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Visualizing tissues in three dimensions provides novel insights into the biology and clinical behavior
pancreatic cancer:

Tissue clearing and 3D reconstruction of digitized serially sectioned slides

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Abstract

Pancreatic cancer is currently the third leading cause of cancer death in the United States. The clinical hallmarks of this disease include abdominal pain that radiates to the back, the presence of a hypoenhancing intrapancreatic lesion on imaging, and widespread liver metastases. Technologies such as tissue clearing and three-dimensional (3D) reconstruction of digitized serially sectioned hematoxylin and eosin stained slides, can be used to visualize large (up to two to three-centimeter cube) tissues at cellular resolution. When applied to human pancreatic cancers, these 3D visualization techniques have provided novel insights into the basis of a number of the clinical hallmarks of this disease. Here we describe the clinical features of pancreatic cancer, review techniques for clearing and the 3D reconstruction of digitized microscope slides, and provide examples that illustrate how 3D visualization of human pancreatic cancer at the microscopic level has closed the gap between bench and bedside. Compared to animal models and 2D microscopy, studies of human tissues in 3D can reveal the difference between what can happen and what does happen in human cancers.

Key words: Pancreatic cancer, clearing, three dimensions, artificial intelligence, digital pathology, machine learning, CODA

Introduction

Pancreatic cancer robs those diagnosed of hope (Wolfgang et al., 2013). It has been estimated that globally 495,773 people were diagnosed with pancreatic cancer in 2020, and that 466,003 died from the disease (Sung et al., 2021). As bad as these statistics are, they appear to be getting worse. Rahib and colleagues have predicted that deaths from pancreatic cancer will become the second leading cause of cancer-related death in the United States by the year 2030 (Carioli et al., 2021; Rahib et al., 2014). New approaches to understanding and treating this disease are needed.

Historically pancreatic cancer has been diagnosed and studied using two-dimensional (2D) microscopy (Bosman, 2010; Hruban, 2007). These studies in 2D significantly under sample the cancers. If the average surgically resected pancreatic cancer measures 3cm in greatest dimension, and if the pathologist exams one 5 microns thick hematoxylin and eosin (H&E) stained slide section for every 1cm of tumor, then pathologists examine only 0.05% of the average surgically resected pancreatic cancer. This incomplete view has several implications. First, the connectivity of cancer cells is lost. Cancers that appear to be separate islands of neoplastic ducts embedded in stroma in 2D, are, in fact, often contiguous branching tubes of cancer cells cut in cross section (Yoshizawa et al., 2020). Second, the prevalence of a feature, such as vascular invasion, will be underestimated as focal microscopic features are unlikely to be captured in the minuscule fraction of the cancer sampled. Third, relationships among cells are lost in thin sections unless the cells of interest happen to fall into the same 5 micron plane. For example, the relationship between an immune cell and a neoplastic cell can not be appreciated unless the two cells happen to fall in the same plane of section (Lin et al., 2022). Fourth, the exact point at which an event occurs is not identifiable in 2D slides. In 2D we can observe cancer cells in a nerve, but we don't know at what point along the full length of that nerve the cells invaded the nerve. Recent technological advances in the microscopic visualization of human tissues in 3D make the visualization of even large volumes at the cellular level possible.

Here we provide a brief clinical overview of pancreatic cancer, we review two broad technologies for 3D visualization of cancers at the microscopic level, and we then show how the 3D visualization of human pancreatic cancer has provided insight into clinically important issues. Looking forward, we envision that true 3D multi-omic analyses of cancers on the subcellular level will soon revolutionize our understanding of cancer growth and metastasis.

Clinical

Signs and Symptoms

Intractable abdominal pain, particularly severe pain that radiates to the back, is one of the most dominant symptoms experienced by patients with pancreatic cancer (Lohse and Brothers, 2020; Schorn et al., 2017; Wolfgang et al., 2013). This pain has been linked to cancer cells invading nerves (perineural invasion) and, when carefully examined, perineural invasion can be found in virtually all pancreatic cancers (Bapat et al., 2011; Ceyhan et al., 2008; Chatterjee et al., 2012; Liang et al., 2016; Schorn et al., 2017). Perineural invasion, in turn, has been associated with poor prognosis in patients with pancreatic cancer (Mitsunaga et al., 2007; Schorn et al., 2017). Many of the nerves in the pancreas arise in either the celiac plexus or the superior mesenteric plexus, and pancreatic cancers can grow along nerves into the connective tissues around the celiac and superior mesenteric arteries (Bapat et al., 2011; Hameed et al., 2010; Mitsunaga et al., 2007). Local recurrence, often in the area of the celiac and superior mesenteric plexuses, occurs in 15-25% of patients with pancreatic cancer who undergo surgical resection, and this local recurrence correlates with perineural invasion (Figure 1) (Conroy et al., 2018; Furuhashi et al., 2020; Groot et al., 2018). Local recurrence is such a significant problem, that some have suggested that the standard surgery (pancreatoduodenectomy) should be extended to include the resection of these nerve plexuses (Klotz et al., 2022).

Signs and symptoms related to pancreatic and bile duct obstruction can also dominate the clinical picture for patients with pancreatic cancer. The main pancreatic duct and the distal common bile duct both pass through the head of the pancreas before they drain into the duodenum, and both ducts are often narrowed by cancers in the head of the gland (Cohen et al., 2014). Obstruction of the bile duct leads to jaundice, while obstruction of the pancreatic duct causes upstream chronic pancreatitis with loss of both exocrine and endocrine cells (Chari et al., 2008; Pannala et al., 2009).

Other signs and symptoms of pancreatic cancer include deep venous thromboses, pulmonary emboli, migratory venous thrombophlebitis, weight loss and nausea (Epstein et al., 2012; Khorana et al., 2022; Seoud et al., 2020).

These signs and symptoms are non-specific, and most patients with pancreatic cancer are not diagnosed until the disease has spread to other organs. The liver is the most common site of metastasis, and close to 80% of patients develop liver metastases (Tanaka et al., 2019; Yachida and Iacobuzio-Donahue, 2009; Zambirinis et al., 2022). The impact of this is devastating as the five-year survival rate for patients with liver metastases is only 3% (Siegel et al., 2022)

Diagnosis

Computed tomography (CT) can be performed to confirm the presence of a pancreatic lesion (Chu et al., 2017). When intravenous contrast is given, the majority of pancreatic cancers are visualized as ill-defined hypoenhancing (relative to the adjacent normal pancreas) intrapancreatic masses (Chu et al., 2017; Chu et al., 2021). Other imaging modalities include magnetic resonance imaging (MRI) and endoscopic ultrasound (EUS) (Chu et al., 2017). EUS has the advantages that a biliary stent can be placed in patients with obstructive jaundice, and that biopsies for pathological examination and for molecular analyses can be obtained (Habib et al., 2021; Kitano et al., 2019). As a variety of neoplastic and non-neoplastic entities can form mass lesions in the pancreas, pathology is the “gold standard” in establishing the diagnosis (Bosman, 2010; Hruban, 2007).

Pathology

Pancreatic cancers grossly form firm, white, ill-defined masses that replace pancreatic parenchyma (Bosman, 2010; Hruban, 2007). At the microscopic level, pancreatic cancers are composed of individual glands haphazardly arranged in dense desmoplastic stroma (Bosman, 2010; Hruban, 2007; Whittle and Hingorani, 2019). One manifestation of this haphazard growth is the diagnostically helpful finding of a neoplastic gland immediately adjacent to a muscular vessel (Sharma and Green, 2004). Vascular invasion, including the invasion of lymphatic spaces as well as the invasion of veins, is identified in 65% of surgically resected pancreatic cancers (Bosman, 2010; Hong et al., 2012; Hruban, 2007). As noted earlier, perineural invasion is seen in virtually all pancreatic cancers (Mitsunaga et al., 2007).

Compared to other cancer types, the stroma is particularly dense in pancreatic cancers (Bosman, 2010; Hruban, 2007; Whittle and Hingorani, 2019). This stroma is rich with active fibroblasts, dense collagen, and immune cells (Grunwald et al., 2021; Whittle and Hingorani, 2019).

Treatment

The optimal treatment for patients with pancreatic cancer depends on the stage of the disease. Surgery, the best hope for a cure, is indicated for patients with low-stage disease (He et al., 2014). Close to 50% of surgically resected patients with stage T1 or T2 disease are alive five years after surgery (Allen et al., 2017). Radiation therapy is often also given, particularly in the United States, to achieve better local control of the disease, and for patients with locally advanced pancreatic cancer to shrink the cancer to the point where surgery is an option (Hill et al., 2022).

Chemotherapy can be given before (neoadjuvant) and after (adjuvant) surgery, or as a primary therapy in patients with advanced disease (Park et al., 2021). Chemotherapy is the mainstay of treatment for patients with advanced disease, and several drug combinations, including FOLFIRINOX (fluorouracil, irinotecan, leucovorin, oxaliplatin), gemcitabine/nab-paclitaxel, and nanoliposomal irinotecan/fluorouracil, have been shown to improve survival by 2 to 6 months in patients with

metastatic disease compared to single-agent gemcitabine (Park et al., 2021). Several investigators have suggested that inadequate drug delivery to the cancers accounts for the poor response of pancreatic cancer to systemic chemotherapy (Maloney et al., 2019; Neesse et al., 2015; Whittle and Hingorani, 2019).

Despite improvements in survival for other cancer types over the past decade, the five-year survival rate for patients diagnosed with pancreatic cancer in the United States is an abysmal 11% (Siegel et al., 2022; Torphy et al., 2020).

Recent advances in 3D microscopy have provided clinically relevant insight into pancreatic cancer, and it is hoped that these insights will translate to improved outcome.

Techniques for 3D Microscopy

Improvements in tissue clearing, artificial intelligence (AI) and microscopy now make 3D visualization of large volumes of tissue at the cellular level possible (Richardson et al., 2021; Vieites-Prado and Renier, 2021). Here we present two techniques, clearing and the 3D reconstruction of serially sectioned and digitized microscope slides, each with strengths and weaknesses, that have been applied to the 3D visualization of human pancreatic cancer (Alkemade et al., 2022; Hong et al., 2020; Kiemen et al.; Kugler et al., 2019; Magee et al., 2015; Noë et al., 2018; Richardson et al., 2021; Schiffer et al., 2021; Song et al., 2013; Tward et al., 2020).

Tissue clearing

Two distinct procedures are necessary for 3D microscopy using tissue clearing. First, cells and tissues of interest are labeled. The principles follow those of 2D immunofluorescence, with the additional complexity of needing to label antigens deeper in the tissues. To accomplish this deeper labeling, the extracellular matrix has to be loosened and cell membranes partially delipidated (Richardson et al., 2021; Zhao et al., 2020). Human pancreatic cancer is especially challenging, as it is

densely fibrotic (Pothula et al., 2016; Whittle and Hingorani, 2019). Permeabilization can be increased through a combination of detergents, collagenases, acids, denaturants, temperature changes and long, up to weeks, antibody incubation times (Hahn et al., 2021; Hong et al., 2019; Molbay et al., 2021; Noë et al., 2018). As the size of antibodies determines their ability to penetrate tissues, nanobodies, which are small (~15kDa versus monoclonal antibodies which are ~150kDa) single domain antibodies found in Camelidae (Alpaca, Llama), are emerging as a versatile option (Chames and Rothbauer, 2020; Jaiikhani et al., 2019; Ramos-Gomes et al., 2018; Rousserie et al., 2015). The next step is to overcome the opaqueness of tissues.

Tissue clearing overcomes the opaqueness of tissues by creating tissues with a uniform refractive index (Richardson et al., 2021; Vieites-Prado and Renier, 2021). Living organisms are composed of cells and tissues with varying refractive indexes. Water has a refractive index of 1.33, while the refractive index of lipids and proteins can range from 1.4 to 1.6 (Ariel, 2017; Tian et al., 2021; Vieites-Prado and Renier, 2021). Light bends as it passes into a medium with a different refractive index. As a result, complex heterogenous tissues become opaque. Tissue clearing overcomes this by reducing the heterogeneity of refractive indices in the tissues being studied (Ariel, 2017; Tian et al., 2021; Vieites-Prado and Renier, 2021). This transparency can be achieved through aqueous based and solvent based approaches (Hong et al., 2019; Richardson and Lichtman, 2015). For the human pancreas, the combination of dichloromethane and dibenzyl ether have proven to be highly effective (Hong et al., 2019; Noë et al., 2018; Pan et al., 2016; Zhao et al., 2020).

The final step is microscopy. Light sheet fluorescence microscopy can be used to image large cleared samples, but other techniques such as two-photon microscopy, stereomicroscopy, near infra-red optical projection tomography (NIR-OPT) and, for higher magnification, laser-scanning confocal microscopy, can also be employed (Hahn et al., 2021; Hahn et al., 2020; Hong et al., 2019; Messal et al., 2021; Molbay et al., 2021; Zhao et al., 2020). Image processing, including surface or spot rendering,

enables further analysis of the 3D images and can be combined with pattern recognition algorithms (Figure 2) (Pan et al., 2019).

Clearing methods are summarized in the STAR methods section.

3D reconstruction of digitized serial microscope slides

Several methods have been developed to create 3D reconstructions of microscopic slides from serially sectioned samples (Kiemen et al.; Kugler et al., 2019; Magee et al., 2015; Schiffer et al., 2021; Song et al., 2013; Tward et al., 2020). Tissues are typically serially sectioned, the slides digitized, and then the digital images are registered to create digital tissue volumes (Kiemen et al.; Kugler et al., 2019; Magee et al., 2015; Schiffer et al., 2021; Song et al., 2013; Tward et al., 2020). Manual segmentation or deep learning can then be used to label components in the digitized images (Figure 3) (Kiemen et al.; Nirschl et al., 2018). In the pancreas as many as ten structures (normal ductal epithelium, pancreatic precancers, pancreatic cancer, islets of Langerhans, vessels, nerves, acini, stroma, lymph nodes, and fat) have been labeled to a resolution of 1-2 microns (Kiemen et al.). Unused intervening sections can be used for immunohistochemistry, imaging mass cytometry, somatic sequencing and spatial transcriptomics, creating 3D integrations of histologic, gene, protein and immune features (Allam et al., 2021; Black et al., 2021; Giesen et al., 2014; Ho et al., 2020; Kiemen et al.; Korehisa et al., 2018; Liu et al., 2020; Rao et al., 2021).

Detailed methods for CODA, a novel approach for the 3D reconstructions of microscopic slides from serially sectioned tissues that has been applied to human pancreatic cancers, are summarized in the STAR methods section.

Strengths and weaknesses

The primary differences between clearing and 3D reconstruction of serial microscope slides relate to the size of tissues, and the number of markers, that can be examined (Table 1).

Clearing can be used to visualize whole organs, including entire human brains (Zhao et al., 2020). However, clearing relies on antibody penetration, and antibodies do not penetrate deeply into densely fibrotic tissues such as human pancreatic cancer (Hong et al., 2019; Noë et al., 2018). By contrast, the potential size of the tissue that can be reconstructed using 3D reconstruction of microscope slides is limited only by the size of the slides (x,y- direction) and by the number of sections cut (axial, or z- direction) (Kiemen et al.). Additionally, since the tissue is sectioned in the generation of serial microscope slides, all tissue is visualized homogeneously; there are no “dark” regions as happens when there is poor antibody penetration using clearing. Several approaches have been developed to overcome the limitations of antibody penetration, including first sectioning larger samples into smaller slabs, clearing and labeling the slabs individually, and then stitching the visualized slabs together using imaging software (Hahn et al., 2021). At the other end of the scale, tissue clearing can also be applied to organoids alone or combined with other tissue engineering approaches (tissue engineered microvessels) (Messal et al., 2021; Okuda et al., 2022). The sizes of tissue that can be examined and resolutions that can be achieved by the two approaches are summarized in Table 2.

In 3D reconstruction of serial microscope slides, AI segmentation allows rapid identification of multiple tissue components that are distinguishable in hematoxylin and eosin (H&E) stained sections (in the human pancreas, this includes 10 labels with CODA) (Kiemen et al.; Nirschl et al., 2018). By contrast, only four or five components are typically identified with clearing; three or four using antibodies with different fluorophores, and one taking advantage of distinct patterns of autofluorescence, such as is seen with vessels (Hahn et al., 2020; Kiemen et al.; Noë et al., 2018).

The main advantage of 3D reconstruction of serial microscope slides is that, because unstained intervening slides are available, this approach is readily integrable with any technique that can be applied to formalin-fixed, paraffin-embedded tissues, such as: immunohistochemistry, image mass cytometry, somatic sequencing, and spatial transcriptomics (Black et al., 2021; Kiemen et al.; Korehisa

et al., 2018). This level of integration is generally not possible with clearing because of the harsh chemicals employed.

Insights from 3D

The 3D visualization of human pancreatic tissues has provided clinically important insights into pancreas pathology (Table 3). Here we use CODA as an exemplar of 3D reconstruction of serial microscope slides, as CODA has recently been extensively applied to the study of large volumes (2-3cm³) of human pancreatic cancer (Kiemen et al.).

In standard 2D H&E stained microscope slides, pancreatic cancer appears as isolated glands haphazardly scattered in desmoplastic stroma (Figure 4A). In 3D, it is clear that invasive pancreatic cancer cells, in fact, can grow as continuous branching tubes (Figure 4B) (Yoshizawa et al., 2020). These tubes do not grow randomly, but sometimes grow along tissue planes that are not appreciated in 2D sections. While the collagen fibers in the stroma of the pancreas appear disorganized when viewed in 2D, in the various planes visualizable in 3D, these fibers can be shown to be well-oriented, particularly around pancreatic ducts and vessels (Figure 5A) (Drifka et al., 2016; Drifka et al., 2015; Fraley et al., 2015; Kiemen et al.; Noë et al., 2018; Puls et al., 2017; Xu et al., 2019). Benias and colleagues recently described previously unrecognized interstitial spaces lined by cells expressing endothelial markers between well-oriented collagen fibers in the biliary tree, and they hypothesized that these spaces are present widely in the body and that these spaces are a pathway for cancer spread (Benias et al., 2018). The patterns of growth observed in 3D suggest that pancreatic cancer invades using the microchannels identified by Benias. Indeed, well-oriented collagen fibers and interstitial spaces in the connective tissue surrounding veins in the pancreas may explain the propensity of pancreatic cancer to grow parallel to veins (Figure 2) (Noë et al.). What appears as random in 2D, when studied in 3D, is found to be a propensity for growth parallel to vessels (Noë et al.).

The growth of pancreatic cancer parallel to vessels may, in turn, explain the high prevalence of venous invasion in pancreatic cancer. Venous invasion is observed in 60-65% of surgically resected pancreatic cancers studied in 2D, but when studied in 3D venous invasion can be identified in nearly 100% of the cancers (Figures 1 and 6) (Hong et al.; Hong et al., 2020; Yamada et al., 2018). The ubiquitous presence of venous invasion in even low-stage pancreatic cancers has important clinical implications. First, since the veins of the pancreas drain into the liver, venous invasion may explain the high prevalence of liver metastases and, ultimately, why pancreatic cancer is so deadly (Hruban et al., 2019). Second, because veins thrombose in areas of venous invasion, venous outflow from pancreatic cancers will be reduced (Hong et al., 2012). With diminished outflow, the inflow of blood into the tumor will be reduced. Venous invasion may therefore also contribute to the hypoenhancement on imaging and the poor delivery of systemic chemotherapies to pancreatic cancers (Hong et al., 2012).

The high prevalence of perineural invasion, noted in 2D studies, has also been observed in 3D studies. In 3D one can appreciate better the length of growth of the cancer along nerves (Kiemen et al.; Schorn et al., 2017). Perineural invasion of 2500 microns has been reported in 2D histologic slides, while in 3D distances as great as 4 mm are readily visualizable (Figure 7A) (Kayahara et al., 2007; Schorn et al., 2017). These distances highlight the role of perineural invasion in local spread of disease and therefore in local recurrence (Figure 1) (Furuhashi et al., 2020; Groot et al., 2018; Kayahara et al., 2007). Similarly, 3D examination of foci of venous invasion have identified foci in which cancer cells grow in continuity inside the vein for @@@ microns before growing out into the stroma (Figure 7B) (Kiemen et al.). These findings highlight venous invasion as a mechanism of intraparenchymal spread of pancreatic cancer.

Studies using 3D microscopy, because they can be used to identify the exact point at which a process occurs, can also help advance our understanding of disease mechanisms (Figure 7A) (Kiemen et al.). For example, studies of the “moment of venous invasion” using tissue clearing have shown that epithelial to mesenchymal transition is only transient during vascular invasion (Hong et al., 2020).

3D analyses have also uncovered flaws in the histologic classification of precursor lesions. In the current classification system, based on 2D microscopy, pancreatic intraepithelial neoplasia (PanIN) lesions are distinguished from intraductal papillary mucinous neoplasms (IPMNs) based on the size of the lesion (Basturk et al., 2015; Hruban et al., 2004). PanINs are, by definition $<0.5\text{cm}$, while IPMNs are, by definition, $\geq 1.0\text{cm}$ (Basturk et al., 2015; Hruban et al., 2004). However, some lesions that meet diagnostic criteria for a PanIN in 2D (they are $<0.5\text{cm}$ in greatest dimension in the plane that they happened to be sectioned), when visualized in 3D, are found to involve $>1.0\text{cm}$ of duct, and thus meet the size threshold for an IPMN (Figure 3) (Kiemen et al.). A classification system based on volumes, as is done with CT, would allow for lesion classification independent of the plane of section.

When lesions are entirely confined to a block of tissue, 3D analyses can also be used to quantify the number of cells in that lesion (Kiemen et al.). Since PanINs and other precursors are a target for early detection efforts, understanding the exact size of PanINs and the number of cells in each PanIN will inform efforts to develop of early detection tests.

The most significant advantage of CODA is that only every third slide has to be used to generate the 3D images (Kiemen et al.). The two intervening slides can be used for molecular or immunolabeling studies (Korehisa et al., 2018). A lesion can be identified in 3D and then the intervening unstained slides of that lesion can be laser capture microdissected and genetically sequenced, providing a 3D map of somatic mutations in the lesion. An understanding of the 3D architecture of somatic genetic alterations in complex branching and bending structures is needed to define molecular heterogeneity.

Similarly, intervening unstained sections can be used for immunolabeling, providing a 3D architecture of the immune reaction associated with a specific lesion (Figure 5B) (Korehisa et al., 2018; Lin et al., 2022).

Summary and conclusions

Visualizing human pancreatic cancers in three dimensions at the microscopic level has provided insights into the biology and clinical behavior human pancreatic cancer (Hong et al., 2020; Kiemen et al.; Noë et al., 2018). We envision two advances that will transform this field even further. First, as spatial transcriptomics technologies are optimized for formalin-fixed paraffin-embedded tissues, detailed 3D spatial transcriptomics will soon be possible (Hu, 2019; Ruiz Tejada Segura et al., 2022). Second, as the resolution of clinical imaging techniques such as computed tomography and magnetic resonance imaging, improve, we foresee the direct, one-to-one correlation of 3D clinical imaging with 3D microscopy (Casamitjana et al., 2022; Walsh et al., 2021). AI will only increase the impact and speed the rate of advance of these analyses (Weisberg et al., 2021).

The earth is not flat. Neither are human tissues.

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Author Contributions

Ashley Kiemen, Alexander Ioannis Damanakis, Laura Wood and Ralph Hruban prepared the first draft of the manuscript. All of the authors reviewed, edited, agreed to submit the manuscript, read and approved the final draft and take full responsibility of its content.

Declaration of interests

The authors declare no competing interests.

Figure Legends

Figure 1: Axial (A) and coronal (B) computed tomography scans of a patient with recurrent pancreatic cancer. The cancer has recurred in the region of the superior mesenteric plexus (arrows).

Figure 2: Cleared human pancreatic cancer, without (panel A) and with (panel B) surface rendering. Note the neoplastic cells (green) growing parallel to a vein (blue) and its associated artery (red). The lumen of the vein is highlighted in yellow. (Light sheet microscopy, 5x. Red = autofluorescence, green = carcinoembryonic antigen labeling). Supplemental Figure 1@@ is a paired video.

Figure 3: Three-dimensional reconstructions of serially sectioned human pancreas using CODA. The precursor lesion (red) measures 1.2cm in one plane and 2.6mm in another. If measured as 2.6mm the lesion would be classified as pancreatic intraepithelial neoplasia (PanIN), but if measured as 1.2cm it would be classified as an intraductal papillary mucinous neoplasm.

Figure 4: Cleared human pancreatic cancer reveals that isolated glands in 2D (A) are actually often longer tubes of cancer cells in 3D (B). (Light sheet microscopy, 6.2x. Red = autofluorescence, green = labeling for cytokeratin 19.). Supplemental figures 3A@@ and 3B@@ are paired videos.

Figure 5: Three-dimensional reconstruction of serially sectioned human pancreas using CODA. In panel A, note how the collagen fibers in one plane appear poorly aligned, and in another appear well-aligned. In panel B the intervening unstained sections of a human pancreatic intraepithelial lesion were

immunolabeled for CD3. In 3D, the intensity of the inflammatory infiltrate in panel B can be seen to range from minimal (blue box) to intense (red box). (A, H&E, B, immunolabeling for CD3)

Figure 6: Tissue cleared human pancreatic cancer. Selected images at serial levels illustrate that vascular invasion (central and right panels) can be missed (left panel) in 2D. (Light sheet microscopy, 10x. Red = autofluorescence, green = immunolabeling for carcinoembryonic antigen. Z-stack distance between left and middle image is 36 μ m; distance between middle and right image is 57 μ m). Pair with supplemental figure 5@@ video.

Figure 7: Three-dimensional reconstructions of serially sectioned human pancreas using CODA. Panel A highlights invasive carcinoma (yellow) invading a nerve (brown) and then extending for a distance of 4mm along the nerve. Note that the “moment” the cancer invades the nerve can be identified in this 3D visualization. In panel B the invasive carcinoma (yellow) extensively grows within a vein (green) for a distance of at least @@mm. Seven points can be identified where neoplastic cells cross the media of the vessel.

Supplemental figure 1: Video of cleared human pancreatic cancer (see figure 1) highlighting cancer growth (green) parallel to a vein (red). (Light sheet microscopy 5x. Red=autofluorescence, green= labeling for carcinoembryonic antigen).

Supplemental figure 3: Video of cleared human pancreatic cancer (see figure 4A) highlighting that the individual ducts of cancer (green)in 2D (video A) connect in 3D (video B). (Light sheet microscopy, 6.4x. Red = autofluorescence, green = labeling for cytokeratin 19).

Supplemental figure 5: Video of 3D clearing showing venous invasion in certain sections only, see also figure 6. Note how early in the video the cancer cells (green) do not appear to involve the vessels, while clear venous invasion can be seen at 7seconds. (serial images, light sheet microscopy, 10x, red = vascular autofluorescence, green = labeling for carcinoembryonic antigen).

Table 1

A comparison of two methods for visualizing human pancreatic cancer in 3D at the microscopic level

Feature	Clearing	3D Reconstruction of serial microscope slides
Size of sample that can be studied	Entire human brains have been studied.	Determined by the size of the microscope slides (x,y plane) and by number of sections cut (z plane) .
Application to densely fibrotic pancreatic cancer	Limited by poor antibody penetration.	Not limited by antibody penetration. Uniform resolution even deep in tissues.
Number of tissue types that can be studied in human pancreatic cancer	Usually three or four fluorophores (antibodies) plus autofluorescence of vessels	10+ distinct tissues labeled with CODA
Application of other technologies such as sequencing	Limited by the harsh chemicals used.	Limited only to technologies applicable to formalin-fixed paraffin-embedded tissues.
Cost	\$	\$\$\$ (proportionate to number of slides sectioned)
Time to process one sample	4-50 days (depending on protocol used, use of unconjugated or conjugated antibodies/nanobodies).	Sectioning / scanning: 1 week Computer processing: 1 week

	Upscaling is easily possible, as samples can be run in parallel.	
Quality of images	Visually stunning,	More easily related to standard hematoxylin and eosin stained sections.
Nuclear detail	Often lost. Propidium iodide can be used to detect nuclei in labelled cells, but details are often lost.	Retained.

Table2

Comparison of resolutions and sample sizes for human pancreatic cancer

	Light sheet microscopy	Serial sections tissue
lateral resolution	>0.3 μm	~ 0.25 (40x) or ~ 0.5 μm (20x)
axial resolution	>1 μm	>4 mm (tissue section thickness)
Sample size size limit in x-, y- dir.	$\sim 15 \times 15$ mm^2 (LSFM)	$\sim 20 \times 50$ mm^2
Sample size size limit in x-, y- dir.	< 10 mm	unlimited
z - continuity	good	moderate

LSFM= light sheet fluorescence microscopy

Table 3

Comparison of Clinical Correlates of 2D and 3D microscopy

Clinical feature	2D microscopy	3D microscopy
Pain	Perineural invasion- visualized for a short distance.	Perineural invasion- visualized for a longer distances (> 4mm).
Hypoenhancement on imaging	Desmoplastic stroma.	Venous invasion resulting in reduced outflow which, in turn, diminishes inflow.
Local dissemination of disease	Perineural invasion.	Perineural invasion and continuous and discontinuous intravenous spread.
Propensity for metastases to the liver	Unclear	Venous invasion is ubiquitous.
Pathology diagnosis	Gland next to vessel is a diagnostic feature, likely secondary to haphazard growth of the cancer cells.	Carcinoma specifically grows in well-oriented collagen planes and parallel to veins.
Screening/early detection	Single cross section of pancreatic intraepithelial neoplasia lesions.	Size of precursor lesions can be determined when contained in a tissue block, and they can be studied using a variety of ancillary technologies.

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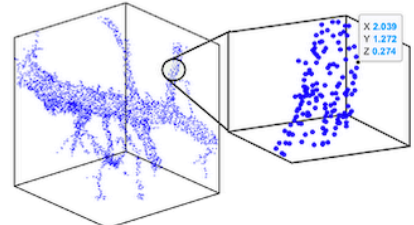
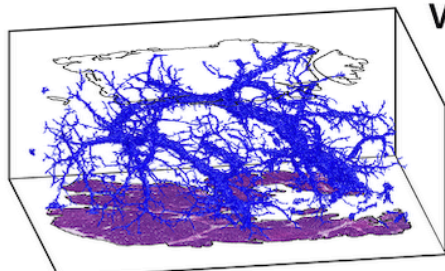
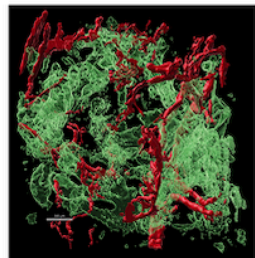
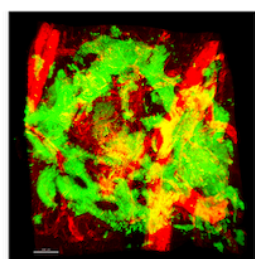
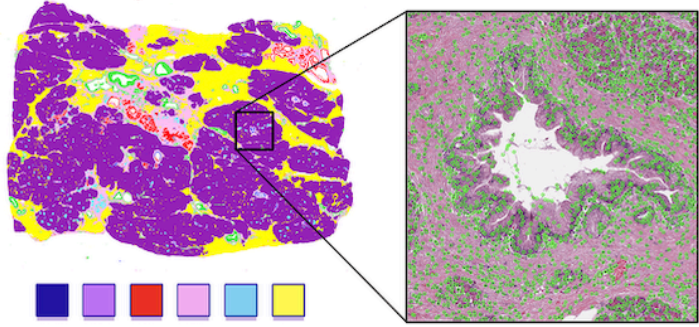
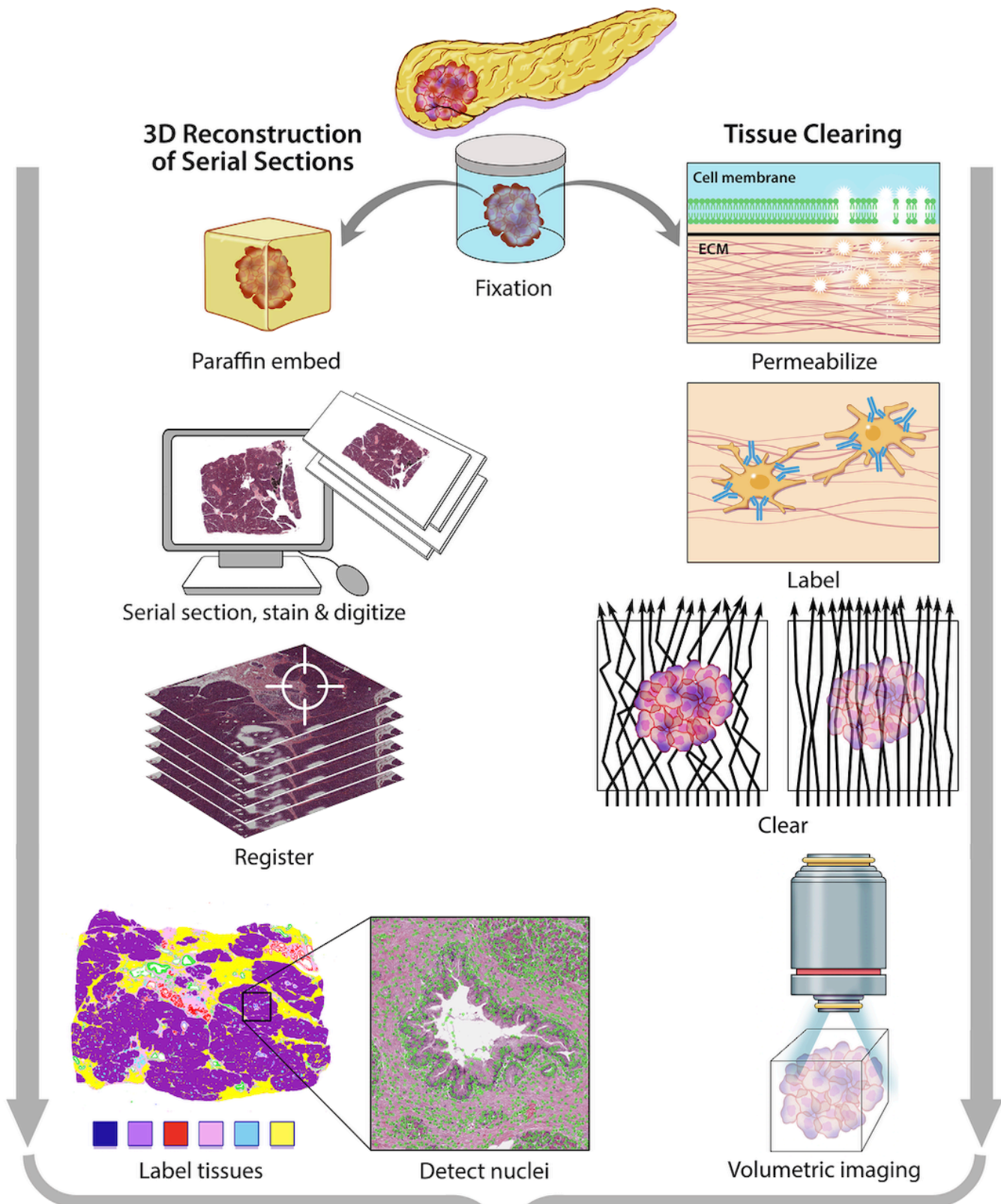
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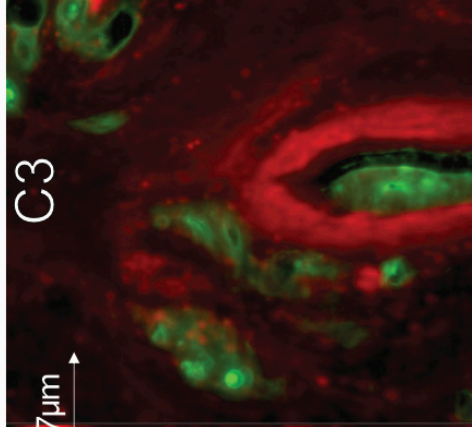
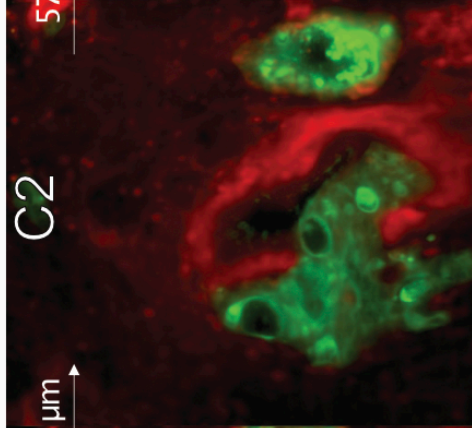
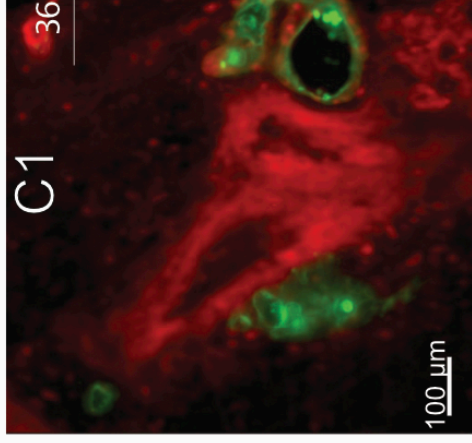
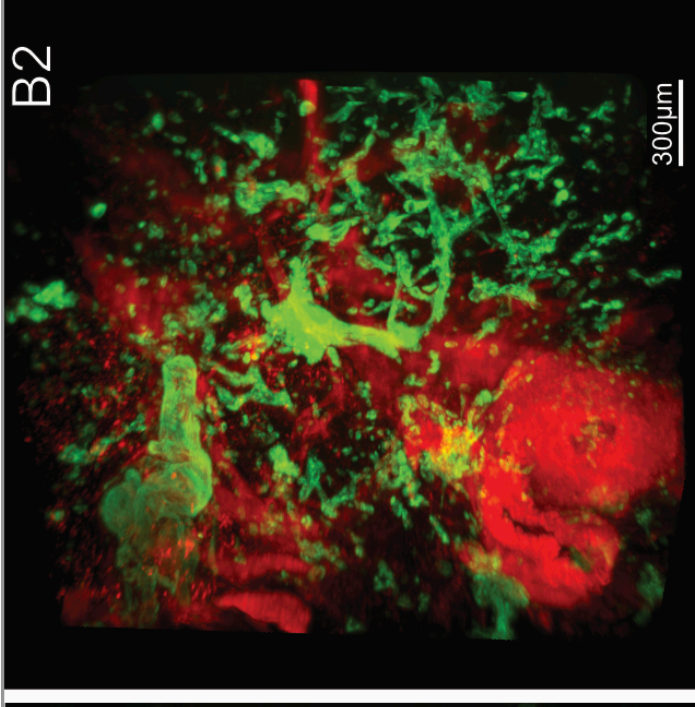
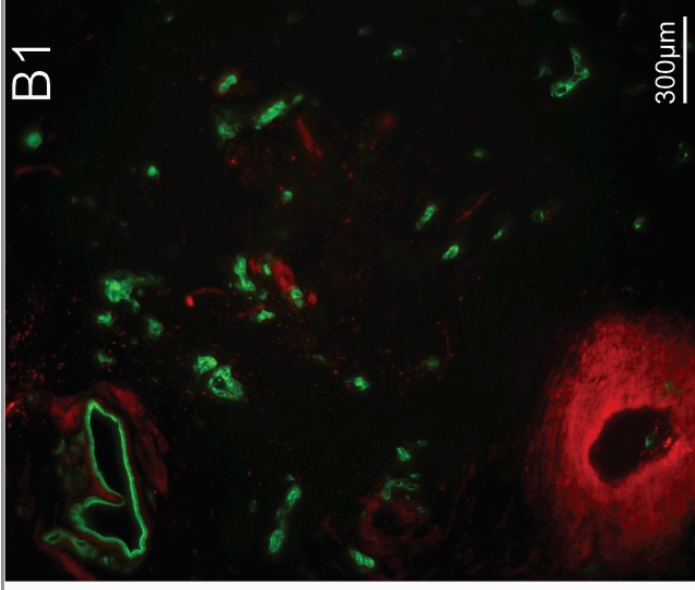
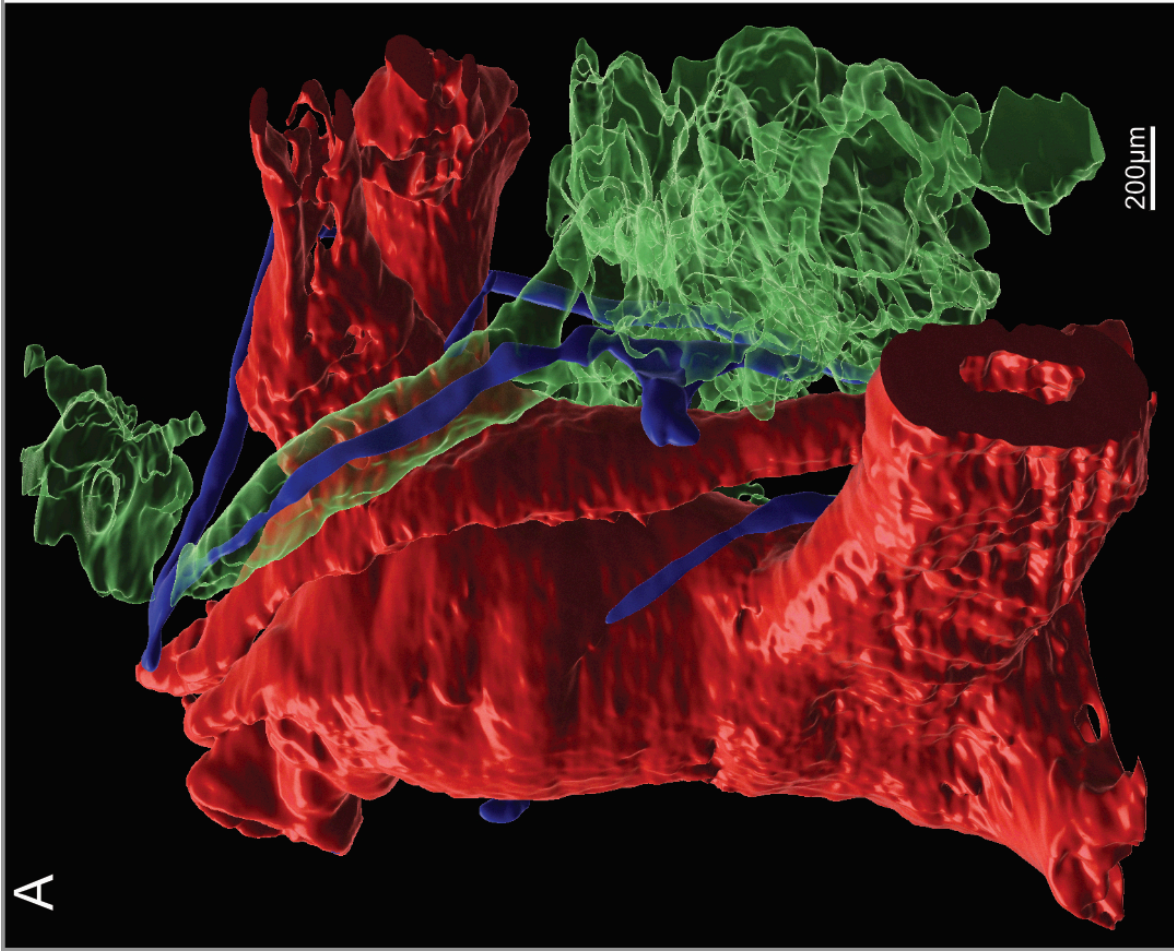
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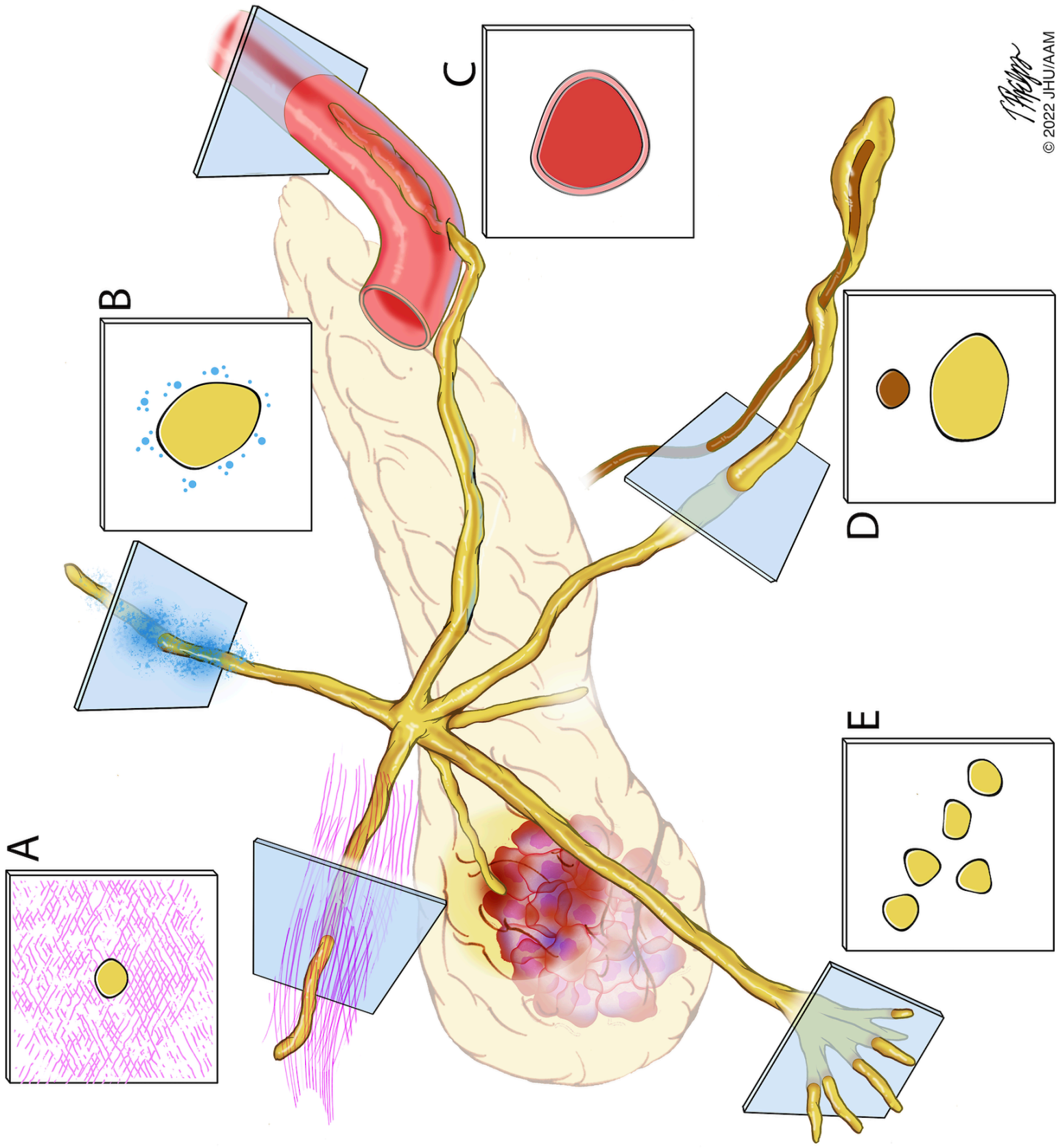
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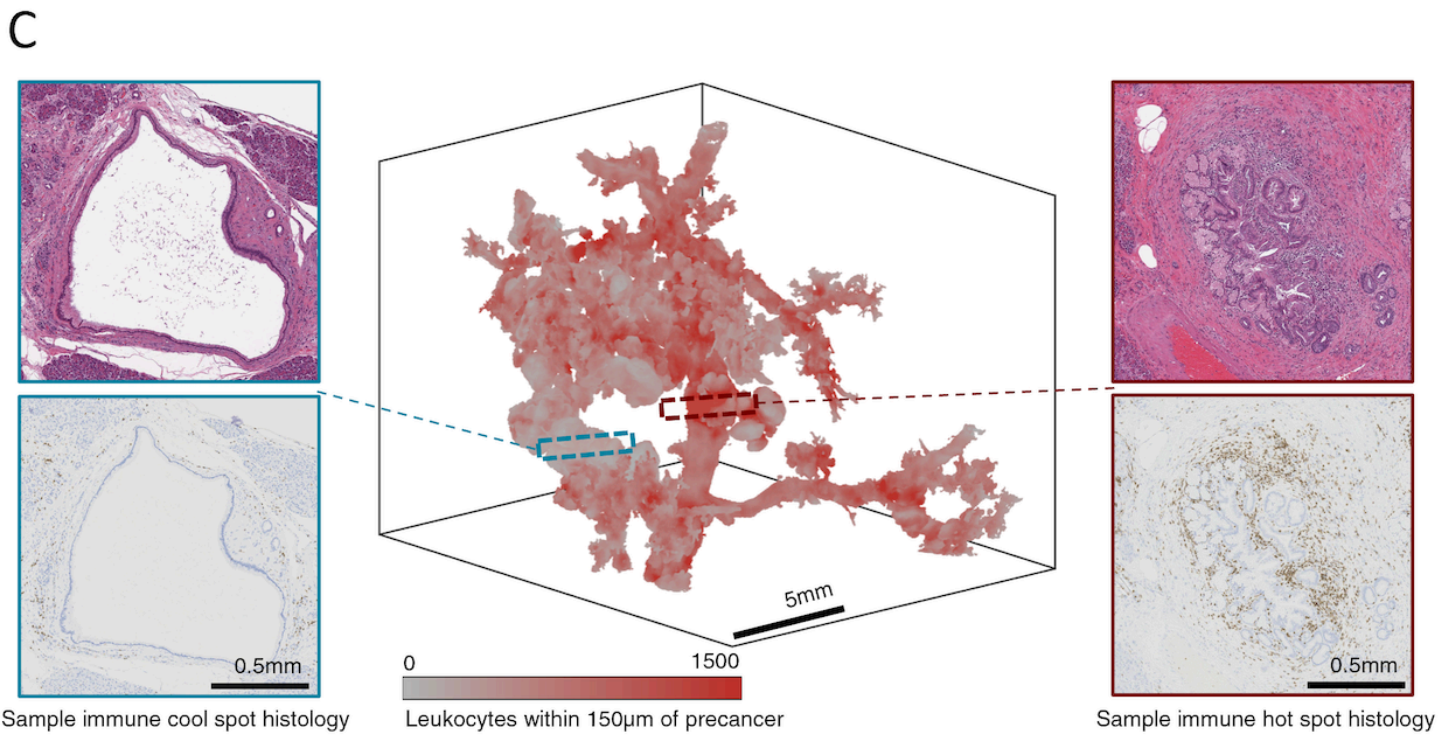
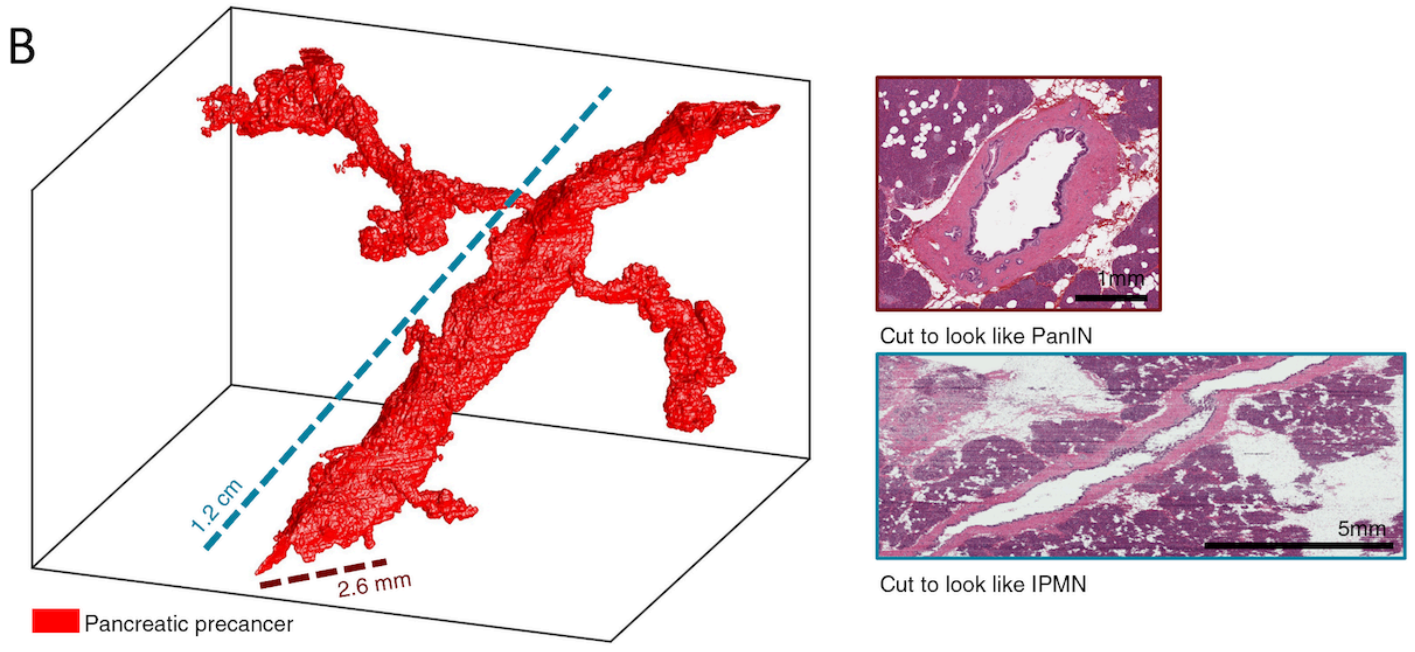
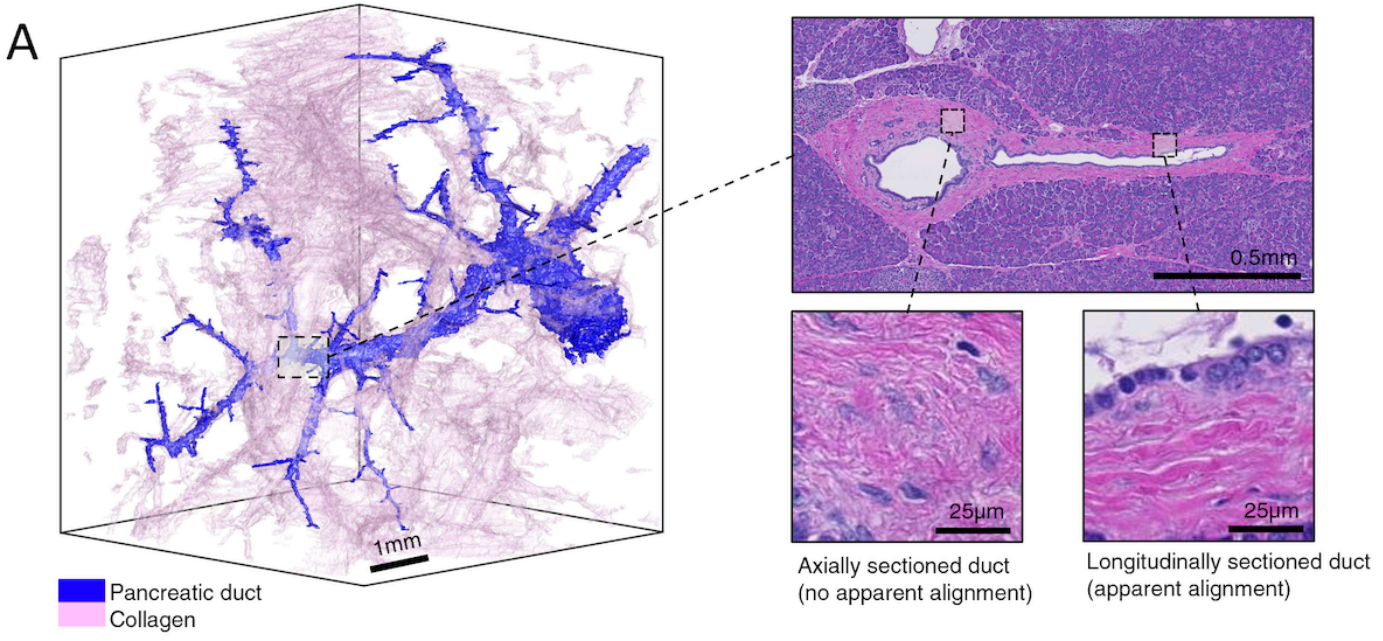
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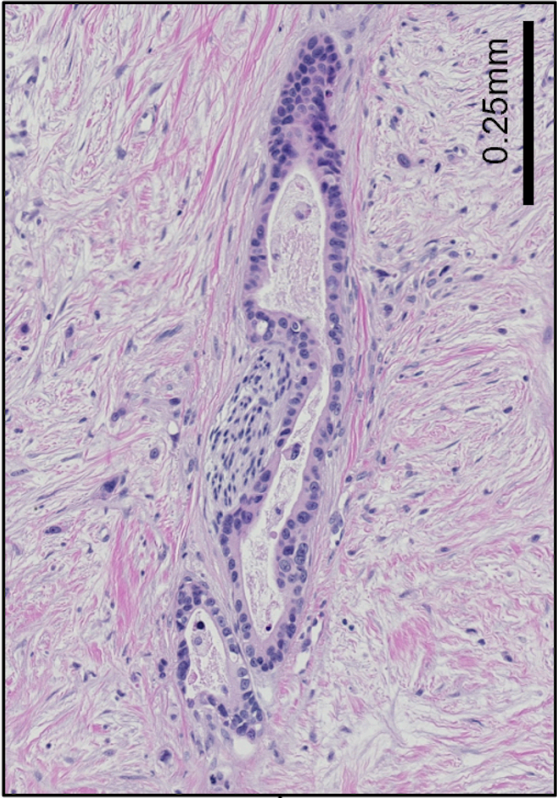
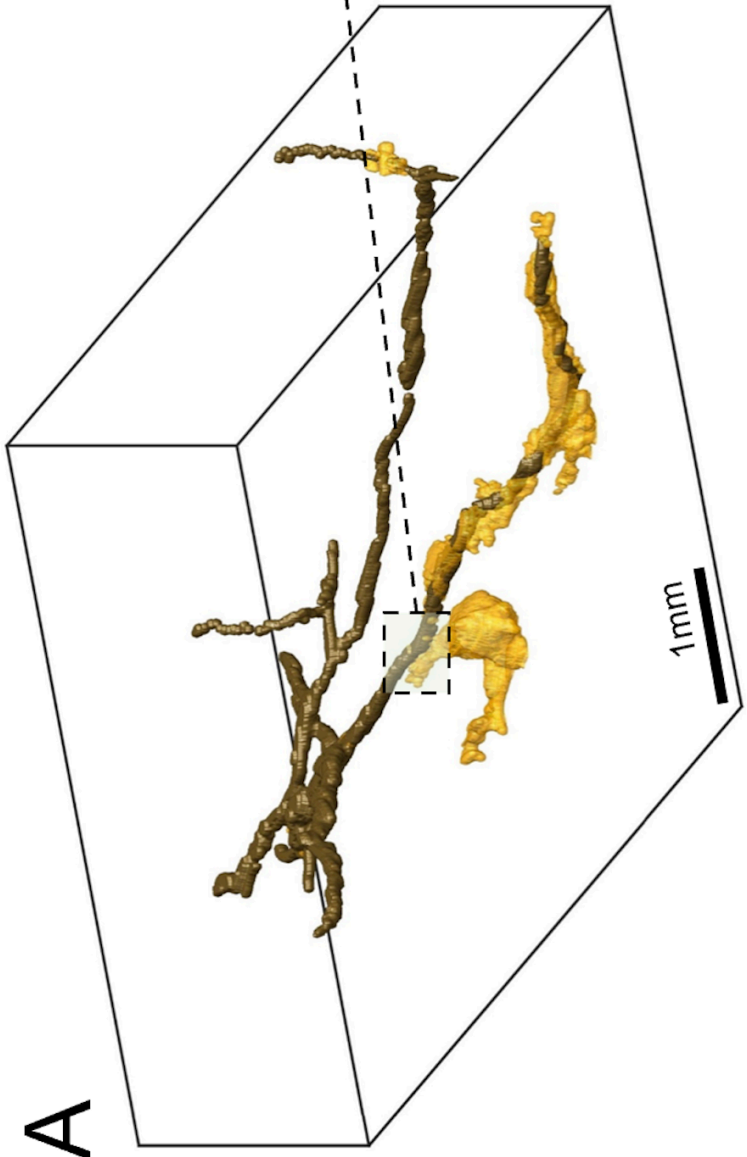








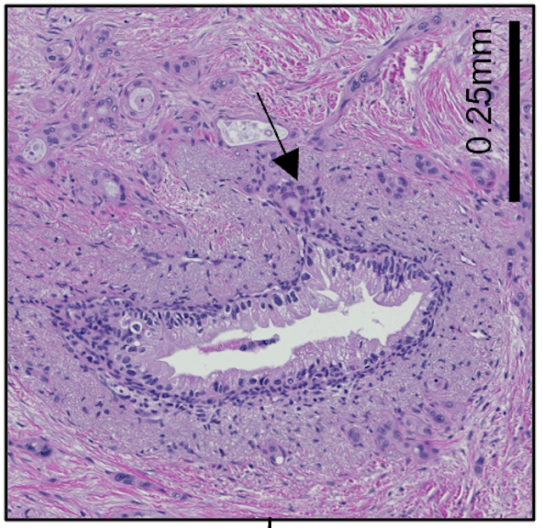
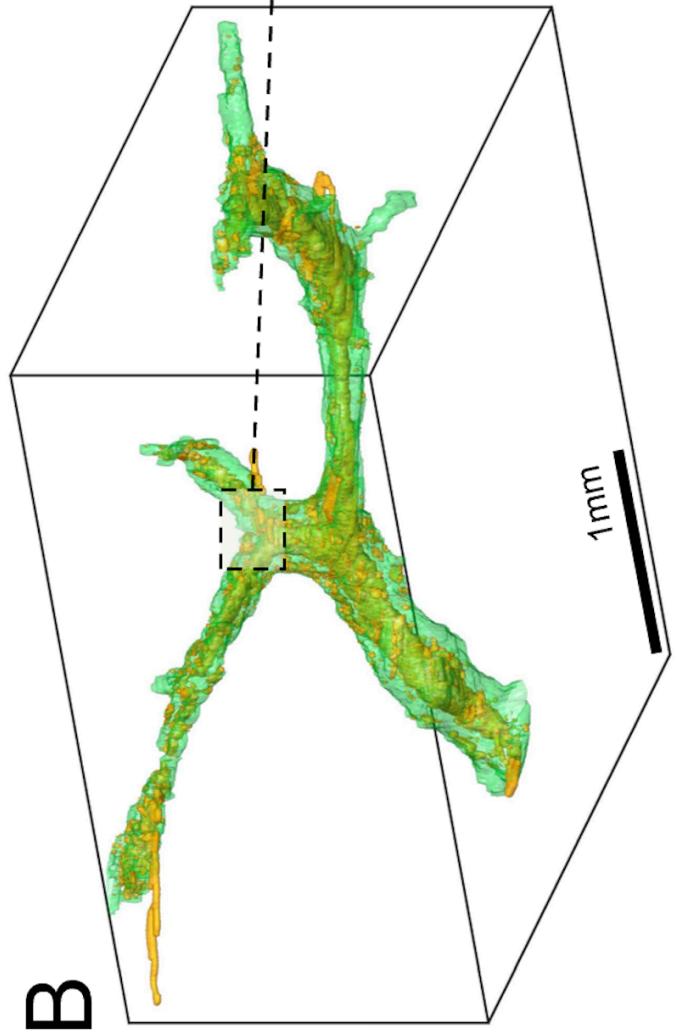
A



Cancer
Nerve

Moment of cancer transition from stromal to perineural

B



Cancer
Vein

Moment of cancer crossing media of vessel