, it can be expected that the tissue structures from one section to the next would remain largely unchanged, with tumor peripheries remaining largely in the same place. As a result, the locations of the MPRs obtained from the H&E sections were translated to the OLIG stained sections to reliably identify their exact position with respect to the tumor.

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