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Abstract: Tissue engineering (TE) holds promise for generating lab-grown patient specific organs which can provide: (1) effective treatment for conditions that require volumetric tissue transplantation and (2) new platforms for drug testing. Even though volumetric structural information is essential for confirming successful organ maturation, TE protocol designs are currently informed through destructive and 2D construct assessment tools (e.g. histology). X-ray phase-contrast computed-tomography (PC-CT) can generate non-destructive, high resolution, 3D density maps of organ architecture. In this work, PC-CT is used as new imaging tool for guiding two TE protocols currently at the in-vitro testing stage. The first (1) involves cell-repopulation of an oesophageal scaffold, with the aim of using the regenerated construct for treating long-gap oesophageal atresia, whilst for the second (2) a lung-derived scaffold is populated with islets for regenerating a pancreas, with the "repurposed" lung offering a platform for diabetes drug testing. By combing 3D images and quantitative information, we were able to perform comprehensive construct evaluation. Specifically, we assessed volumetrically: (1) the cell-distribution within the regenerated oesophagi and (2) islet integration with the vascular tree of the lung-derived scaffold. This new information was proven to be essential for establishing corresponding TE protocols and enabled their progression to more advanced scale-up models. We are confident that PC-CT will provide the novel insights necessary to further progress TE protocols, with the next step being in-vivo testing. Crucially, the non-destructive nature of PC-CT will allow in-vivo assessments of TE constructs following their implantation into animal hosts, to investigate their successful integration.

Introduction: TE holds promise for generating lab-grown patient specific organs which can provide: (1) effective treatment for conditions that require volumetric tissue transplantation and (2) new platforms for *in-vitro* and *in-vivo* drug testing. In the former case (1), such conditions can be caused by trauma, cancer resection, or congenital malformations [1]–[5], with an example being long-gap Oesophageal Atresia (OA), affecting 1:4000 births [6]. To provide a significant example of the latter case (2), discovering a drug for diabetes treatment would significantly improve the quality of life of people suffering from this condition, currently affecting (type1) 5.9:10000 [7] and (type2) 606:10000 [8], globally.

Even though volumetric structural information is essential for confirming successful organ maturation, TE protocol designs are currently being informed through destructive and 2D construct assessment tools such as histology.

X-ray PC-CT can generate high resolution, 3D density maps of organ architecture in a non-destructive manner. In this work, PC-CT is used as a new imaging tool for guiding the progression of two TE protocols currently at the preclinical testing stage. The first (1) involves the cell-repopulation of a piglet-derived oesophageal scaffold, with the aim of using the regenerated construct for treating long-gap OA. For the second (2), a rat-derived-lung scaffold is populated with pancreatic islets, aiming to create a transplantable endocrine organ to treat type 1

diabetes. Beyond the aforementioned objective, successful generation of this organ can also offer a realistic platform for drug discovery in diabetes.

In the case of the oesophageal TE protocol (1), PC-CT was established as a new tool for performing volumetric visual and quantitative (density- and morphology-based) construct evaluation, enabling for protocol optimisation and subsequently successful construct generation [9]. The protocol was at the early preclinical testing stage with 3kg piglet as the animal model, and currently we are in the process of "scale-up" to a clinically relevant size, transitioning first to 10kg piglet and finally to 25kg pigs as large animal models. In this work, we present the initial findings from the imaging of a regenerated 10kg piglet oesophagus and discuss potential changes to the protocol design, in order to achieve similar outcomes to the successfully regenerated 3kg piglet oesophagi. Furthermore, we are preparing for the *in-vivo* large animal testing stage, which entails a regenerated oesophagus being transplanted into a size-matching pig. Surgical stents are a key component for the successful transplantation of regenerated oesophageal constructs, and to this point their optimisation was hampered by the unavailability of accurate design specifications (e.g. dimensions). PC-CT datasets of a native (as extracted from the 25kg pig) oesophagus, were converted into 3D "drawings" and given as input to a 3D printer in order to print a real-size silicon model, to be used as a reference for guiding the fabrication of the surgical stents.

Following the successful integration of PC-CT in the aforementioned oesophageal TE protocol [9], the application of our construct assessment platform was extended, to inform the regeneration of a pancreatic tissue construct (2), based on the integration of pancreatic islets within a decellularized scaffold produced via decellularization of a rat lung left lobe. The preliminary results presented in this report indicate that, also in this case, PC-CT can deliver valuable information which is essential to inform protocol progression, such as the reliable detection of islet and endothelial cells engraftment, and their topological distribution throughout the sample.

Methodology

The oesophageal samples (1) were generated as described in Savvidis, S *et al*. [9]; it should be noted, however, that there is currently no established protocol for producing a 10kg piglet- or 25kg pig-based regenerated oesophagus, and its generation was based on a linear scale-up of that previously used for the 3kg piglet model. Their imaging was performed using the UCL edge illumination (EI) PC-CT set-up [10] using the "Phase-Shift Computed Tomography" acquisition mode [11].

The engineering of the pancreatic construct (2), which entailed the repopulation of a rat-decellularised-lung left lobe scaffold with pancreatic islets, was performed based on the protocol described in Citro, A *et al*. [12]. The constructs were imaged at the I13-1 (coherence branch) beamline of the Diamond Synchrotron Radiation facility [13], with system components and acquisition details described in Vitoria, F.A *et al*. [14] and Diemoz, P *et al*.[15], respectively.

Results

An example of recellularised pig oesophagus is shown in fig. 1. The visual assessment was performed through "virtual dissection" of the sample at perpendicular planes. This enabled us to identify individual cell injection points (IP), and revealed that significant portions of the sample remained cell-free. This absence of cells is due to portions of the sample which received no IP or unexpected, "gaps", cell-free regions between IPs.
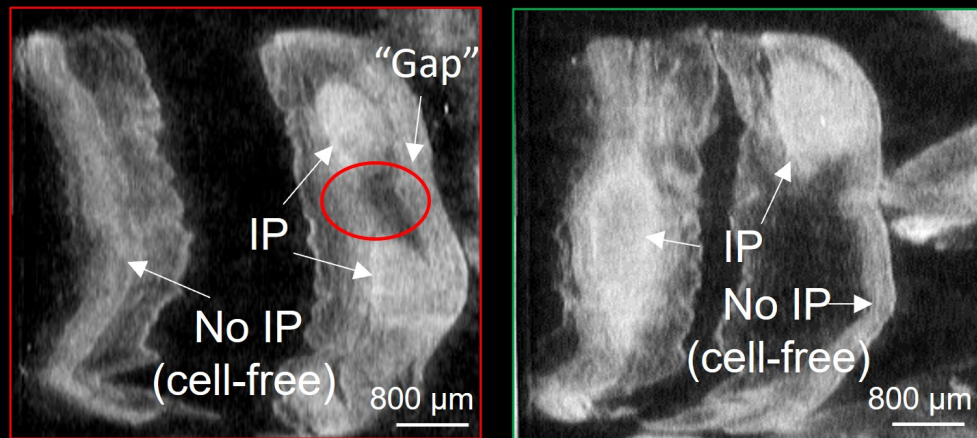
Fig 1 | Visualisation of cell distribution in a recellularised pig oesophagus:, Sagittal (red) and coronal (green), planes are used for assessing the cell distribution along the sample's length; enabling the distinction between cell-populated and cell-free regions. Cell-repopulation is achieved through the "seeding" cell injections, with individual injection points, IP, visualised in the images. Labelled as "gap" is an unexpected cell-free region between IPs.

Fig 2a shows the volumetric PC-CT images of a native (as extracted from the animal) pig oesophagus. These images were used as "input drawings" for 3D printing an oesophageal silicon model (fig. 2b) with realistic specifications in terms of dimensions and morphology. This model will be used to fabricate surgical stents *ad hoc*. This will play an essential role in the *in-vivo* stages, as these are expected to offer an essential structural support to the regenerated construct following transplantation within the host.
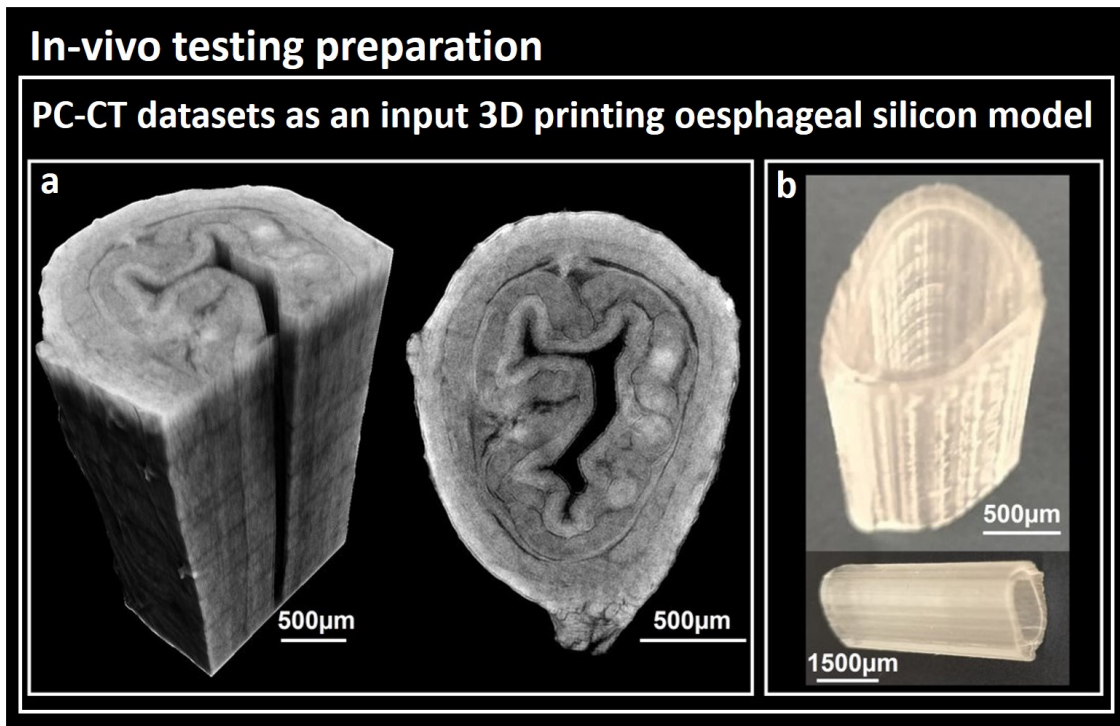
Fig 2 | PC-CT dataset used for 3D printing an oesophageal muscularis silicon model: The volumetric PC-CT dataset of a native oesophagus (**a**) - "input drawings" for 3D printing an oesophageal silicon model (**b**) to be used for fabricating surgical stents.

Fig 3 shows orthogonal views (in 3 anatomical planes) of a regenerated endocrine construct. This enabled the visualisation of pancreatic islets (examples indicated by green arrows), and highlighted variability in terms of their distribution across the sample. Furthermore, inherent vasculature extending to the pancreatic islets (examples shown by blue arrows) is also visualised.
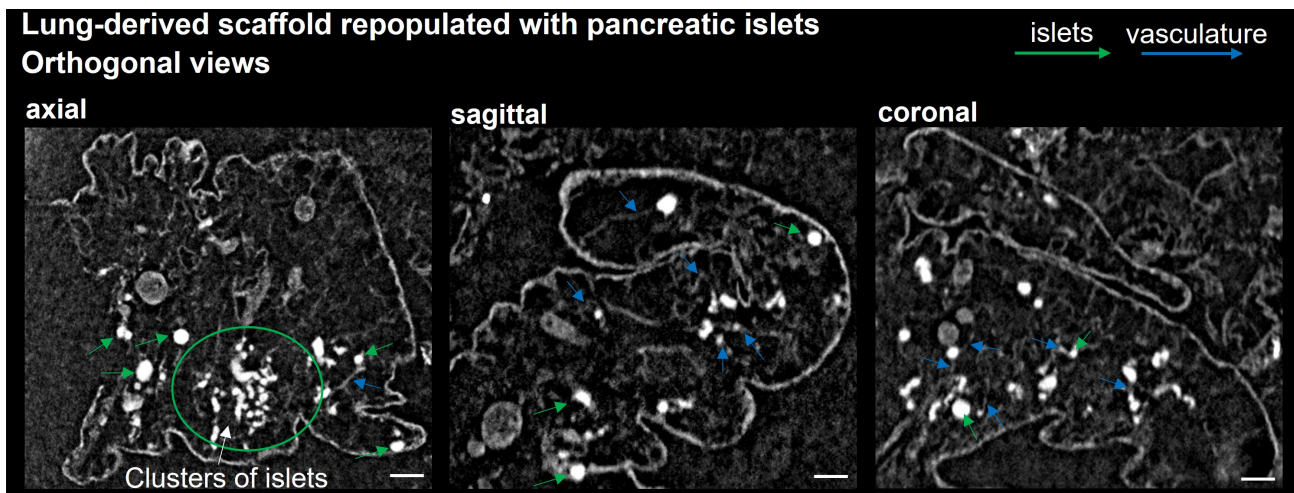
Fig 3 | Visualising pancreatic islet integration and vasculature within a lung-derived scaffold: The regenerated pancreatic-like endocrine construct is visualised using orthogonal anatomical planes (axial, sagittal and coronal). Examples of pancreatic islets are indicated by green arrows whilst examples where inherent lung

vasculature extends into the islets are indicated by blue arrows. A cluster of islets with a relatively dense topological distribution is identified in the axial plane (green circle). The scale bars represent 50 µm.

Discussion

In this work, we demonstrated the use of PC-CT as a new construct assessment tool in an oesophageal (1) and a pancreatic (2) TE protocol. The non-destructive nature of this methodology delivers novel insights, which are used as feedback for adjusting protocol parameters and enabling protocol design optimisation.

The imaging of a recellularised pig oesophagus (fig. 1) demonstrates that simply scaling up the original 3kg piglet-based protocol [9] is not sufficient to produce a "successfully regenerated" sample. Indeed, this fails to satisfy the requirements of having a uniform cell-repopulation throughout the construct. The volumetric visual assessment (fig. 1) allowed us to identify: (a) regions with no IPs and (b) a cell-free "gap" between IPs. Issue (a) can be attributed to erroneously not delivering IPs uniformly throughout the sample, whilst (b) is an unexpected outcome, as in the original (piglet-based) protocol cells from adjacent IPs were seen to migrate and merge, eliminating cell-free gaps in between. We believe the gaps observed in the 10kg pig case could be due to differences in the matrix microenvironment (possibly in terms of stiffness) compared to the 3kg piglet derived scaffolds. We therefore aim to adjust the protocol parameters to address this issue, with the initial changes entailing an increased cell concentration (cell numbers) per IP and/or a reduced spacing between IPs. To better define the nature and extent of the problem, a more comprehensive evaluation of the IPs will also be performed through density-based information, extracted by established PC-CT data processing tools [9].

In addition, we demonstrated the use of PC-CT for guiding the design of surgical stents to be used at the *in-vivo* stage. Stents are a crucial component of the procedure, as they offer structural support to the regenerated constructs whilst in the host. For ensuring such functionality, it is of paramount importance for the stent to be fabricated at the correct specifications (i.e. dimensions and morphology). As shown in fig. 2, the non-destructive nature of PC-CT enabled us to accurately create volumetric digital "drawings" which were used to produce a realistic 3D printed silicon-based replica of the analysed oesophagus. This can be used as accurate physical model for stent fabrication.

The outcome from the volumetric assessment of the regenerated endocrine pancreas (2) was compared against the requirements for classifying such constructs as successfully engineered. These are: (a) achieving an even distribution of pancreatic islets throughout the lung scaffold – essential for organ functionality; and (b) ensuring the integration of inherent vasculature in the proximity of the pancreatic islets - crucial to islet survival. With regards to (a), PC-CT revealed an uneven islet distribution and the presence of a densely populated "cluster", evident only in one anatomical plane (fig. 3, axial view). Based on this information, the parameters for the organ regeneration protocol will be adjusted by introducing changes in islet number and/or size (diameter) in an attempt to address this issue. The inherent lung vasculature (b) is also visualised in 3D and, based on the presented results, vessels appear to integrate with the pancreatic islets. Requirements of this type are currently assessed using 2D validation tools such as histology, which restrict their assessment to a single anatomical plane and require destroying the sample. For a comprehensive evaluation of islet distribution, volumetric visualisation is highly more informative with respect to an assessment based solely on 2D images; even in the limited dataset presented here, this is emphasised from the fact that the islet "cluster" could be identified only in one anatomical plane, which was arbitrarily defined in the virtual dissection of the PC-CT dataset as "axial". Histological assessment would have been able to provide this information only in the case where sample dissection (determined by the organ orientation during wax embedding) matched the "axial" plane shown in fig. 3. Extracting comprehensive construct vascular information (b) also requires a volumetric imaging tool such as PC-CT, which enables for the visualisation of vascular structure continuity in 3D; here we showed how this proved essential to evaluate the extent of pancreatic islet vascularisation.

In the immediate future, we will be implementing the suggestions obtained from PC-CT construct assessment, to improve the design of both oesophageal and endocrine tissue engineering protocols. Samples obtained with the updated protocols will be imaged again for continuing protocol refinement. We believe PC-CT will not only enable the progression of the discussed protocols, but it will eventually become a new construct assessment platform that will unlock true potential of the field of TE, eventually making the use of personalised organs a reality.

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