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Basic Neuroscience

Combining micro-computed tomography with histology to analyze biomedical implants for peripheral nerve repair☆

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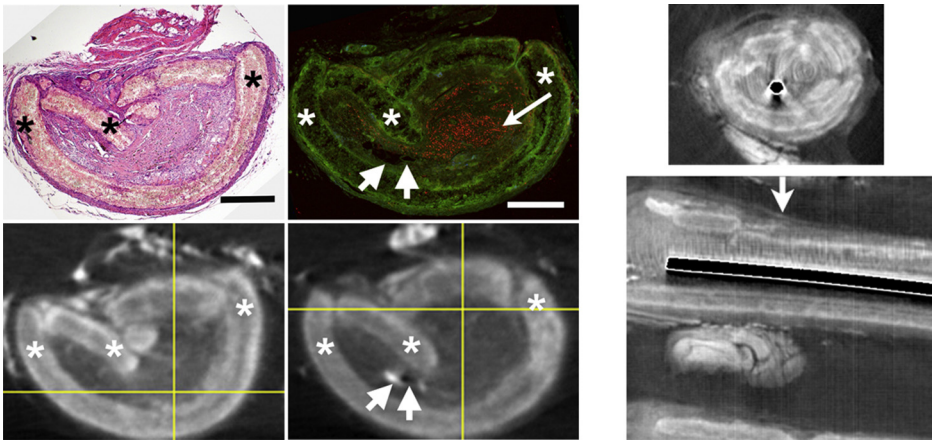
HIGHLIGHTS

- Imaged ex vivo iodinated normal and repaired nerves with micro-CT.
- Removed iodine with sodium thiosulfate, allowing subsequent histology.
- Removal of iodine did not alter immunostaining or cellular morphology.
- Matching CT to staining improved studies of nerve regeneration through conduits.
- Micro-CT with iodine allowed imaging of soft tissues adjacent to metal implants.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Biomedical implants used in tissue engineering repairs, such as scaffolds to repair peripheral nerves, can be too large to examine completely with histological analyses. Micro-computed tomography (micro-CT) with contrast agents allows ex vivo visualization of entire biomaterial implants and their interactions with tissues, but contrast agents can interfere with histological analyses of the tissues or cause shrinkage or loss of antigenicity.

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; anti-NF, antibody to neurofilament protein 200; DICOM file format, digital imaging and communications in medicine file format; DTI, diffusion tensor imaging; GLUT-1, the glucose transporter-1 protein; H&E, hematoxylin and eosin; I^-, iodide ions; IKI or I_2KI, iodine potassium iodide; LM, light microscopy; micro-CT, micro-computed tomography; I_2, molecular iodine; MRI, molecular resonance imaging; PBS, phosphate buffered saline; PET, positron emission tomography; PCL, poly(caprolactone); PCR, polymerase chain reaction; KI, potassium iodide; STS, sodium thiosulfate; TRITC, tetramethylrhodamine isothiocyanate; Ti, titanium; I_3^-, triiodide ion; US, ultrasound.

☆ Portions of this work have appeared previously in abstract and poster format.

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New method: Soft tissue, ex vivo micro-CT imaging using Lugol's iodine was compatible with histology after using a rapid (48 h) method of removing iodine.
Results: Adult normal and repaired rat sciatic nerves were infiltrated ex vivo with iodine, imaged with micro-CT and then the iodine was removed by incubating tissues in sodium thiosulfate. Subsequent paraffin sections of normal nerve tissues showed no differences in staining with hematoxylin and eosin or immunostaining with multiple antibodies. Iodine treatment and removal did not alter axonal diameter, nuclear size or relative area covered by immunostained axons ($p > 0.05$). Combining imaging modalities allowed comparisons of macroscopic and microscopic features of nerve tissues regenerating through simple nerve conduits or nerve conduits containing a titanium wire for guidance.
Comparison with existing methods: Quantification showed that treatment with iodine and sodium thiosulfate did not result in tissue shrinkage or loss of antigenicity.
Conclusions: Because this combination of treatments is rapid and does not alter tissue morphology, this expands the ex vivo methods available to examine the success of biomaterial implants used for tissue engineering repairs.

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1. Introduction

Biomedical implants are of significant interest in tissue engineering and medical repairs. The pre-clinical work that is crucial for proceeding to translation involves careful evaluation of the implant, including determining its efficacy in supporting repair, how well the implant integrates into the tissue location and how the tissues respond to the implant. If the implant is biodegradable, then the extent of degradation and tissue reactions to the degradation products must also be evaluated, at both the macroscopic and microscopic levels. Because most surgical implants are relatively large compared to cellular dimensions, then in pre-clinical trials only a small portion of the implant can reasonably be sampled by the normal destructive methods used for histological analyses. Nerve repair is a good example of this because nerves are long, thin, cylindrical structures and repairs can involve nerve gaps of up to several centimeters in length. Histological sectioning, especially in the axial plane, would require significant resources to thoroughly examine the entire implant. Our research focuses on using biomaterial scaffolds to repair injured peripheral nerves, to identify alternatives to autografts, the current gold standard in nerve repair, because autograft use requires additional surgery and nerve loss and current results still do not result in complete restoration of function (Kuffler, 2009; Pfister et al., 2011; Kuffler, 2014). Research has shown that nerve repair is functionally possible using biomaterial nerve conduits (hollow nerve guides) to connect gaps in nerves by placing the cut nerve stumps into the conduits, but only if the nerve gaps are smaller than 2 cm; autografts are still required for longer nerve injury gaps (~2.5 cm or greater) (Pfister et al., 2011; Kuffler, 2014). Thus, research is ongoing to overcome this clinical limit. Studies in adult rats focus on repairs of the sciatic nerve, which has diameter of around 1–2 mm, and a maximal possible repair length of ~2.8 cm. Random or limited sampling using histological means introduces the potential to miss important features, such as breaks in the conduit, identification of the presence of a continuous strand of regenerating tissues, or identification of the front of incompletely regenerated tissues.

Non-histological imaging techniques are increasingly being used to study nerve regeneration, and some are able to be used in living animals, including magnetic resonance imaging (MRI), diffusion tensor imaging (DTI), ultrasound (US) and positron emission tomography (PET) imaging (Rangavajla et al., 2014; Tseng et al., 2014). Micro-CT imaging has been used far less frequently, because most nerve repair scaffolds and conduits are not dense enough to scatter X-rays. It is possible to use micro-CT on soft tissues (after animal sacrifice) if the tissues are infiltrated with contrast reagents, giving excellent differentiation of different types of soft tissues, as shown in recent studies where multiple reagents were compared (Metscher, 2009a; Metscher, 2009b; Degenhardt et al., 2010). This technique can more readily provide a greater resolution per time of imaging than the other techniques and can be cheaper and easier to access than MRI techniques, which are the ones providing the next best resolution (Rangavajla et al., 2014). We explore here the use of one of these contrast reagents, elemental iodine, for micro-CT imaging of nerves and nerve injury repairs using biomaterial implants.

While useful, the use of contrast reagents for soft tissue micro-CT imaging can add additional challenges. Three relatively well-studied contrast agents are osmium, phosphotungstic acid and elemental iodine. Direct comparisons showed that iodine gave the best rate of tissue infiltration without losing tissue differentiation, it does not require toxic waste disposal and the depth of penetration is greatest, even fully penetrating structures that are 10s of mm^3 thick (Metscher, 2009a; Metscher, 2009b; Stephenson et al., 2012; Pauwels et al., 2013; Vickerton et al., 2013). A further advantage of iodine is that, if the penetration is incomplete, the tissues can be repeatedly returned to the iodine solution to allow greater penetration (Jeffery et al., 2011). Osmium infiltration for micro-CT has one of the poorest tissue penetration depths (Metscher, 2009a; Gregg and Butcher, 2012). But an advantage of osmium is that it has been used to combine micro-CT imaging with post-imaging histology at both the light and electron microscopic levels (Handschuh et al., 2013; Sengle et al., 2013; Scheller et al., 2014). With iodine, post-imaging histological studies have been limited, except for very superficial analyses of tissue structure, because iodine treatment renders soft tissues dense and brittle and imparts a yellow color (Jeffery et al., 2011). One group was able to remove iodine from tissues after micro-CT imaging by extensive soaking and rinsing, but the process took many weeks (Stephenson et al., 2012). Thus, because we sought to combine the advantages of micro-CT using iodine as a contrast reagent with subsequent histology, we sought to identify a more optimal method of removing the iodine after imaging.

A frequently used iodine solution employed for tissue contrast is "Lugol's solution", in which molecular iodine (I_2) is combined with potassium iodide (KI) in water resulting in formation of the triiodide ion, I_3^-, with an equilibrium constant of 698 at 25 °C (Palmer et al., 1984). The triiodide ion, which gives the solution a characteristic blue-black color and the tissues a yellow color, binds differentially to various tissue types, providing excellent contrast in micro-CT imaging (Jeffery et al., 2011). The terminology surrounding Lugol's solution, first described in the 1800s, can be confusing. As classically defined, "Lugol's solution" refers only to the fact that the solution contains a certain amount (weight) of