### Implantable Tissue Isolation Chambers for Analyzing Tumor Dynamics In Vivo

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Sarah Ruthven
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Dear Editor,

We are submitting for your consideration our manuscript titled: “Implantable Tissue Isolation Chambers for Analyzing Tumor Dynamics In Vivo”.

Drug discovery and validation of potential therapeutic strategies in cancer requires functional assays that provide the appropriate anatomy, microenvironment and tissue-level physiology of human tumors. Unfortunately, much of this work is still being performed in vitro with highly simplified or artificial cell environments. More reliable information can be obtained in mouse models, but even with transparent windows and sophisticated microscopy techniques, it is often difficult to track the underlying processes involved in cancer progression such as cancer and stromal cell interactions or fibrogenesis.

To address these problems, we developed a new hybrid in vitro – in vivo system based on tissue isolation chambers made of polydimethylsiloxane (PDMS, a biocompatible material widely used in microfluidics and cell culture experiments), which we implant in mice. This approach allowed us to partially confine and monitor the interactions between the tumor and host tissue and “untangle” the tumor vascular supply. The tumor construct is vascularized within days and starts migrating out of the TIC within the first two weeks. At all time points, the interactions between host stroma and the tumor are easily visualized in a pseudo-2D geometry below the glass cover, allowing detailed analyses of extracellular matrix deposition, host cell infiltration and vascularization. We were able to observe the growth of vascular sprouts and loops, the evolution of smooth muscle covered arteries and the change in vessel morphology as vessels enter the tumor.

Using this methodology, we discovered a previously unreported mechanism of neovascularization -- matrix guided sprouting – that facilitates tumor angiogenesis. Rather than digesting the extracellular matrix to create tunnels as they move, the angiogenic sprouts attach to, and migrate on, matrix fibers that are in tension.

We believe that this work would be of great interest to the readership of Lab on a Chip as it 1) paves the way for new applications for PDMS technology, 2) offers unprecedented in vivo insight into tumor dynamics in a native environment and 3) provides a new tool for cancer research by bridging the gap between cell cultures and animal models that will be valuable for both basic research and clinical work. The technology can be customized for drug screening as well as other tissue engineering applications.
such as artificial organs that require isolation from the host tissue while maintaining a functional blood supply.

We look forward to your response.

Sincerely,

Lance L. Munn
Implantable Tissue Isolation Chambers for Analyzing Tumor Dynamics In Vivo

Gabriel Gruionu1,2, Despina Bazou1, Nir Maimon1, Mara Onita-Lenco1, Lucian G. Gruionu1,4, Peigen Huang1, Lance L. Munn1,*

Recruitment of new blood vessels from the surrounding tissue is central to tumor progression and involves a fundamental transition of the normal, organized vasculature into a dense disarray of vessels that infiltrates the tumor. At present, studying the co-development of the tumor and recruited normal tissue is experimentally challenging because many of the important events occur rapidly and over short length scales in a dense three-dimensional space. To overcome these experimental limitations, we partially confined tumors within biocompatible and optically-clear tissue isolation chambers (TICs) and implanted them in mice to create a system more amenable to microscopic analysis. Our goal was to integrate the tumor into a recruited host tissue – complete with vasculature – and demonstrate that the system recapitulates relevant features of the tumor microenvironment. We show that the TICs allow clear visualization of the cellular events associated with tumor growth and progression at the host-tumor interface including cell infiltration, matrix dynamics and angiogenesis. The tissue within the chamber is viable for more than a month, and the process is robust in both the skin and brain. Treatment with losartan, an angiotensin II receptor antagonist, decreased collagen density and fiber length in the TIC, consistent with the known activity of this drug. We further show that collagen fibers display characteristic tumor signatures, and play a central role in tumor angiogenesis, guiding the migration of tethered endothelial sprouts. The methodology combines accessible methods of microfabrication with animal models and will enable more informative studies of the cellular mechanisms of tumor progression.

Introduction

Drug discovery and development of new therapeutic strategies would benefit from functional assays that provide the appropriate anatomy, microenvironment and tissue-level physiology of human tumors. To control experimental conditions, much of this work is still being performed in vitro with highly simplified, artificial cell environments such as tissue culture plates or reconstituted biogels. These assays are useful for identifying and dissecting molecular pathways, but they lack crucial cell-cell and cell-matrix interactions that regulate tissue biology in vivo. Moreover, in vitro methodologies for creating vascularized tumors with nutrients supplied by flowing blood still being developed. There is now widespread acknowledgment that cancer progression is inextricably linked to the stromal microenvironment of the tumor and its vascularization1-4, and their absence makes it difficult to interpret in vitro studies of tumor biology.

More clinically relevant information can be obtained from in vivo mouse models, which allow measurement of growth rates or assessment of distant metastases using spontaneous, induced, or implanted tumors. Florentine reporter constructs have greatly enhanced the power of animal studies, allowing identification of specific cell populations and visualization of gene expression in vivo5-9. However, even when using transparent windows and sophisticated microscopy techniques, it is often difficult to track the underlying stromal remodeling processes involved in cancer progression such as fibrogenesis or the collagen between host and cancer cells. This is due to two major limitations. First, the limited depth penetration of optical microscopy generally restricts observations to a few hundred micrometers below the surface, thus missing important cellular dynamics that occur beneath the growing tumor. And second, structures and cell motion that extend in the z direction are difficult to detect or deconvolve when they overlap in the 3-D space.

To overcome these limitations, we implanted silicone elastomer tissue isolation chambers (TICs) beneath transparent windows in the brain or skin of mice. The implants are biocompatible and transparent, allowing long-term observation. They partially confine a centrally-placed tumor so that host-tumor interactions only occur in a relatively thin layer of tissue near the surface. The goals of the present study were to determine i) how the host tissue responds to contact with the PDMS of the TIC, ii) whether a tumor placed in the TIC can recruit host tissue and vasculature into the device, and iii) whether the stroma created within the device responds appropriately to pharmacological treatment.

We show that over a period of two weeks, host macrophages and fibroblasts enter the chamber and create the stromal matrix for the angiogenic vasculature, which integrates into the growing tumor. The TICs allow clear visualization of the events associated with tumor growth and progression at the host-tumor interface including cell infiltration, matrix dynamics and angiogenesis. The tissue within the chamber is viable for more than a month, and the process is

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robust in both the skin and brain. Treatment with losartan, an angiotensin II receptor antagonist, decreased collagen density and fiber length in the TIC, consistent with the known activity of this drug.\textsuperscript{10,11}

We also demonstrate how the methodology can reveal processes that were previously difficult to discern, including distinct tumor collagen patterns and the direct association of angiogenic blood vessels with collagen fibers as they migrate to vascularize new tissue.

Results

Three TIC designs were used in this study (Fig. 1). All devices were constructed from PDMS, a biocompatible, optically clear elastomer using standard techniques of soft lithography.\textsuperscript{12-15} To partially isolate a growing tumor, three approaches were used. First, in the “raft” design, a flat rectangular piece of PDMS (1.6x2.7mm) was placed directly on the tissue under the glass of the dorsal window chamber, and a small fragment of an AK4.4 explant was placed on top (Figs. 1a and 2, Supplementary Videos 1a and b). Second, in the “hole” design, a PDMS disk (10mm in diameter) was used for the bottom layer, and polystyrene beads were placed on top to maintain a fixed gap between the PDMS and glass cover. Holes were punched in the bottom layer to allow access to host cells and vessels (Figs. 1b and 3, and Supplementary Videos 2 and 3). This design does not require alignment of multiple PDMS layers and allows the chamber to move relative to the coverslip. Third, in the “pillar” design, a disc of PDMS is placed against the tissue, and a donut-shaped PDMS spacer is placed on top to create the inner chamber that contains the tumor (Figs. 1c, 4-9, Supplementary Figs. 1-3 and Videos 4-6). The system is closed with a PDMS cover and then a glass coverslip, which acts as the imaging window for the mouse chamber. PDMS pillars in the base layer extend upward and contact the gasket, allowing access of host cells from the periphery, around the pillars. These pillars also provide a structural array to support the collagen network that forms. This design has the advantage that host tissue can only infiltrate the chamber from the periphery, so the time course of tumor integration is easy to follow. After implantation into the mouse skin or brain (Fig. 1d) were able to observe the dynamics of macrophage and fibroblast infiltration, vessel sprouting, collagen and vessel remodeling, and formation of a stable vasculature in these systems.

**Blood vessels and stromal cells are recruited to the host-PDMS interface**

We first analyzed the initial reaction after contact with PDMS by placing a rectangular raft on the tissue in the dorsal window chamber. Within 48hrs, there is dilatation of host capillaries, migration of host cells to the PDMS surface, and deposition of matrix at the interface. By day 13, tissue structures containing vascular bundles create bridges between the host tissue and the PDMS surface (Fig. 2). Using αSMA-/-DsRed/Tie2-/-GFP/FVB mice, it is possible to identify αSMA- and Tie-2-expressing cells using intravital microscopy. αSMA is generally expressed in vascular smooth muscle cells and myofibroblasts, while Tie2 is a marker of endothelial cells and macrophages. The bundles of perfused vascular loops have many outflow vessels that lead back to host capillaries and fewer inflow vessels that connect to arterialized capillaries with αSMA positive walls (Fig. 2). Thus, there was a surprisingly rapid transformation of capillary segments into an arteriole, likely accomplished by pruning of side branches and formation of the vascular wall. These new arterioles also express high levels of Tie-2 (Fig. 2). The new arterioles can be traced back to connections with high pressure arteries, far from the locations in the capillary bed where the blood exits the vascular structure. This ability to observe arterIALIZATION and quantitatively trace the topology of the forming network is a major advantage, and it shows how the system develops in a way that ensures a large pressure difference to drive and flow through the new looping vasculature (Supplementary Videos 1a and 1b). The newly formed vascular bundle is well anchored in the tumor, forming a bridge that flexes during skin movement (Supplementary Video 1b). These observations showed interesting features of vascular morphogenesis and maturation, but also revealed a limitation of this “raft” design: the PDMS is able to drift relative to

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**Figure 1. Implantable PDMS rafts and Tissue Isolation Chambers.** a) A rectangular slab (“raft”; 1.6x2.7mm) of PDMS attracts host cells and vasculature when placed against mouse tissue. b) In the “hole” design, cancer cells are placed on top of a layer of PDMS. Host cells and vessels enter the chamber through holes (200-500 μm diameter) punched in the bottom PDMS layer. Polystyrene beads (200-300 μm diameter; Polysciences Inc., Warrington, PA) were used to create the space between the bottom PDMS layer and glass coverslip. After placing the tumor, the assembly is implanted in the mouse window preparation (dorsal skin or cranial). Host stromal cells including macrophages and fibroblasts (blue) enter the chamber and create an extracellular matrix that is anchored in the normal tissue. Blood vessels (red) migrate through the gap at the periphery created by the pillars to vascularize the tumor. c) In the “pillar” design, the TIC is formed from a base PDMS layer, which contains the molded pillars, a spacer with a central void that creates the tumor chamber and PDMS cover. d) Transparent window models allow visualization of the tissue in the dorsal skin (top) or brain (bottom).
a).	See supplemental videos 1a, b.

The venule, and originates at an artery far from capillary bed is evident. The single arteriole (blue arrow) runs parallel to number of places. Some dilation and morphogenesis of the existing smaller draining venules. At the leading edge, flow is stagnant in the magnification view of the indicated region in (a). The looping system of new arterialized associated with the new tissue structure is invested vessels appear red (middle panel). Note that one of the vessels of the P line. A vascular bundle reaches from the skin and extends over the surface window chamber. The boundary of the PDMS is indicated by the dotted line -2 expressing cells are also identified in this mouse by GFP expression (bottom panel). There is colocalization of strong Tie-2 expression with the new arterialized cSMA+ vessel in the new vasculature. b) Higher magnification view of the indicated region in (a). The looping system of vessels consists of the feeding arterial highlighted in (a) and a number of smaller draining venules. At the leading edge, flow is stagnant in the blind-ending sprouts, and purple deoxygenated blood is visible (blue arrowhead). c) Region of origin of the new tissue structure. The large venule draining the new structure (*) enters the host capillary bed in a number of places. Some dilation and morphogenesis of the existing capillary bed is evident. The single arteriole (blue arrow) runs parallel to the venule, and originates at an artery far from the capillary bed (arrow in a). See supplemental videos 1a, b.

the tissue over a period of weeks or days, introducing stresses in the forming tissues.

Creation of stroma within tumor chambers

To limit motion of the PDMS relative to the tissue, we next developed larger, circular devices with access holes or supporting pillars (Fig. 1h, c). These were then tested to determine whether the vasculature will extend sufficiently above the PDMS to interact with a co-implanted tumor. In the TICs with the “hole” design, vascular sprouts appear within the holes as early as three days after implantation and continue to emerge and mature throughout the observation period. The sprouts form in a radial pattern above the PDMS layer, connect with each other and support the growth of a tumor placed on the PDMS (Fig. 3). Significant vascular remodeling is also observed in this system, and a predominant pathway usually evolves between adjacent holes by flow-driven vascular adaptation while smaller vessels are pruned. The developing vascular network advances by extension of sprouts and their subsequent connections to form loops. This progressive addition of loops maintains a blood supply to the system during angiogenesis. Pulsating flow is even observed in blind-ending sprout cells (Supplementary Videos 2, 3).

These are all features of angiogenesis described in other models such as the chick CAM and developing retina, indicating that the process observed in the TIC recapitulates angiogenesis. Thus, these experiments validated the process of angiogenesis in the devices and showed that it is possible to form an extensive tumor vasculature above the PDMS layer.

We next analyzed the host cells and matrix components during the process of chamber vascularization. The pillar design was more appropriate for these studies, because all host cells and vessels enter from the periphery, and then migrate radially into the central chamber. This geometry is more amenable to tracking the dynamics than the hole design, which allows access at multiple points under the tumor chamber. In the pillar design, the height of the chamber (50µm) is sufficient to allow a few cell layers and formation of a complex collagen matrix while minimizing overlap of structures in the z direction. Within 7 days after implantation, red cSMA+ cells (likely myofibroblasts) appear at the edge of the chamber and produce collagen, imaged using second harmonic generation (SHG) microscopy (Fig. 4a). The collagen establishes a structural matrix that originates in the host tissue, anchors to the PDMS in some locations (Fig. 4b), and spans the height of the chamber (Supplementary Video 4).

Along with the cSMA expressing cells, Tie-2 positive cells (likely macrophages) enter the chamber, independent of the vasculature (Fig. 4c; Supplementary Video 4). This cell mobilization and formation of fibrosis recapitulates the early stages of tumorigenesis in fibrotic tumors such as pancreatic, breast and liver. As the activated stromal cells and collagen matrix populate the periphery of the chamber, the vascular sprouts follow behind (Fig. 4e). The sprouting tip cells extend from perfused loops at the leading front as they move into the device (Fig. 5a, b; Supplementary Videos 2 and 3). In the hole design, angiogenic sprouts advance and make new connections, building an extensive network progressively at the leading edge.

Remodeling of the extending vascular network, and its maturation, happens continuously as the vasculature extends. Within 7-10 days, both the vasculature and collagen network have
remodeled to resemble a typical stroma (dense and aligned collagen fibers and differentiated arteries and veins) in the region near the TIC entrance, away from the tumor (Fig. 5c and d).

The arterIALIZATION seen in the early bridging structure (Fig. 2) is also seen here, as evidenced by the smooth muscle cell-invested walls (Fig. 5a, D6, arrow). These new arterioles are functional, exhibiting vasomotion (Supplementary Video 5). In later stages, the network of vessels and collagen assumes normal morphology (Fig. 5c, d). Interestingly, the distribution of collagen around the pillar structures often appears asymmetric, suggesting that the matrix fibers are under tension (Fig. 5c arrowhead). Indeed, if the fibers are cut with a high-intensity laser, the surrounding network relaxes (Supplementary Fig. 1). This indicates that there are activated myofibroblasts contracting with the collagen, just as in fibrotic tumors in other models.¹¹

The host stroma integrates into the tumor tissue in the central tissue chamber

Within 7-14 days, the host stroma contacts the tumor in the central chamber. In the mosaic overview image of Fig. 6 (showing day 7 after implantation of a MMTV tumor fragment in the cranial window TIC), the collagen network originates in the host tissue where it is still integrated with the underlying stroma. It extends over the edge of the PDMS and interfaces with the tumor, extending into, and encircling it.

The host stroma becomes vascularized by sprouting capillaries from the tumor (Fig. 7a). Intussusceptive microvascular splitting occurs (Fig. 7b, e). In regions where the collagen network has become sparse, the attachment of migrating sprouts to collagen fibers is more evident (Fig. 7d, e). Associated with the extension of sprouts is the appearance of tissue structures within the trailing network that resemble intussusceptive microvascular splitting (Fig. 7d and e).
This journal is the periphery of the device, the vasculature matures, developing a new vascularization pattern. The tumor (Fig. 6) represents a significant angiogenic process, allowing for better nutrient delivery and growth. To determine whether the sprouts preferentially migrate along collagen fibers, we measured the angles between the fibers and the extending sprouts (Supplementary Fig. 2a). We found that the angles clustered tightly around a relative angle of 0, indicating strong alignment (Fig. 7f, control). In Losartan treated mice, there were fewer sprouts, and a wider range of relative angles (Fig. 7f). We also found that Losartan decreased the collagen content (Supplementary Fig. 2b), as expected \(^\text{19}\). The anisotropy index increased (indicating less fiber alignment) from 7 to 14 days independent of Losartan treatment (Supplementary Fig. 2c). The length of sprouts decreased in the group treated with Losartan at day 14 (Supplementary Fig. 2d), whereas sprout diameter was not significantly different between groups (Supplementary Fig. 2e).

This demonstrates how the TIC can be used to distinguish cell-matrix interactions during tissue morphogenesis. The results show that angiogenic contact guidance requires specific structural and mechanical properties of the collagen matrix, and these properties were modified with Losartan treatment.

The new vasculature adapts into a hierarchical arterial-venous tree at the periphery but not inside the tumor.

In traditional mouse models of cancer, tumors are implanted in mice in direct contact with the host tissue, and the resulting angiogenic vasculature accumulates at the periphery and under the tumor (Supplementary Fig. 3a). In the Tie2-GFP cSMA-DsRed mice, it is possible to identify the large feeding vessels, but the thick tumor tissue obscures the tumor microvessels (Supplementary Fig. 3b). In contrast, tumors growing in the TIC have a well-delineated, pseudo-2D vascular network that enters radially from the edge of the chamber (Fig. 8a) and infiltrates the tumor (Fig. 8b). Feeding vessels can be tracked from their origin in the host tissue to the tumor, and blood flow can be observed throughout the network (Fig. 8c, Supplementary Video 6a-c).

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The new collagen matrix exhibits the expected range of tumor associated collagen signatures

The production of collagen in and around tumors has attracted attention because of its potential role in tumor progression and invasion\(^1\). In fibrotic tumors, infiltrating macrophages and fibroblasts cooperate to create a dense collagenous matrix that changes the mechanical properties and biology of the growing tumor. It has been shown that the amount of collagen – and its organization – correlate with tumor malignancy, and that tumor collagen can be classified into three “tumor-associated collagen signatures” (TACS)\(^2\). TACS-1 indicates highly dense tumor collagen, and in TACS-2, tense collagen fibers wrap around the tumor tangentially\(^3\). Perhaps most interesting is TACS-3, where collagen fibers are aligned normal to tumor boundary. Collagen fibers configured in TACS-3 potentially support cancer cell invasion, and this tumor collagen signature is associated with poor disease-specific and disease-free survival in breast cancer patients\(^4\).

![Figure 8. MMTV tumor vasculature in the cranial window pillar TIC. a. b) MPLSM/SHG images of the indicated regions in the brightfield image (c). Near the edge of the PDMS, the vasculature extends radically into the central chamber (a). At this time point (day 7) after implantation, the vasculature is mature and has normal morphology in the regions far from the tumor. Four feeding arterioles (red arrowheads) and three venules (blue arrowheads) are indicated. These vessels have significant flow (see Supplementary Video 6a), and have acquired smooth muscle cells in their walls (red, \(\alpha\)SMA\(^\ast\)-DsRed). Note that the arterioles generally have more \(\alpha\)SMA signal than the venules, as expected. b) The vasculature near the growing tumor has dramatically different morphology and flow (see Supplementary Video 6b,c), as observed in other animal models and human tumors. The tumor was not fluorescently labeled in this group, but is visible as the whitish mass extending from the central tumor (T) in (c).](image)

Interestingly, the ECM within our chambers displays these same collagen signatures (Fig. 9). Collagen organization appears diffuse and cross-linked far from the tumor, but there is highly dense, “loose” collagen within the tumor (TACS-1). Collagen fibers are also stretched around the main tumor mass (TACS-2) and there are often tense fibers extending radially into the tumor (TACS-3; Figure 9, arrowheads).

Discussion

Identification of the tissue interactions responsible for tumor progression has been difficult because of the challenges in analyzing cell and matrix dynamics in thick, 3D tissues. As the host tissue and tumor interact, there is mixing of extracellular matrix (ECM), vasculature and cells, all overlapping in 3D space, making the individual events difficult to track or interpret\(^5\). To deconvolve these processes, we combined microfabricated chambers with intravital imaging to visualize matrix and vascular topologies in a pseudo-2D system. Microfabricated devices have proven valuable for creating specific geometries or mechanical properties to probe cell behavior in vivo\(^6\), 15, 28. Unfortunately, it is not yet possible to faithfully reproduce the correct anatomy and physiology in vitro. By introducing microfabricated devices in vivo, we are able to specify the design, but populate it with vasculature and stroma supplied by the host tissue. In addition to providing a new tool for analyzing tumor dynamics, this technology also allows careful monitoring of the self-assembly of tissues in vivo and will be valuable for developing novel approaches to tissue engineering.

The advantages of two dimensional systems for studying cell dynamics and vascular development have long been recognized by the research community, as evidenced by the wide-spread use of the retinal vasculature and the chick chorioallantoic membrane models\(^9, 13, 15, 28\). These models are naturally two dimensional and can be manipulated so they are accessible for longitudinal studies. Systems with similar geometry – but designed for versatile studies of tumor progression in non-embryonic, adult tissue – would accelerate cancer research.

In our hybrid in vitro – in vivo approach, all of the constituents within the PDMS construct are native, including growth factors produced by the tumor and the stromal cells that respond to them. Furthermore, as opposed to in vitro studies that impose artificial matrices such as collagen, fibronectin or matrigel, the ECM in our chambers is entirely fabricated by native stromal cells and remodeled by endogenous mechanisms. These early events – vessel sprout infiltration and matrix deposition – can be initiated by the chamber itself\(^10, 28\), thus accelerating the process of tumor integration. Similar approaches could be used to encourage vascularization in tissue engineering applications.

The sequence of events that result in tumor angiogenesis in our chambers closely follows that observed in spontaneous mouse and

![Figure 9. Collagen organization in the TIC. a) Isolated collagen fiber image from Figure 5, showing the differences in collagen morphology in the chamber. In addition to normal collagen far from the tumor, we observe TACS 1-3 around the tumor. b) Magnified view of box in (a). Elongated matrix fibers are often observed entering the tumor (TACS-3), maintaining connections to the external ECM (arrowheads). It is thought that these allow invasion of tumor cells\(^7\).](image)
human tumors: the infiltration of activated stromal cells, the deposition of extracellular matrix, and the extension of new vasculature into the tumor. The time course of vascularization is also similar to that in embedded tumor models in mice, where the vascular network reaches a maximum density within one or two weeks. In the TICs, the vascular network is also extensive by day 14 and exhibits the same vascular tortuosity, vessel density variations, and flow abnormalities documented extensively in tumors.

An interesting observation made in this study was the close association of migrating sprouts with taut bundles of collagen fibers. Guidance of cell migration by contact with matrix fibers has been documented previously in cell culture assays. In addition, angiogenesis by matrix guidance has been described in other systems, including wound healing in the rat mesentery and in collagen gels in vitro. However, evidence of matrix-guided sprouting has not been previously reported in the context of tumor angiogenesis. In the TICs, it was possible to detect and document the association between blood vessels and collagen fibers concomitantly with tumor growth.

We have shown that the system allows analysis of processes that are poorly accessible with other methods due to their low frequency, rapid dynamics or the short distances involved. For example, the migration of the host stromal cells into the tumor occurs over a distance of ~two millimeters in our device. In a tumor developing in a tissue bed, this distance might be only a few micrometers. Thus, by slightly increasing the physical distance between the tumor and host, we increased the observation field and extended the vascularization and stroma migration processes for more detailed studies of host cell recruitment. Although not present in clinical or traditional in vivo animal models, this separation between the host and tumor tissues did not prevent the recruitment of the appropriate host cells, and can be controlled by modifying the device design.

Similarly, the collagen dynamics seen in our system would be difficult to detect in a tumor that is directly in contact with the tissue, as the network would be situated in a smaller space between the tumor and normal tissues. The vasculature is especially amenable to analysis in the device because the extended distances needed for angiogenesis allows careful imaging of sprout cells and their interactions with matrix fibers and trailing vasculature. The 2D geometry also forces vessel branches to align in the same plane. This allows clear identification of intussusceptive processes, which generally penetrate the lumen perpendicular to the plane of a bifurcation. In 3D tissues, these might be hidden; in our 2D geometry, many are visible.

Silicone elastomers have been used in the clinic for chronic catheters, biosensors and tissue reconstruction since the 1960s. As with any implanted, non-native material, there is a potential for the host tissue to respond to implanted elastomers in a “foreign body response,” which involves macrophage and fibroblast recruitment, and fibrosis at the interface. The foreign body response (FBR) can result in biofouling, diminished function of the implant, or chronic inflammation at the site. Although silicone elastomers such as PDMS are highly biocompatible, further information about host-implant interactions should provide new opportunities and applications for microfabricated devices.

Our results show that, although stromal cells and matrix components are recruited to the implant, there is also extensive vascularization of the deposited matrix that coats the PDMS. This is not generally observed in the FBR, and could greatly benefit implantable microsensors that need to maintain contact with blood-borne metabolites or other chemical species. Importantly, the vascularization was observed in both tissue sites – skin (Fig. 2 and Supplementary Fig. 3) and brain, (illustrated in the rest of the figures and supplementary videos) so appears to be robust. Furthermore, the PDMS did not produce inflammation in the host tissue.

Another potential application of this methodology is the development of vascularized tissues for transplantation. A major challenge in tissue engineering is the lack of a supporting vasculature to supply the tissue quickly after implantation. In our system, a stable, mature vasculature developed and remained functional for more than two months post TIC implantation. Interestingly the vascular network develops continuously and dynamically with the tumor, and small sprouts continue to form as late as 24-25 days post implantation (Fig. 7a, b and c). It is possible that a similar strategy could be used to vascularize other engineered tissues designed to replace patient tissues. Although the process is robust in our tumor samples, further studies are necessary to determine whether other tissue types can be adequately vascularized in this way.

Many laboratories around the world study tumor biology using transparent window models, and even more groups have the ability to fabricate PDMS devices. The procedures here represent a straightforward integration of these technologies, and should be accessible to many researchers. We have presented three simple designs for isolating a tumor (a “raft,” a “strainer”- a disk with access holes, and a “sandwich”), but more sophisticated chambers could, for example, include subcompartments for collecting specific cell populations or access ports with varied dimensions to control gradients of tumor- or host-derived chemokines. Thus, this technology is easily adaptable and versatile, and has the potential to facilitate new discoveries in the fields of tumor biology and tissue engineering.

Materials and Methods

All animal experiments were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care. αSMA-DsRed/Tie2-GFP/FVB and Tie2-GFP/Rag1 (for TIC1 tumor implantation) mice were used in these studies. In these transgenic strain, blood vessels express GFP under control of the Tie2 promoter, while αSMA-expressing cells express DsRed. The mice were implanted with transparent dorsal skinfold chambers (DSC) or cranial windows (CW) as described previously.

Tumor fragments

The AK4.4 pancreatic (Fig. 2, 3, Supplementary Fig. 3 and Supplementary Videos 1a and b, 2 and 3), 4T1 (Figs. 5c, d, 7a-e, Supplementary Fig. 1) and MMTV (Figs. 6, 8, 9 Supplementary Fig. 2a and 4, Supplementary Videos 6a-c) breast cancer tumors were developed in a FVB/N (AK4.4 and MMTV) background and implanted in αSMA-DSRed/Tie2-GFP/FVB mice or developed in a Rag1 and implanted in Tie2-GFP/Rag1 mice (4T1). For implantation in the chambers, the source tumors from donor mice were surgically excised and placed in a petri dish in sterile HBSS cell culture medium. The tumors were then cut into small fragments using sterile surgical scissors. Small fragments (~2mm in diameter) were placed in the center of the bottom layer of the TIC, covered with HBSS cell culture media, and either the glass coverslip or the PDMS cover was placed on top.

In vivo tissue isolation chambers (TICs)

TICs were fabricated from poly (dimethylsiloxane) (PDMS, Sylgard 184, Dow Corning) at a ratio of base to curing agent of 12:1. A silicon wafer master was constructed using soft lithography to form the negative relief features. An electrospriner was used at 800 rpm to create thin 75-100 mm PDMS sheets. The PDMS sheets were then heated to 60°C overnight. The PDMS “raft,” a 1.6x2.7mm rectangular piece (Fig. 2 and Supplementary Videos 1a and b)
was obtained by cutting a flat portion with PDMS with a sterile surgical scalpel. For the “hole” design, a PDMS disk of 10mm diameter was punched out with a circular punch (Fig. 3 and Supplementary Video 2 and 3). A variable number of access hole were then punched out with a 500micron biopsy punch. The resulting holes were 200-300micron in diameter due to the elasticity of the PDMS material. Finally, for the “sandwich” design, PDMS disks were carefully cut out from the master using biopsy punches of various diameters and then sterilized by exposure to UV light for ~30 min (Figs. 4-9, Supplementary Figs. 1, 2 and 4, and Supplementary Videos 4-6). Three separate components, each 4 mm in diameter, were created for each device: i) a bottom layer with an array of circular pillars oriented vertically (each 200 µm wide, 50 µm in height, separated by 500 µm); ii) an annular middle layer with a 3 mm diameter void, which served as a spacer and defined the central chamber; and iii) a top cover, comprised of a PDMS disk with no features; this layer contacted the glass coverslip. The tumor fragment was placed on the pillars of the bottom layer and inside the annulus of the middle layer. At the periphery of the device, the pillars contact the spacer layer, creating a 50µm high gap through which host cells can enter the chamber.

**Intravital Microscopy**

**Multiphoton laser microscope:** A custom-made multiphoton laser microscope was used at 810nm excitation wavelength. Three dichroic mirrors and three filters of 405, 535 and 610 nm were used to simultaneously image second order harmonic generation (SHG), GFP and Ds-Red fluorescence signals respectively.

**Stereo microscope:** Bright field and fluorescence images were acquired with a Nikon SMZ1500 stereomicroscope (Nikon Instruments Inc., Melville, NY, USA) equipped with a Nikon D90 SLR photo camera and a QIClickTM digital CCD camera (QImaging, Surrey, BC, Canada). The microscope was equipped with GFP and Ds-Red fluorescence filters, allowing visualization of the vasculature and the labeled RBCs in vivo. This setup was also used to acquire the supplemental intravital videos.

**Visualization of the host vasculature in live mice:** To assess the perfusion status of the vessels and visualize the blood flow in vivo, animals were perfused via retro-orbital injection with 100 µl of either FITC- or rhodamine- dextran solution or labeled red blood cells. For labeling, RBCs were collected from a donor mouse, labeled with highly lipophilic carbocyanine dyes (DiO or DiD) (InvitrogenTM Life Technologies, Grand Island, NY). The RBC solution (100µl, 50% hematocrit) was injected into the animal retro-orbitally. Fluorescence from the labeled RBCs was sufficiently bright to allow imaging for approximately 15 days.

**Pharmacological treatment:** Mice implanted with cranial window chambers and MMTV tumor fragments were treated with Losartan, an angiotensin receptor blocker at 40mg/kg (daily, day 1-7) and then 80µg/kg (day 8-15), or an equal amount of PBS (control group) intraperitoneally.

**Collagen fiber analysis:** The orientation of the collagen fibers and blood vessels was determined using FibrilTool for ImageJ. The algorithm calculates the angle of aligned structures (fibers or vessels) and an index of anisotropy, which indicates the uniformity of the alignment. First, a z-projection was obtained from the entire stack of Fluoview images. Two similar size regions of interest (ROI) were chosen to include a sprout and the collagen fibers at the top of the sprout. The software reports the predominant angle of the fiber structures in the ROI (either the collagen fibers or the vessels) and an index of anisotropy. The results are reported as the absolute value of the difference between the vessel and corresponding fiber angles and isotropy indices.

After defining a region of interest (ROI, yellow box), the FibrilTool plugin for ImageJ returns the average angle of fibers and displays a red line with that orientation. The length of the line is proportional to the anisotropy of the fibers. Repeating this procedure over the entire image gives a map of local collagen angles and anisotropy (Fig 5d). Relative angle between sprouts and ECM fibers measured with the FibrilTool at day 7 (D7) and day 14 (D14) as described in the Supplementary methods section. Collagen fraction was calculated from the SHG images as the percentage area of collagen fibers in five equal fields of view oriented at four corners and the center of the image. The anisotropy index is a measurement of fiber alignment and is calculated with FibrilTool. The length of sprouts is measured from the top of a vascular loop to the tip of the vessel sprout. Only the sprouts which were entirely located in the visual field were included in the length measurements. The diameter of sprouts was measured as the inner diameter marked by the fluorescent marker inside the sprout lumen.

**Laser ablation of collagen fibers:** The multiphoton laser microscope was used to ablate selected collagen fibers in the chambers. A preablation image was taken to identify the location and thickness of the collagen fibers. The laser beam was then programmed to scan a small rectangular region perpendicular to the collagen bundle. The laser power was then increased to the maximum voltage of 3V. The laser beam was focused to scan the collagen fibers repeatedly until the collagen fibers were interrupted as evidenced by empty space in between fibers. No visible damage was noticed in the adjacent tissue. The laser was returned to imaging range and a post-procedure image was acquired.

**Statistical analysis:** The numerical data in Figure 6 and Supplementary Figure 2 are expressed as mean ± SEM. Analysis of means was performed with a two-tailed paired (longitudinal comparisons) and unpaired (between groups) t-test (GraphPad Prism software, San Diego, CA, USA). Differences were considered significant at P values less than 0.05.

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**References:**


Supplementary Figure 1. Collagen tension around the TIC pillars. a) Brightfield image of collagen stretching around a pillar structure in a TIC with a 4T1 tumor. b) Corresponding fluorescence image of the αSMA-expressing cells embedded in the collagen. c, d) SHG images before (c) and 85 minutes after (d) laser ablation of a selected bundle of collagen fibers (red arrow) near a pillar structure (*). Some of the collagen fibers relax following laser ablation (yellow arrow heads). Scale bar = 200 µm.
Supplementary Figure 2. Effect of losartan on fiber and sprout properties. Measuring relative angles and the effect of Losartan treatment on collagen and sprouts. a) Using the ImageJ plugin FibrilTool plugin, we create regions of interest (ROIs) that extend past the tip of an identified sprout process (top right panel). The algorithm was applied to the SHG (collagen) channel, and returned the average angle of fibers in each ROI as well as the index of anisotropy. The orange line in the figure indicates the average fiber angle, and the length is proportional to the anisotropy of the fibers. The angle of each sprout was then determined manually using the angle tool of ImageJ (bottom left panel). The relative angle is then calculated by subtraction (bottom right panel). The results using this method are presented in the main text, Figure 6f. b) Collagen fraction, measured by autothresholding the SHG images and calculating the fraction of positive pixels, significantly decreased with Losartan treatment at Day 14. c) The anisotropy index was also measured over the entire collagen matrix (rather than only at sprout tips). Anisotropy increased with tumor growth, indicating more fiber alignment. This enhanced directional alignment of fibers was not affected by losartan treatment. d) The length of sprouts decreased in the losartan group at day 14, compared to buffer-treated controls. e) Sprout diameter was not significantly different between groups.
Supplementary Figure 3. Tumor growth in the dorsal window chamber without a TIC. a) AK4.4 pancreatic tumor (T) imaged through the window chamber of a αSMA-DsRed/Tie2-GFP mouse. There is significant angiogenesis at this time point (day 4), and new vessels accumulate at the tumor periphery. b) fluorescence imaging of the same tumor, showing αSMA-DsRed-positive arterial walls, which appear yellow. Vessels appear red due to blood hemoglobin. Identifiable arteries and veins are indicated (* and ◊, respectively). Some angiogenic processes are visible, but most are obscured by the thick tumor tissue. Scale bar: 1mm.
**Supplementary Figure 4.** Regions of the TIC imaged in Supplementary Videos 6a-c. The MMTV tumor appears as the white, diffuse mass. The mouse was treated with Losartan for 7 days and perfused with DiO labeled RBCs to visualize the vasculature.
Supplementary Videos

**Supplementary Video 1a.** Vascular bundle advancing over the edge of the PDMS on day 13 after implantation with AK4.4 tumor fragment and PDMS “raft”. The venules terminate at the capillary bed, out of the field of view at top. The single arteriole is fed by a distant artery to the left (see Figure 2, main text). The diameter of the larger venules is in the range 30-50µm.

**Supplementary Video 1b.** Lower magnification video of the vascular structure in Video 1a. The view shows the connections of the venules with the host capillary bed; the flexing motion is due to muscle contractions in the skin underlying the PDMS.

**Supplementary Video 2.** Intravital imaging of blood flow in an advancing angiogenic network. On day 21 in a DSFC chamber with a AK4.4 tumor, sprouting tip cells (visualized by Tie-2 GFP signal) can be observed extending from perfused looping structures are produced by progressive connection of sprouts at the leading edge. The process ensures that the tip cells are always near a supply of flowing blood (visualized by injection of DiO-labeled RBCs). Note that the sprouting cells quickly develop patent lumens, and platelets or RBCs can be observed pulsating with the structures.

**Supplementary Video 3.** Intravital imaging of blood flow in an advancing angiogenic network at lower magnification. On day 21 in a chamber with an AK4.4 tumor, multiple tip cells (visualized by Tie-2 GFP signal) extend from the edge of the new network. To visualize the blood flow, the mouse was injected with DiO labeled RBCs.

**Supplementary Video 4.** Animated z-stack of an image volume near the entrance to the TIC without a tumor implant. The dark shadow marks the inner boundary of the PDMS spacer. On Day 1, fibroblasts (red, αSMA-DsRed) have entered the chamber and a collagen matrix is evident (SHG signal, white). Angiogenic sprouts (FITC-dextran, green) are migrating within the collagen. The depth of the stack, 45µm, spans the height of the middle chamber of the TIC. Width of
field=630x630microns.

**Supplementary Video 5.** Time lapse movie of the maturing vascular network in a TIC in a Tie2-GFP/α-SMA- DsRed mouse at day 8 with no tumor implant. Blood appears green due to DiO labeled RBCs. One of the new vessels, an arteriole transporting blood from the periphery towards the center of the TIC, has several αSMA+ cells incorporated in its wall and undergoes vasoconstriction (arrow head). In contrast, the large venule at bottom has more sparse αSMA+ cell coverage. Tie2+ macrophages are often very active, migrating on the surface and the matrix.

**Supplementary Video 6a.** Intravital imaging in a TIC with a MMTV tumor and treated with Losartan (Day 7). Arrows indicate blood flow direction in the major arterioles and venules, and the dashed line marks the boundary of the inner chamber. The video starts near the edge of the chamber and scans to a region near the tumor (see the map in Supplemental Figure 4 and Figure 8 of the main text). Note the normal vascular morphology and blood flow near the TIC entrance, and the change in these features as the vasculature approaches the tumor. Blood flow is visualized by injection of DiO-labeled RBCs.

**Supplementary Video 6b.** Intravital imaging in a TIC with a MMTV tumor and treated with Losartan (Day 7). This video is within the tumor, at the interface of the avascular tumor core (see Supplemental Figure 4). Note the characteristic tumor vessel morphology with highly dilated and tortuous vessels and non-uniform blood velocities. Blood flow is visualized by injection of DiO-labeled RBCs.

**Supplementary Video 6c.** Intravital imaging in a TIC with a MMTV tumor and treated with Losartan (Day 7). This video starts within the tumor, where vessel density is non-homogeneous and there are avascular regions. It scans down to the entrance of the chamber, where the angiogenic vessels interface the tumor. Here, we see the typical dense accumulation of vessels associated with tumor angiogenesis. (see map in Supplemental Figure 4). Blood flow is visualized by injection of DiO-labeled RBCs.
Reference: