WILEY-VCH

DOI: 10.1002/((please add manuscript number))
Article type: Communication

Nanoparticle Based Imaging of Clinical Transplant Populations Encapsulated in Protective Polymer Matrices

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Abstract: A recent clinical trial proved that autologous olfactory mucosal cell (OMC) transplantation improved locomotion in dogs with naturally occurring spinal injuries comparable to human lesions. However, not all dogs responded to the treatment, likely due to the transplantation procedures involving injections of cell suspensions that are associated with cell death, uneven cell distribution and cell washout. Encapsulating cells in protective hydrogel matrices offers a tissue engineering solution to safely achieve 3-D growth of viable transplant cells for implantation into injury sites, to improve regenerative outcomes. We show for the first time that canine OMCs can be propagated with high viability in 3-D collagen matrices. Further, we describe a method to incorporate canine OMCs pre-labelled with clinical grade iron-oxide nanoparticles into the constructs. Intra-construct labelled cells could be visualised using magnetic resonance imaging, offering substantial promise for in vivo tracking of cOMCs delivered in protective matrices.

1. Introduction

Spinal cord injury (SCI) has high associated costs for healthcare systems,[1] with a critical need to investigate emerging therapies. Cell therapy using olfactory mucosal cells (OMCs; a mixture of olfactory ensheathing cells [OECs] and olfactory fibroblasts) can promote axonal regeneration following SCI[2,3] and offers an autologous, low-risk cell source, with limited ethical and immunological implications.[4,5] In a 2012 trial in domestic canines with chronic naturally occurring SCI, autologous transplantation of canine OMCs (cOMCs) could restore locomotor coordination between thoracic/pelvic limbs.[6] OMCs have also been safety tested in human trials, with no recorded adverse events one year after transplantation.[7,8]

In the canine trial, only 12/23 dogs responded to transplantation demonstrating the need to refine OMC therapy for improved regenerative potential.[6] Low cell survival using standard injection of cell *suspensions* is likely a major factor in poor functional outcomes; <5% of injected neural progenitor cells and ca. 29% of oligodendrocyte precursors survive transplantation procedures.[9,10] In rodents, OMC survival was only 3.1 ± 1.4% three weeks post-transplantation.[11] Hydrogels may offer a protective cell delivery solution, possessing tissue-mimetic mechanical properties that confer biocompatibility and allow survival/3-D cell growth in a mouldable matrix[12]- hydrogels offer better transplant survival and distribution in injury sites.[10,13] Rodent OMCs have been successfully cultured in hydrogel matrices,[14,15] with OMCs and Matrigel constructs able to support neurite outgrowth *in vitro*.[15]

A major requirement for cell therapy translation is non-invasive cell tracking post-transplantation to correlate cell biodistribution with functional/safety outcomes. Extensive progress has been made in non-invasive tracking of *suspensions* of cells,[16] but this is overwhelmingly overlooked for cells encapsulated in implantable matrices. However, hydrogel delivery of transplant cells poses a unique set of considerations for cell tracking.

These include the ability of contrast agents to track cells in biomaterials with various physicochemical properties, immune cell infiltration into biomaterials and possible accumulation of signalling agent in immune cells (leading to false positives) and how cell division and subsequent dilution of signalling agent is affected by encapsulation. One study proved that neonatal rat astrocytes could be safely labelled with magnetic nanoparticles (MNPs), incorporated into collagen hydrogels and imaged using magnetic resonance imaging (MRI) up to 14 days, demonstrating the translational potential of this approach.[17] To our knowledge, this is the *only* example of cell specific tracking after their encapsulation into biomaterials using non-invasive methods. It is crucial therefore to test and refine such an imaging approach for clinical cells and materials. In this report, we describe the first combination of these two bioengineering technolgies for *clinical* transplant cell populations. Accordingly, we demonstrate safe and homogenous 3-D encapsulation of cOMCs in type I collagen hydrogel matrices is feasible and that clinical grade nanoparticles combined with MRI can be used to detect 'intra-construct' cOMCs.

2. Experimental Section

Expanded methods are in Supplementary Information. Briefly, cells preserved during the Cambridge cOMC transplantation trial[6] were used here. Cells were mixed with collagen before setting to form 3-D cultures. cOMC labelling was achieved by incubation (24 h) with 5 μg/mL Lumirem (siloxane based, clinical contrast MNPs) before trypsinisation and encapsulation in collagen.

Cells were immunostained with p75 (OECs) and Fn (fibroblasts) and morphologically characterised to classify their relative proportions, as previously described.[18] Nuclear staining, LIVE/DEAD staining and the Click-iT® EdU assay were performed to quantify cell numbers, viable cells and proliferation respectively. Perl's staining was used to detect MNPs

in neutral red counterstained cells. All images and z-stacks of live/fixed cell-constructs were acquired using an Axio Observer Z.1. Quantification was performed from regularly spaced planes of analysis (PoA) of a minimum of three z-stacks from each gel/experiment. Coronal MRI sections were obtained using a Bruker 9.4 T Avance III HD instrument.

Data were analysed by one-way ANOVA with Bonferroni's multiple comparison test or an unpaired t-test as appropriate using Prism (Graphpad, San Diego). Data are presented as mean ± standard error of the mean. 'n', refers to the number of independent cultures used, each derived from a different companion dog.

3. Results

3.1. cOMCs can be safely incorporated and distributed throughout collagen hydrogels

Microscopy of cOMCs grown on glass showed healthy cells, displaying normal attachment and morphologies associated with a mixed population of cells (**Figures 1A&B**). p75 positive, bi-polar cells identified as canine OECs (cOECs) made up 50.0 ± 2.9% and Fn positive, rounded cells identified as fibroblasts made up 22.2 ± 6.9% of the population (Figure 1B). 27.8 ± 4.8% cells did not fit either category and were unclassified. This proportion of cOECs correlates well with our previous study (47.6 ± 3.1%)[18] and the reported cOEC densities used for the clinical trial for which these cells were derived (49 ± 6.8%).[6] Following incorporation into collagen, the majority (≳ 95%) of cells expressed a fusiform morphology and were p75 positive, with a small proportion (< 5%) remaining unstained or displaying a rounded morphology (**Figures 1C&D; Supplementary Video 1**). Collagen densities of 1.2 and 2.4 mg/mL could support 3-D cOMC growth with a mean gel depth of 1133 μm (range: 885-1434 μm) (Figures 1C&D). At all gel densities and timepoints, cell number was greater at the base versus the rest of the matrix. However, there was no statistically detectable difference

between cell numbers within the remainder of the gel (**Figure 1E&F; Supplementary Video 2**). LIVE/DEAD staining indicated high cell viability (> ca 85%) across all experimental conditions (**Figure 2A&C; Supplementary Videos 3&4**). EdU positive cells were clearly visible within the matrix with no statistical differences in proliferation profiles across all experimental conditions (**Figures 2B&D**).

3.2. MNP labelled cOMCs can be visualised in 3-D hydrogels using histology and MRI techniques

Successful pre-labelling of cOMCs was achieved on glass with cells displaying blue accumulations indicative of MNP presence 24 h post labelling, absent in controls (**Figures 3A&B**). Labelled cells displayed similar morphology and adherence to controls, with cell number/viability unaffected by MNP labelling (**Figures 3C&D**). Hydrogel incorporated, labelled cells displayed elongated morphologies similar to those on glass. MNPs were detected by Perl's staining and 90.9 ± 1.3% intraconstruct cOMCs were labelled (**Figure 3E; Supplementary Video 5**). Extracellular MNPs were detected (Supplementary Video 5), however, the majority (ca. > 95%) appeared to be intracellular. Gels containing unlabelled cOMCs showed a weak increase in signal intensity versus agarose. By contrast, gels containing labelled cells displayed intense and discreet spots of hypointense contrast distributed throughout the gel depth (**Figure 3F; Supplementary Videos 6-8**).

4. Discussion

We show for the first time that a clinical transplant population can be safely and effectively labelled with clinically approved MNPs, grown throughout collagen matrices and

subsequently imaged non-invasively. Our results support the feasibility of developing a mouldable, implantable "plug" of cOMCs encapsulated in a protective matrix and not subject to lysis and clumping during injection.[19] Moreover, cell survival and distribution in the matrix can be verified *ex vivo* before surgical procedures. As MNP labelling of transplant cells has significant clinical potential as a safe method to facilitate MRI tracking,[16] adaption of these simple yet effective techniques to other clinical cell populations (including human cells) seems feasible and warrants further investigation.

The key parameters for feasibility evaluation of hydrogel encapsulation were cell safety and capacity of gels to maintain cells in 3-D conformations. For clinical injury, cOMCs are cultured for up to four days pre-transplantation[6], justifying our time-points to ensure that cells survive the requisite time in the *ex vivo* construct. Collagen is the main component of the extracellular matrix,[20] possesses cell adhesion domains for axonal growth,[21] and collagen products are employed as medical sealants in neurosurgery for dural repair (e.g. Duragen®)[22], justifying its use.

NSCs can be safely labelled with Lumirem;[23] indeed, siloxane coated MNPs are safe for clinical MRI in humans[24] and we detected no obvious effect on cOMC health post-labelling suggesting protocol safety. Effective labelling was achieved without potentially cytotoxic uptake-enhancing strategies (e.g. transfection agents or electroporation), likely due to the high endocytotic activity of cOMCs resulting in avid MNP uptake.[18] These cells therefore appear well suited for the combined encapsulation and non-invasive imaging approach described here, highlighting its potential for clinical cell therapy. However, there are well-known issues in the field of cell tracking in terms of MNP labelling and non-invasive imaging. One of these is transplant cell death and release of the tracking agents which are subsequently taken up by host macrophages, leading to false positives.[16] Implantation of cells within a biomaterial

matrix has been shown to improve cell survival and also provides a protective matrix to the encapsulated cells.[10,13] Therefore, there may be advantages of using our technique in terms of maintaining cell survival and reducing false-positives arising from transplant cell death. However, as yet, this is still speculative and would need to be tested in in vivo studies.

At both gel densities used, cell numbers were greatest at the base of the gel, indicating some degree of cell sinkage. From a translational standpoint, it is unclear what the significance of these "bottom-heavy" constructs could be. Indeed, it may be of some value to know where in the construct the highest numbers of cells are, such that if one region of a heterogeneous lesion was "more damaged", the gel could be orientated accordingly. Conversely, this may be undesirable in terms of achieving even distribution, in which case there may be some value to investigating means of preventing cell collection at the base, such as inverting the well plates more frequently during gelation through the development of robotically-assisted automation systems, or simply removing the base of the gel pre-implantation.

Acknowledgements: MRI was performed by Professor Harish Poptani and Dr Arthur Taylor at the Centre for Preclinical Imaging (Institute of Translational Medicine, University of Liverpool, UK). This work was funded by an Engineering and Physical Sciences Research Council Landscape Fellowship (CFA) and a Comparative Clinical Science Foundation and Royal College of Physicians bursary (AMD). Christopher Adams and Alexander Delaney contributed equally to this publication.

Keywords: Canine olfactory mucosal cell, spinal injury, cell transplantation, hydrogel, magnetic nanoparticle

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

Supplementary Video 1: Z-stack of double labelled cOMCs within a 2.4 mg/ml collagen gel at 4 days in vitro, stained with Hoescht and p75.
Supplementary Video 2: Z-stack of nuclear labelled cOMCs within a 1.2 mg/ml collagen gel at 4 days in vitro.
Supplementary Video 3: Z-stack of LIVE/DEAD stained cOMCs within a 1.2 mg/ml collagen gel at 4 days in vitro.
Supplementary Video 4: Z-stack of LIVE/DEAD stained cOMCs within a 2.4 mg/ml collagen gel at 4 days in vitro.
Supplementary Video 5: Z-stack of magnetic nanoparticle labelled cOMCs stained with Perl's within a 2.4 mg/ml collagen gel at 2 days in vitro.
Supplementary Video 6: Coronal MRI sections of collagen hydrogels containing (1) unlabeled cOMCs and (2) magnetic nanoparticle labelled cOMCs from "sample 1".
Supplementary Video 7: Coronal MRI sections of collagen hydrogels containing (1) unlabeled cOMCs and (2) magnetic nanoparticle labelled cOMCs from "sample 2".
Supplementary Video 8: Coronal MRI sections of collagen hydrogels containing (1) unlabeled cOMCs and (2) magnetic nanoparticle labelled cOMCs from "sample 3".

Received: ((will be filled in by the editorial staff))
Revised: ((will be filled in by the editorial staff))
Published online: ((will be filled in by the editorial staff))

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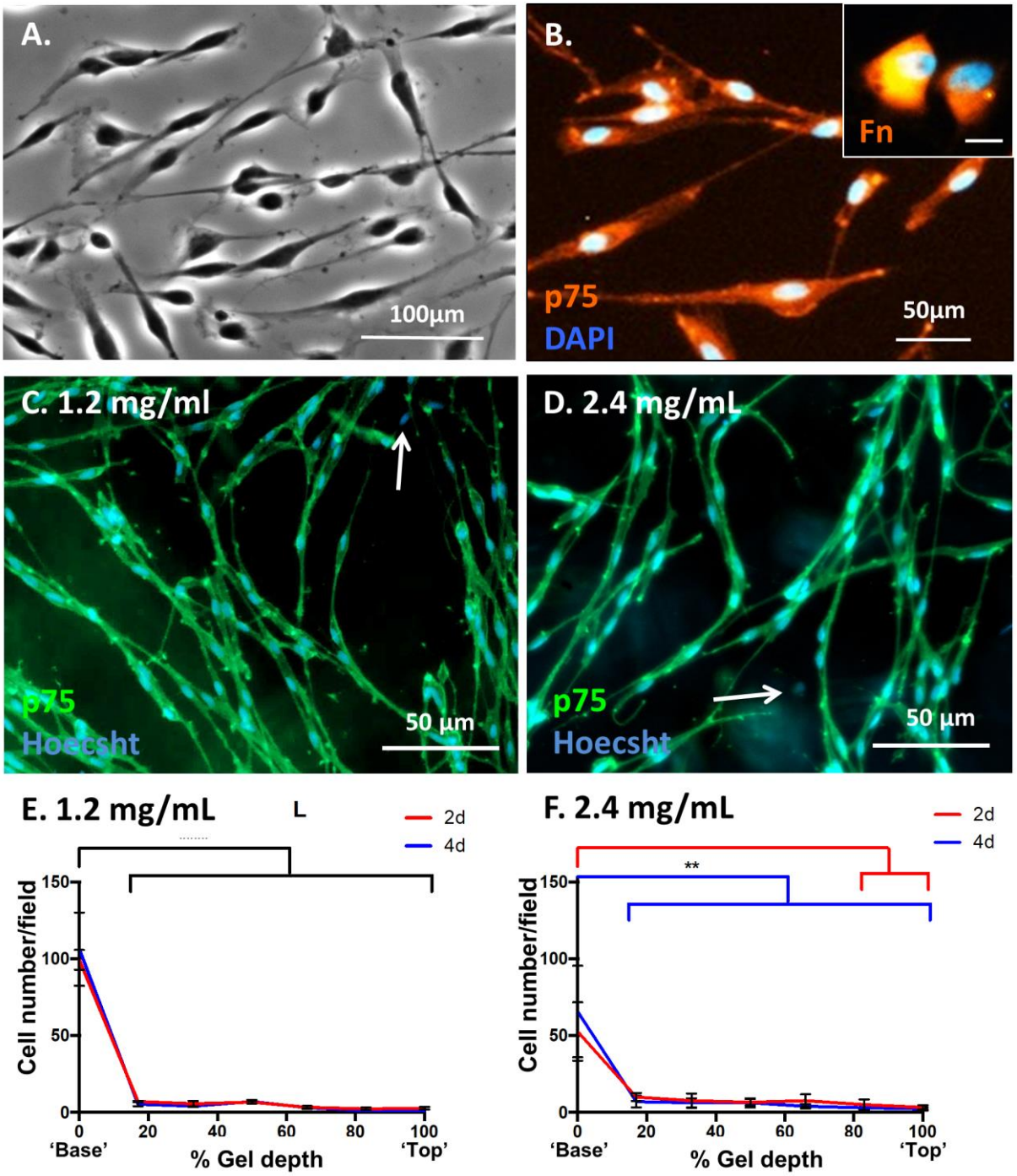
Figures

Figure 1


A.
100µm
B.
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p75
DAPI
50µm
C. 1.2 mg/ml
p75
Hoecsht
50 µm
D. 2.4 mg/mL
p75
Hoecsht
50 µm
E. 1.2 mg/mL
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Cell number/field
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'Base'
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% Gel depth
F. 2.4 mg/mL
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% Gel depth

Figure 2

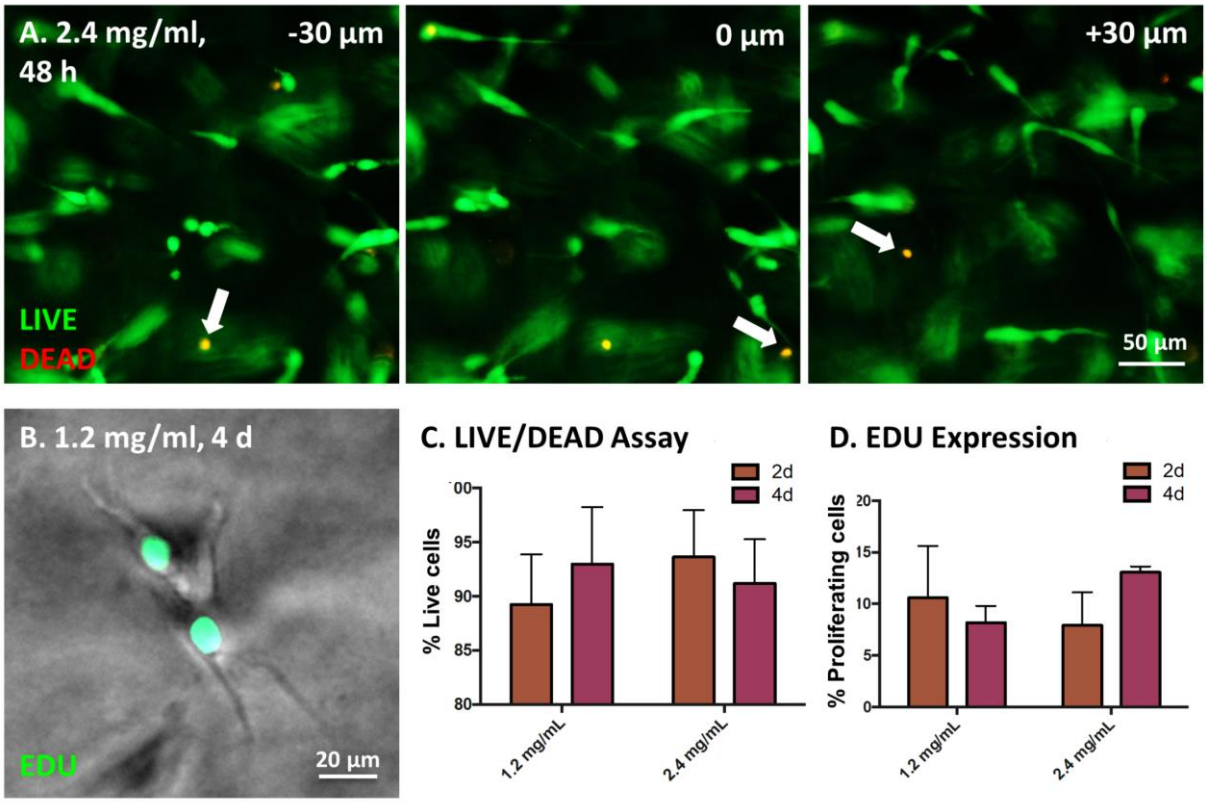
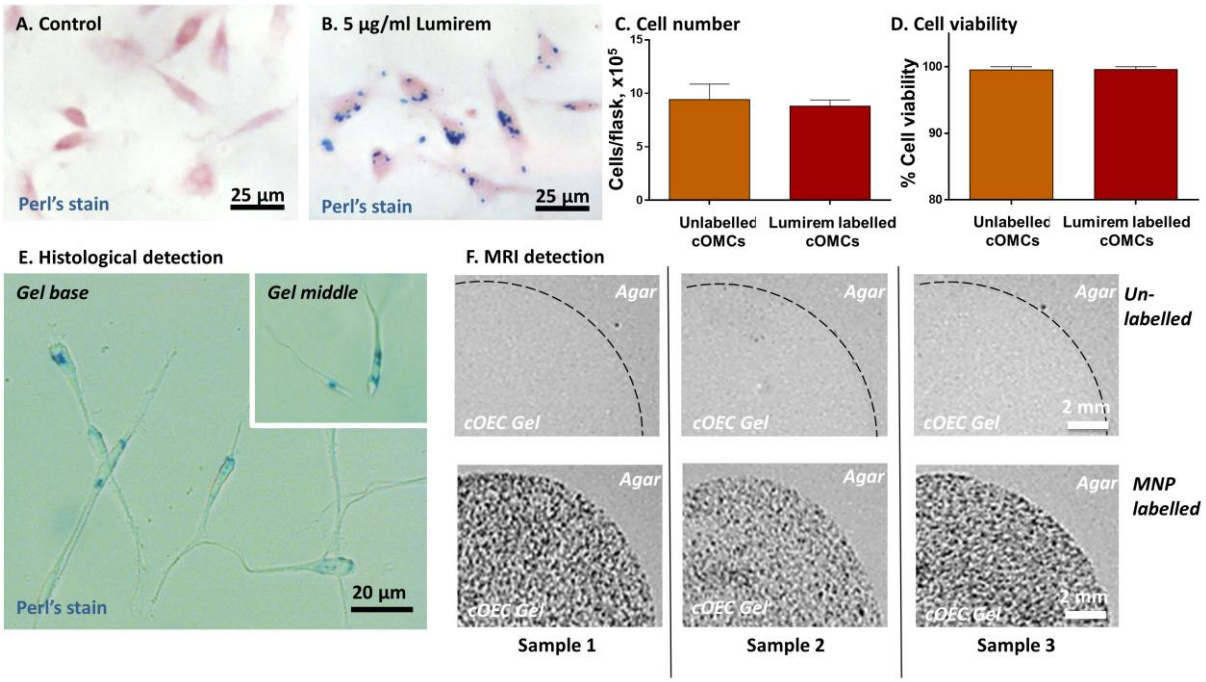


Figure 3



Captions

Figure 1 cOMCs were successfully cultured within collagen hydrogels. *(A) Representative phase image of cOMCs cultured on glass. (B) Representative double-merged fluorescent images of p75 positive, bipolar cells (OECs) and Fn positive, rounded cells (fibroblasts) (inset; 10 µm scale bar) on glass. Representative fluorescence images of cOECs, 4d within a (C) 1.2 mg/mL and (D) 2.4 mg/mL collagen gel. The majority of cells are p75 positive, although unstained cells were identified (arrows). Graphs showing the number of cells counted at each regularly spaced PoA throughout (E) 1.2 mg/mL and (F) 2.4 mg/mL collagen gels. *P<0.05, **P<0.01 and ****P<0.0001 versus PoA1, n=3.*

Figure 2 cOMCs survive and proliferate in 3-D culture. *(A) Representative z-stack images (75, 105 and 135 µm above gel base) showing LIVE (green) and DEAD (red; arrows) cell staining within a 2.4 mg/mL collagen gel. (B) Representative double-merged (phase/fluorescence) image of EDU positive cOMCs within a 1.2 mg/mL collagen gel. Bar charts displaying proportions of (C) LIVE cells and (D) proliferating cells in each experimental condition (n=3).*

Figure 3 cOMCs were safely labelled with MNPs and visualised in hydrogels using histology and MRI. *Representative Perl's and neutral red staining of (A) unlabelled cOMCs and (B) Lumirem labelled cOMCs on glass. Bar charts depicting quantification of (C) cell number and (D) cell viability in unlabelled and labelled samples (n=3). (E) Representative light microscopy images of Perl's stained hydrogel containing labelled cOMCs taken at the (E) base and (E-inset) middle of the gel. (F) Representative MRI coronal sections of collagen hydrogels from individual dog samples containing (top) unlabelled cOMCs and (bottom) MNP labelled cOMCs.*

Injection of cell suspensions for clinical transplantation results in cell death, washout and uneven distribution. To address this, we show canine olfactory mucosal cells can be propagated safely throughout implantable, protective collagen matrices. In addition, we show biomaterial encapsulation of clinical transplant populations can be combined with nanoparticle imaging technologies to non-invasively visualize intraconstruct cell populations using magnetic resonance imaging.

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