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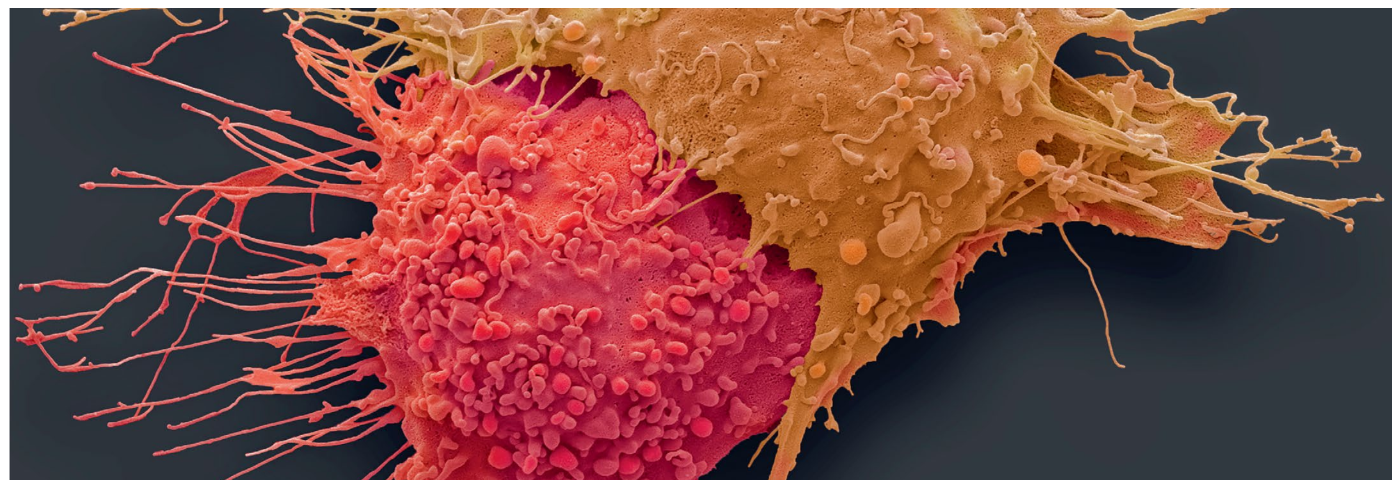

What occurs in cancer's earliest stages? Combining methods may help characterize those beginnings and find ways to thwart cancer earlier. The scanning electron micrograph shows ovarian cancer cells; ovarian cancer is often diagnosed only in its later stages.

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Researchers dig into cancer niches

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The tumor microenvironment is a source of cancer's strength. Multi-omics and new spatial techniques stand to find ways to weaken it.

By Vivien Marx

Cancer screening, especially for people who have undergone treatment and wish to know if it is helping, is an anxiety-ridden time. For such moments, MD Anderson Cancer Center suggests people think of cancer screening as maintenance and see screens as ways to reduce the potential need for extensive care. Another recommendation: "Deep breaths or easy mindfulness exercises can help you stay in the moment and stop those 'what if?' questions taking over."

What can also quell anxieties is to know that basic cancer researchers are exploring new ways to characterize how cancer settles into its microenvironment. This could help to anticipate its later moves and indicate how one might thwart cancer earlier. One side effect is the potential for less anxiety during screening. Among the approaches scientists are exploring are new ways to combine single-cell analysis and spatial transcriptomics or to infer, from two-dimensional information, three-dimensional detail of a tumor and its microenvironment[1–6].

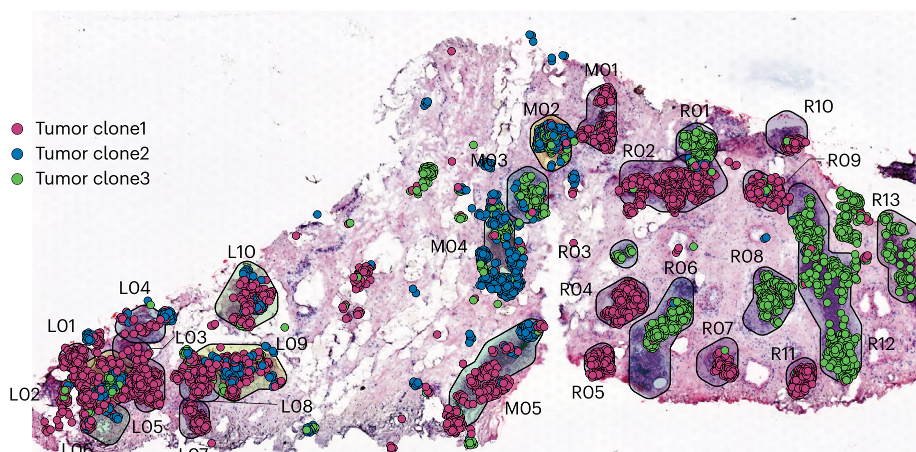
Integrated view of cancer

To get a deeper view of cancer's early moves and metastasis, MD Anderson Cancer Center's Nicholas Navin and his team develop and use single-cell techniques. One project is on ductal carcinoma in situ (DCIS), which can be a disconcerting and flummoxing finding in a breast cancer screening[7]. Ducts of the breast harbor tumor cells, which could be the beginning of a niche from which invasive breast cancer develops. As Navin and his team point out, however, of the more than 50,000 people in the United States diagnosed annually with DCIS, invasive breast cancer will develop in fewer than half. Yet DCIS is often overtreated. With single-cell techniques meshed with spatial transcriptomics, the Navin lab parses differences between DCIS and invasive breast cancer.

Runmin Wei, who is a quantitative postdoctoral fellow of the Damon Runyon Cancer Research Foundation in the Navin lab, developed a method called CellTrek[4], which maps single cells onto the spatial coordinates of tissue sections. Although, he says, researchers could use spatial deconvolution for this, such analysis can be constrained by cell taxonomies and hierarchies such as cell type and cell state level. CellTrek is a way for scientists to fully explore single-cell information – both of the categorical and the continuous kind – in a spatial manner. The method integrates single-cell RNA-sequencing data prepped with the Seurat software package and spatially resolved transcriptomic data.

The scientists applied copyKAT, a software tool of theirs that applies copy number changes to separate tumor cell data from those of healthy cells, but DCIS and invasive breast cancer appeared to reveal few differences.

When they analyzed more than 6,000 single cells and over 1,500 spots of spatial transcriptomics data, they saw hints of transcriptionally heterogeneous tumor subclones. More detail about these clones came from CellTrek-based analysis. The tumor cells mapped to where the hematoxylin and eosin (H&E)-stained slides showed DCIS cells, but not uniformly. One early clone was spread across many regions; another, later-emerging clone dominated in the middle breast duct; another late one occurred more in the right duct.



CellTrek maps single cells onto the spatial coordinates of tissue sections and integrates single-cell RNA sequencing and spatial transcriptomic data. Here it has analyzed the tumor cell–immune cell microenvironment in DCIS and mapped tumor and immune cells near the regions with tumor clones.

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When they mapped tumor and immune cells near the regions with tumor clones, transcriptional signatures with distinct spatial patterns emerged. They found ecosystems of cell states that might indicate shifts to invasive breast cancer initiation and progression. These spatial gene modules are detected with spatially weighted gene-correlation analysis, says Wei, and CellTrek-based analysis avoids issues that can crop up when directly analyzing spatial transcriptomics data due to co-expression patterns shaped by cell composition bias.

Says Navin, genomic signatures alone were insufficient to make predictions about DCIS, but insight came from tumor-intrinsic genomic programs, transcriptional signatures and the interaction of tumor cells within the local microenvironment. This analysis is mainly for demonstrating CellTrek and based on just a few samples from patients. "This work should be run subsequently in a larger cohort of patients to make more general claims," says Navin; the work is ongoing in his lab.

To those who might try CellTrek, Wei recommends reading the tutorial and tinkering with parameters to see how they shape results. "Always try multiple ways of analyzing the data and see whether they can agree with each other," he says. Plotting the data from intermediate steps can also be a good "sanity check." Given how heterogeneous tumors are, it's advisable to match tumor cells for analysis.

Cancer travels

For metastasis to occur, a tumor must shed a cell that travels to a distant site in someone's body and the cancer cell then must settle into that niche. As tumor cells travel through the bloodstream, they can change their phenotype and adapt to a new environment, says Cyrus Ghajar, a researcher at the Fred Hutchinson Cancer Research Center.

Given that cancer is a systemic disease, tumors in one location can also shape events in a distant microenvironment, says Ghajar. For example, David Lyden at Weill Cornell Medicine and his team have shown a tumor can shed molecules into the bloodstream, some of which are packaged into microvesicles that make distant organs more hospitable to arriving tumor cells.

Ghajar also finds fascinating how Xiang Zhang and colleagues at Baylor College of Medicine used a CRISPR clock to phylogenetically map endocrine-dependent, estrogen-receptor-positive breast cancer cells. These cells, when resident in bone marrow, can become 'triple negative'. They then lack estrogen and progesterone receptors and do not make HER2 protein, which are traits that lend them a fitness advantage once they leave the marrow and arrive, say, at the lung.

A labeling system called Cherry-niche[1,3] can light up the tumor microenvironment. It reveals the early neighborhood of a traveler cancer cell that has arrived in a potential niche. The label was developed by Luigi Ombrato, Ilaria Malanchi and colleagues during a lengthy journey, says Ombrato. At the time, he was a postdoctoral fellow in the Malanchi lab at the Crick Institute that focused on metastasis, and he has since started his own lab at the Barts Cancer Institute at Queen Mary University of London.

The scientists had wanted to pick apart the molecular events taking place early in a niche. "It's something very peculiar, and possibly very different from what we see in advanced-stage, big bulk metastases," says Ombrato. But there was no good tool to parse these peculiarities. There are ways to study a tumor microenvironment once it is well established, but the idea here was to render the niche visible early.

Says Ombrato, they started from this thought: "Let's make a fluorescent protein that moves in and out of cells." They lacked experience with tool development of this kind, but knew they wanted a label that eschewed receptor-mediated internalization to enable an unbiased gaze at the neighborhood, and so, he says, "we played around." After trying several fluorescent proteins, they chose mCherry mainly because it was monomeric and thus smaller than green fluorescent protein (GFP).

Perhaps, he says, it helped they lacked label development expertise, which let them think freely about the probe's desired behavior instead of, say, focusing on the label's structure. As the team scoured the literature, they found a lipid-permeable transactivator of transcription-κ (TATk) peptide and generated a fluorescent mCherry protein that contained a secretory peptide and the TATk peptide LP, which enables its uptake in cells. Classically, says Ombrato, TATs are retained in the endoplasmic reticulum and the Golgi.

They engineered breast cancer cells to coexpress GFP and sLP-Cherry and secrete liposoluble mCherry, which is taken up by neighboring cells and stored in vesicular bodies. It has a bright fluorescent red signal.

When injected into the tail of a mouse, the engineered cancer cells set about their metastatic behavior. As the label diffuses out of the cells, it lights up its vicinity of healthy cells about to be tainted. This is a tumor-supportive microenvironment in the making.

In mice, the researchers tracked metastasis of engineered breast cancer cells to the animal's lung tissue. There, a surprise awaited: epithelial cells in the tissue emerged as active participants in the metastatic niche. The scientists call the cells cancer-associated parenchymal cells.

Parenchymal cells are known inflammation mediators, but, says Ombrato, their role in cancer is murky. Furthermore, these parenchymal cells morph in the metastatic niche and take on stem-cell-like traits. The active roles of these parenchymal cells in the tumor microenvironment, also perhaps furthering metastasis, are yet to be explored, he says.

Beyond this in vivo use, Ombrato says he sees labs using Cherry-niche to study tissues such as liver niche, bone marrow niche and brain metastases. And it can be used to study stem cell niches beyond those in cancer, which takes optimizing the system, he says, but involves basically the same strategy.

One could imagine, says Ombrato, getting a virus to express Cherry-niche. Then scientists could assess the moments when a virus first infects cells. Given that the label is unbiased, he says, and labels all cells close to the cell secreting the label, no prior knowledge about the cells it will interact with is needed.

To parse the molecular traits of a cancer cell's neighborhood, he says, it's also thinkable to use Cherry-niche in combination with single cell-sequencing, which has become mature and refined. It could be combined with in silico techniques such as CellChat or NicheNet with which intercellular communication can be assessed in the context of gene expression changes.

Cherry-niche helps to locate cells in the niche, and single-cell sequencing will help to dig into characterizing these cells and how they change. That in turn could yield hints about intercellular interactions that one can study with yet more tools. "I think it's a lot about combining state-of-the-art tools more than optimizing one single tool," he says.

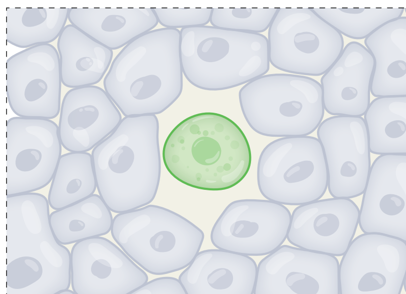
Ombrato and his group are now exploring how the label could use an inducible promoter so it can be switched on and off. With a tunable Cherry-niche system, one could capture very early events and then characterize a subsequent wave of events. A related idea – one he, his colleagues and Malanchi have worked on, as have others – is to make a mouse model with endogenous labeling of a cell of interest. A Cherry-niche mouse model could help developmental biologists who look at particular embryonic stages. For that kind of work, a mouse model in which one can activate the endogenous label for lineage tracing is particularly critical, he says. He points to work from others, who have, for instance, used Cherry-niche to develop what they call a Cre-induced intercellular labeling protein (CILP) to label neighboring cells of a predefined cell type.

Cherry-niche is a "very cool approach," says Ghajar, and he sees a number of labs using it. He also likes labeling approaches based on TurboID, an enzyme-catalyzed proximity-labeling tool.

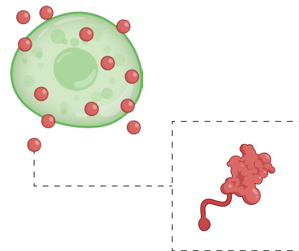
Spatial, 3D, 4D cancer

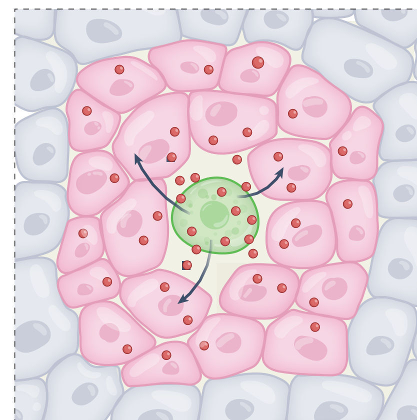
One can look at cancer with a 2D or a 3D view or add in the time factor to make a 4D assessment.

To model metastasis, one can label a cancer cell with green fluorescent protein (GFP). But what remains hidden is how the cell will begin to taint its neighbors in a tissue.

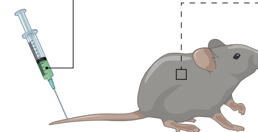


The Cherry-niche labeling system is a secreted fluorescent mCherry protein with a modified lipid-permeable transactivator of transcription (TATk).





A breast cancer cell coexpresses GFP and Cherry-niche.



Cancer cells engineered with Cherry-niche can render visible the tumor microenvironment in different tissues, such as the lung to which a cancer cell has migrated (top right).

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In the spatial transcriptomics class, Ghajar thinks highly of MERFISH, which came from the lab of Harvard University researcher Xiaowei Zhuang and is being commercialized by her lab's spinout Vizgen. And he highlights NanoString's CosMx.

The challenges for users, however, are both technical and computational, says Ghajar. "Even with CosMx, every lab is not going to have the computational expertise to thoroughly analyze the data and explore these rich datasets at the level of depth they deserve to be." Technically, it's challenging to image the environment of a rare or dormant cancer cell.

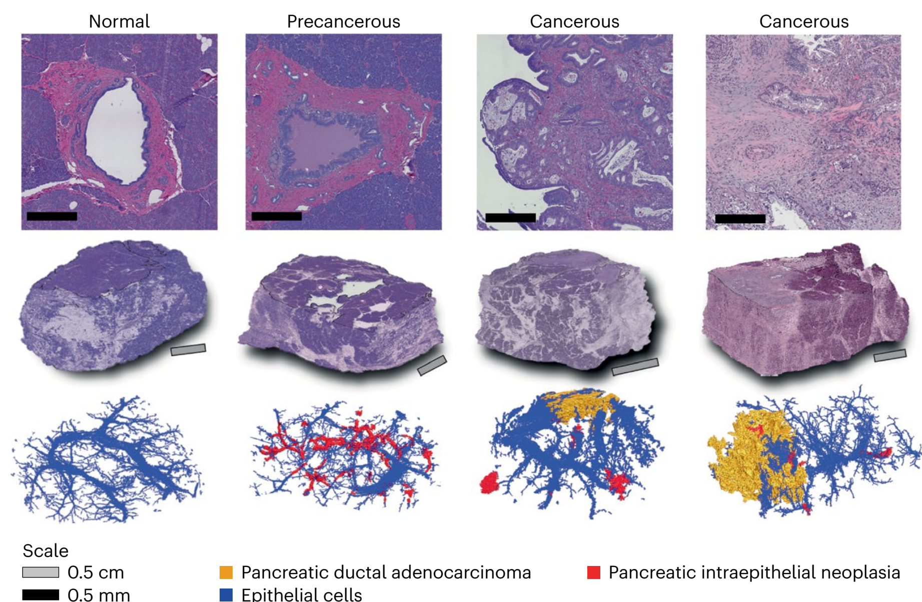
"I agree that 4D is an amazing thing to strive for," says Ghajar. "But making these technologies broadly accessible may be an even greater challenge."

Accessibility of spatial transcriptomic data generation is an aspect important to Curio Bioscience. The company's technology is based on Slide-seq[8] and Slide-seq2 (ref. 9), developed by Evan Macosko, Fei Chen and colleagues at the Broad Institute of MIT and Harvard. The technique lets scientists detect and quantify RNAs transcriptome-wide. With Slide-seq2, the resolution reached near single-cell resolution, and it's the version Curio is commercializing, says Christina Fan, Curio's chief technology officer. "It's the same technique, largely, but with improvements, and we will continue to improve upon that," she says.

The technique uses dense arrays of DNA-barcoded beads. Starting from tissue sections, RNA is transferred onto array surface and sequencing delivers a readout, as with RNA-sequencing. Any researcher with access to high-throughput sequencers, such as in a core facility, can use this method. As a former graduate student in the lab of Stanford University researcher Stephen Quake, she knows that researchers try to avoid needing an expensive instrument or facing a steep learning curve. "I want to be able to jumpstart on my experiment as soon as I can," says Fan.

While in the Quake lab she developed a method to capture and sequence cell-free DNA from a pregnant woman's blood to analyze the fetal genome for potential aneuploidy. She also co-developed a platform at the company Cellular Research to do massively parallel single-cell mRNA sequencing from mRNAs captured on beads barcoded with oligonucleotides. Cellular Research was bought by BD Biosciences.

Over the last two years, Fan, the Curio team and the academic method developers have addressed how to manufacture Slide-seq's surface of barcoded beads; these are around ten



CODA is a platform that uses data from H&E-stained tissue slices — in this case, prostate cancer samples — to computationally generate a three-dimensional rendering of the sample's microarchitecture.

micrometers in diameter, which corresponds to a one-to-two-cell diameter, she says. Users receive the barcoded array, which is manufactured as a consumable. Overall, the technique addresses the lack of spatial context when performing single-cell analysis of disassociated cells, says Fan. This omission can lead to problematic bias when characterizing tumors in a spatially resolved way — for instance, when a researcher is looking at the degree to which immune cells are invading a tumor.

What also stands to help cancer researchers, says Fan, is that this application of Slide-seq is spatial and it gives a whole transcriptome-view. A scientist might use it to focus on clonal expansions of tumor cells, or to assess T cells in a tumor or at its margins. The read-out can deliver insight into the V(D)J regions of immune cell genomes, which shape how the immune system reacts to a tumor or to treatment. Such data, says Fan, are not what FISH or other types of spatially resolved in situ hybridization-based approaches can deliver. For now, the Curio Bioscience analysis is two-dimensional, but in principle it can be applied to three-dimensional spatial analysis by stacking data from tissue slices and merging them into a whole picture.

To better characterize cancer and factors at play in the tumor in its microenvironment and during metastasis, a team at John Hopkins University and its School of Medicine along with colleagues at other universities have developed CODA[5], a computational platform for 3D renderings in biology that have both cellular and tissue-level resolution. "Once you see it in 3D, you never go back," says Johns Hopkins University researcher Denis Wirtz, who led the project and is also the university's vice provost for research. The team applied CODA to pancreatic cancer, which has a disheartening survival rate.

The CODA process starts with resected pancreas tissue samples from patients, which contain healthy tissue and tumor tissue. These are sectioned and stained with H&E, a stain "as old as medicine," as is tissue sectioning itself, he says. The process continues with registering the slides to a common coordinate system. Registration is crucial, says Wirtz, because slight errors will propagate and the final 3D rendering of a tumor can end up quite wrong. "Registration is not a trivial task," he says.

Next is color analysis and a deep-learning workflow to segment the slides into facets of various types that allow subcellular analysis. Among the findings of their CODA-enabled 3D reconstruction of pancreatic tumors are aspects related to the tumor microenvironment. For example, pancreas precursor tumors can develop in spatially independent ways.

The CODA-based 3D-reconstruction of pancreatic cancer also revealed that cancer cells move out from the bulk tumor and creep along collagen fibers that follow the winding 3D paths of ducts and lobes in the organ, as well as blood vessels and nerves.

An emerging theme in cancer biology research is the contribution of nerves to the tumor microenvironment, says Ghajar. At Albert Einstein College of Medicine, Paul Frenette, who passed away recently, was, Ghajar says, "the pioneer in describing sympathetic and parasympathetic innervation of prostate tumors and the disparate effects this had on tumor phenotype." Other work on this, such as by Frank Winkler at the German Cancer Research Center (DFKZ), involves glioblastoma. "Neurons and gliomas form functional synapses, which has broad effects on the tumor's aggressiveness," Ghajar says.

An emerging theme in cancer biology research is the contribution of nerves to the tumor microenvironment, says Cyrus Ghajar.

The implications of their CODA analysis, says Wirtz, such as the findings on the collagen fibers, will depend on ongoing research in their lab and others. Overall, the results suggest that 2D analysis can miss important aspects, and perhaps some published papers are therefore wrong. Depending on the angle, a 2D cross section of a collagen fiber or nerve might not reveal that cancer cells are traveling along its length.

His colleagues, and others, too, says Wirtz, view immune cell infiltration and the role of branching ducts and blood vessels in and around a tumor as three-dimensional concepts that deserve 3D assessment but are routinely characterized in 2D.

Ghajar thinks highly of CODA, which is amenable to a situation in which a researcher only has access to tissue slides. When they have whole tissue, however, in his view, "tissue clearing and light sheet imaging approaches are just stunning," he says. One can, for instance, clear the whole body of a mouse, image throughout the animal and find cells by using fluorescent labels.

Indeed, says Wirtz, tissue clearing has many positives. But the protocols can be lengthy and must be tailored to each organ under study. Labels used in these cleared tissues can work inconsistently. "Perhaps an area is dark

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not because there are no cells of interest but because the label didn't reach that area of the cleared tissue," says Wirtz.

Another issue he sees with clearing is that one must predetermine what one wishes to study. For example, a scientist might focus on pancreas ducts and use a label specific to them. A later question might emerge about the interaction of ducts and blood vessels. But with cleared tissue, there is no second pass. Says Wirtz, "once you have imaged, it's finished; you can't image again." "Every sample is super-precious," he says, especially those from a person's biopsy or surgery.

With the proper permissions in place, CODA allows one to return to previously analyzed samples, as well as those in biobanks. Another issue is coaxing quantitative data from cleared samples. In parallel to CODA, Wirtz and the team applied tissue clearing and battled distortions such as tissue shrinkage.

3D next

The CODA team, says Wirtz, is being flooded with requests for collaboration in cancer, heart disease, pediatric conditions and more, as well as with requests from biopharmaceutical companies eager to move beyond a classic assessment of pathology samples to thinking in 3D — for example, companies want to set up CODA in their internal pipeline.

A next step for the CODA developers could be to spin out a company that offers CODA-based analyses. An alternative could be to build a community-based pipeline, such as one on the cloud-based Galaxy platform, and invite tool integration from other labs. Perhaps both options are possible, he says.

Wirtz remembers over a decade ago when a colleague and CODA co-developer, pathologist Ralph Hruban, took him to the basement of the campus's pathology building. Hruban and colleagues had painstakingly registered and aligned tissue sections manually using toothpicks. But, even with a steady hand, that approach is precise only to a millimeter or so. And the toothpick method destroys tissue.

When pancreatic cancer researcher Laura Wood joined the CODA discussions, she and her team were keen on gaining a 3D view of this cancer. She and others, he says, wonder if the approach might one day predict immune cell infiltration — which matters in immuno-oncology, in which a patient's immune cells are engineered to fight a cancer.

What also matters in CODA's next phase is ground-truthing their 3D reconstructions. "We need quantification," says Wirtz. The three-dimensional parameters must be checked for their fidelity to the organ of origin. A tissue engineer might use CODA to build an organ model and would need true-to-life microarchitecture and cellular composition.

> "We need quantification," says Denis Wirtz.

From his dissections, says Wirtz, the sixteenth-century anatomist Andreas Vesalius knew about the pancreas, and much of his speculation about the pancreas's role in digestion has turned out to be true. Were Vesalius to return today and check out CODA, he might say something like, "I knew what the pancreas looks like; I couldn't see inside it," says Wirtz. He would know he had missed the fine organization and structure of the tissue and organ.

Being able to take H&E-stained slides into the 3D realm, as the organ was originally in the body of a person, can aid cancer biologists in many ways. Not only is H&E beautiful, says Wirtz, these stained images hold molecular information. By using deep learning and algorithmic approaches, one can tease out transcriptomic and proteomic profiles, as well as receptor expression levels. CODA as is "is just the beginning," he says.

When it comes to pulling back the curtain on the tumor microenvironment, Ghajar sees a number of promising approaches. "To me the goal is to apply technology that yields accurate, actionable biological insight," he says. "So however one gets there the fastest, I am all for."

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