

1 **Functional angiogenesis requires microenvironmental cues balancing endothelial cell migration and proliferation**

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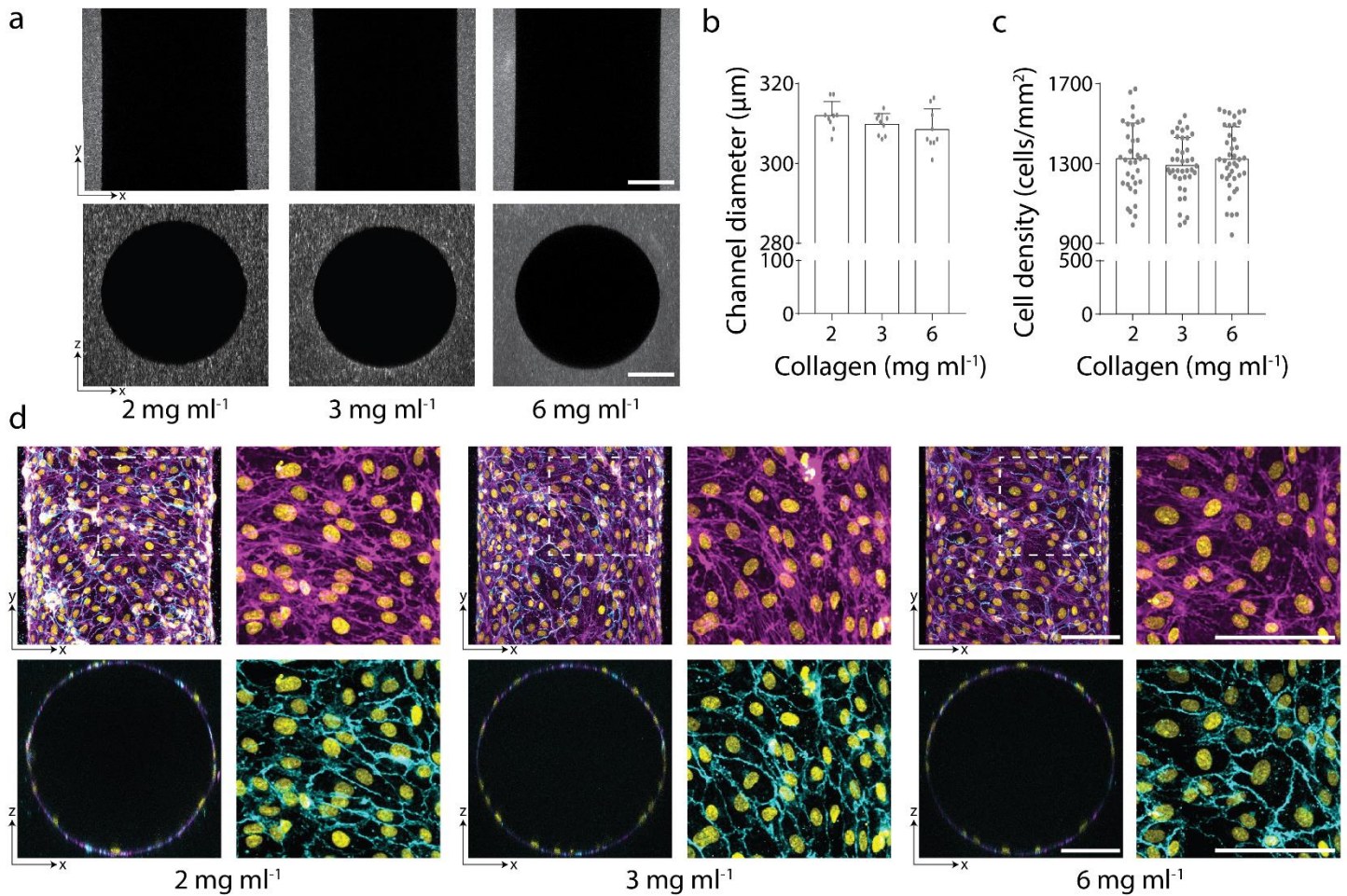
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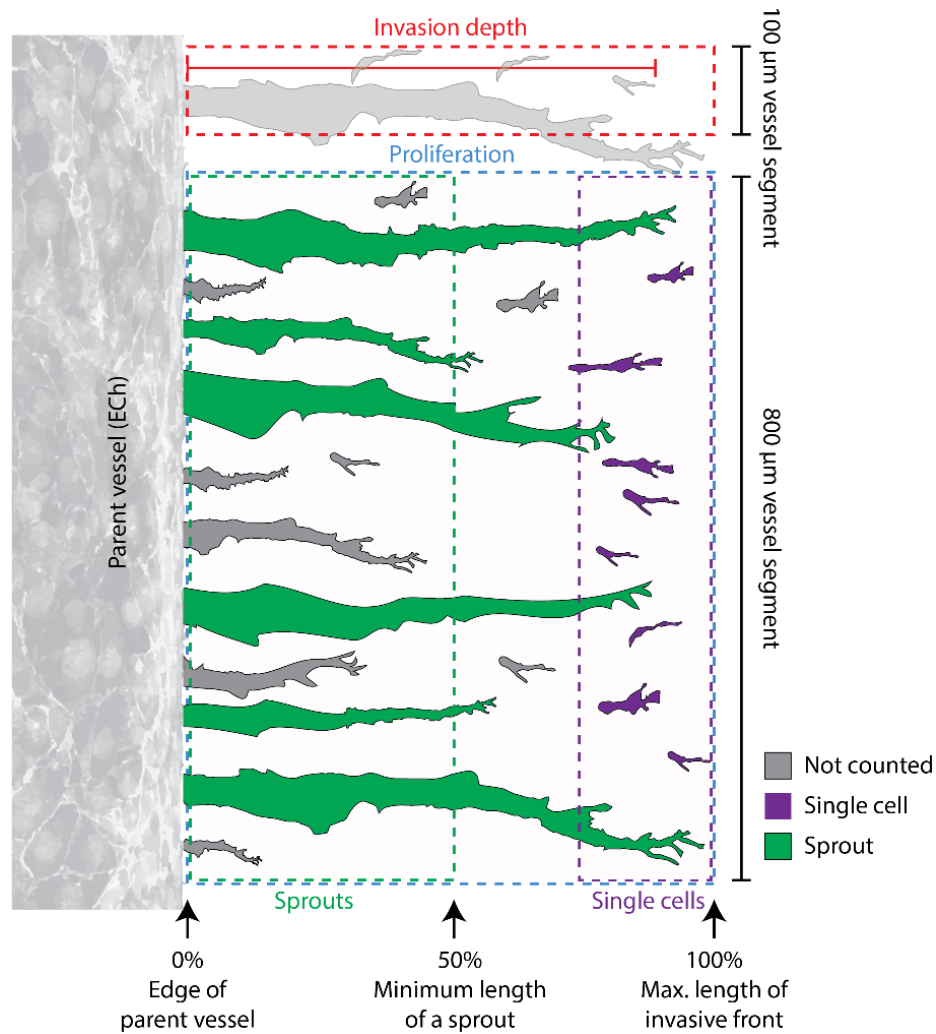
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8 **SUPPLEMENTARY MATERIAL**

9 The Supplementary Material includes 6 Supplementary Figures, 1 Supplementary Table, and 5 Supplementary Movies.

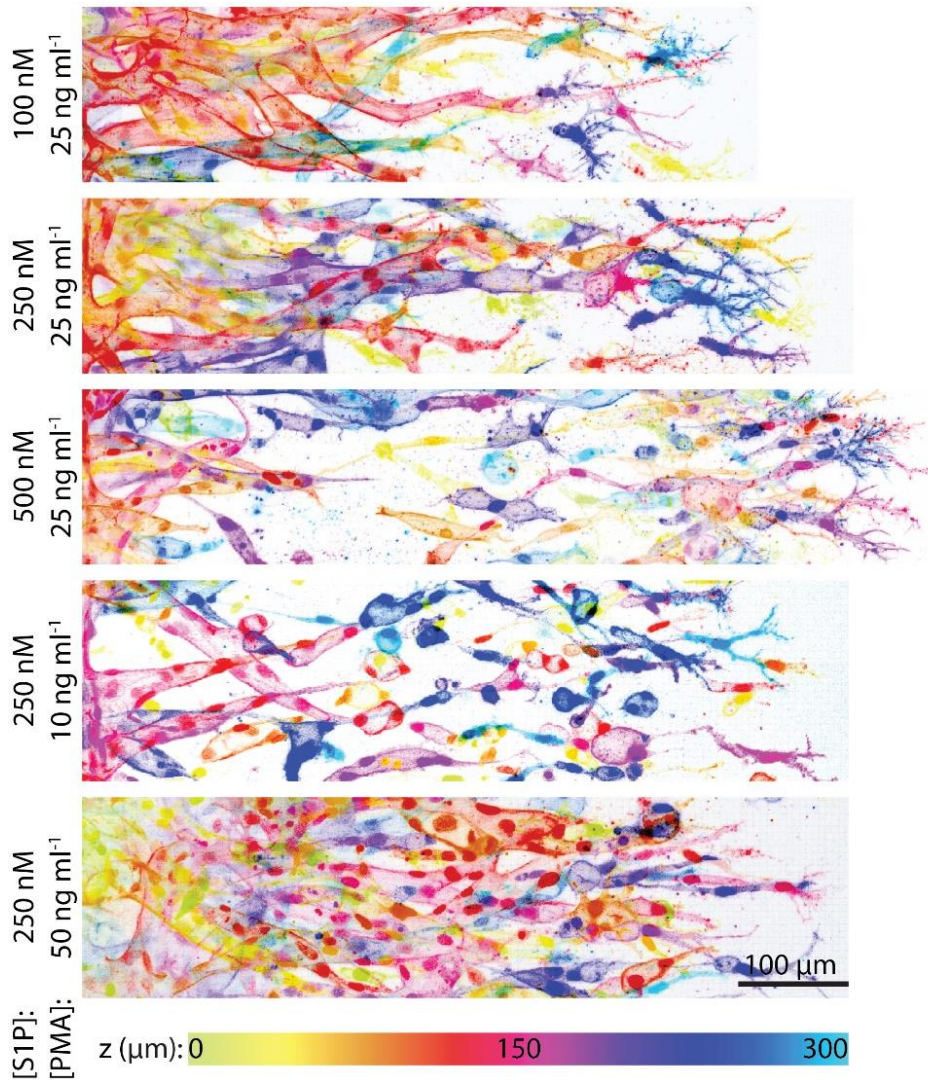


10 **Supplemental Figure 1 | Parent vessels with consistent diameter and cell density.** **a**, Representative images of x-y and
11 x-z orthogonal views of 3D channels generated in indicated collagen density. **b-c**, Quantifications of channel diameter and
12 parent vessel endothelial cell density with varying collagen concentration. Cell seeding density was varied as EC
13 attachment efficiency was dependent on collagen density (1.5 M/ml for 2 mg ml⁻¹, 2 M/ml for 3 mg ml⁻¹ and 5 M/ml for 6
14 mg ml⁻¹). All data presented as mean ± s.d.; * indicates a statically significant comparison with P<0.05 (one-way analysis
15 of variance). n≥8 channels (**b**) and n≥34 vessel segments (each 400 µm length) (**c**) per condition. **d**, Representative images
16 of x-y (max intensity projection) and x-z (single slice) orthogonal views of parent vessels generated with varying collagen
17 concentration after 24-hour culture. Insets indicated with dashed white lines. Nucleus (yellow), F-actin (magenta), and VE-
18 cadherin (cyan).
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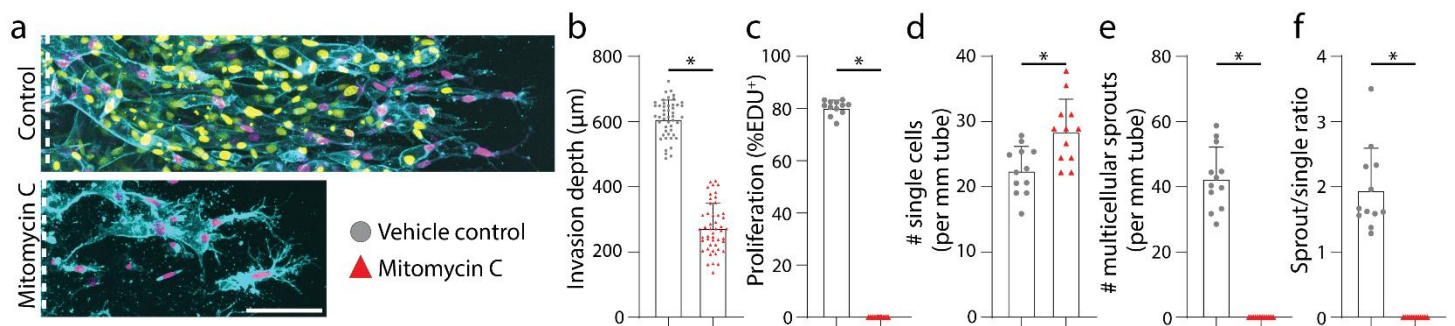


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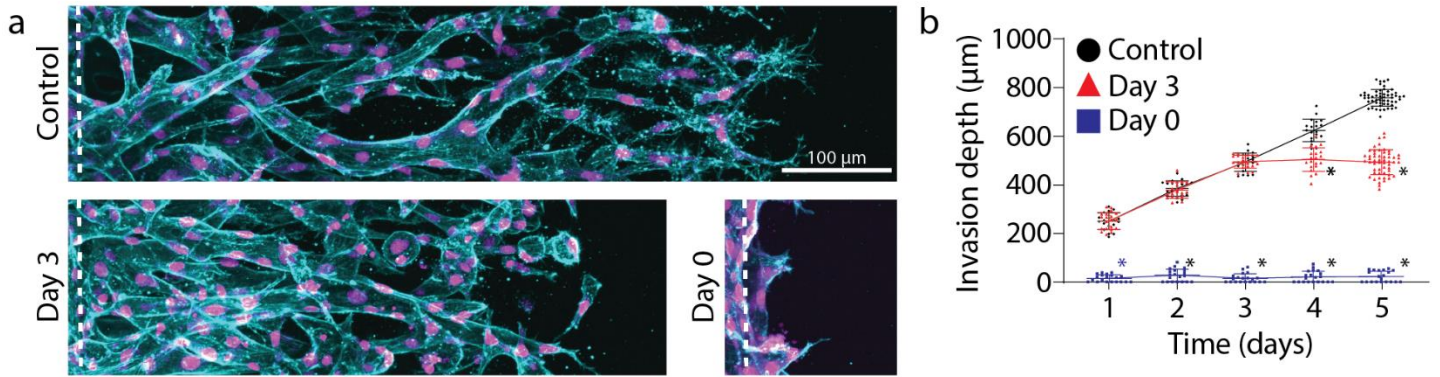
Supplemental Figure 2 | Schematic of quantification metrics. To quantify invasion depth (red), images were segmented into 100 μm segments along the parent vessel and measurements were taken from the ECh edge to the tip of the endothelial cell that invaded furthest within that segment. To quantify proliferation (blue), images were segmented into 800 μm segments along the parent vessel, and the percentage of EdU⁺ nuclei was measured only in endothelial cells that had invaded into the extracellular matrix from the ECh edge to leading invasive front. To quantify leading edge single cells (purple), images were segmented into 800 μm segments along the parent vessel and isolated single cells were quantified within 150 μm of the leading invasive front. To quantify multicellular sprouts (green), images were segmented into 800 μm segments along the parent vessel, and quantified connected endothelial sprout structures (from ECh edge to sprout tip) with a minimum length of half the max invasion depth. Single cells and sprouts outside of these criteria were not included in the analysis (grey).



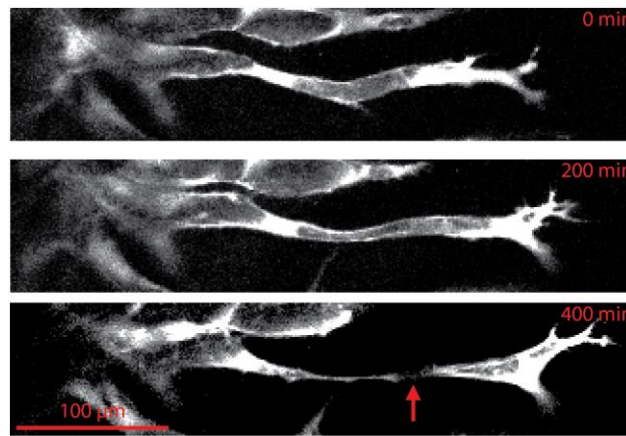
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32 **Supplemental Figure 3 | Z-depth encoded projections.** 300 μm max intensity projections from Figure 2 pseudo-colored
33 with z-position. Z-stack image files were processed in FIJI using “Temporal-Color Code” function to assign each z-slice an
34 individual color. “Physics” color scale was utilized and inverted.
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37 **Supplemental Figure 4 | Proliferation inhibition abrogates multicellular sprouting.** **a**, Representative images (max
38 intensity projection) of endothelial cell invasion in response to mitomycin C proliferation inhibition with S250:P50 and
39 3mg ml^{-1} collagen. **b-f**, Quantifications of invasion depth, proliferation, and morphology of invading endothelial cells as
40 single cells or multicellular sprouts. All data presented as mean \pm s.d.; * indicates a statically significant comparison with
41 $P < 0.05$ (two-tailed Student’s t-test). For invasion depth analysis (**b**), $n = 48$ vessel segments (each $100 \mu\text{m}$ length) per
42 condition. For proliferation and migration mode analysis (**c-f**) $n = 12$ vessel segments (each $800 \mu\text{m}$ length) per condition
43 from $n = 2$ devices/condition (technical replicates) over $n \geq 4$ independent studies (biological replicates).



44 **Supplemental Figure 5 | S1P receptor inhibition abrogates S1P-driven EC invasion.** **a**, Representative images (max
 45 intensity projection) of invading endothelial cells in response to 100 nM FTY720 treatment with S250:P25 and 3mg ml⁻¹
 46 collagen. Day 0 conditions were composed of ECs pre-treated with FTY720. F-actin (cyan), nucleus (magenta). **b**,
 47 Endothelial cell invasion depth over time in response to 100 nM FTY720 treatment. All data presented as mean ± s.d.; *
 48 indicates a statically significant comparison with P<0.05 (one-way analysis of variance). n≥20 vessel segments (each 100
 49 µm length) per condition.
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52 **Supplemental Figure 6 | Tip cell breakage from stalk cell.** Time-lapse series demonstrating the separation of tip and stalk
 53 cell connectivity of a sprout cultured within 3 mg ml⁻¹ collagen with 250 nM S1P and 25 ng ml⁻¹ PMA. Time-lapse imaging
 54 began on day 2 of sprout culture. Red arrow indicates tip and stalk cell breakage.
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Figure #	[S1P] (nM)	[PMA] (ng ml ⁻¹)	[Collagen] (mg ml ⁻¹)
1c-e	0	0	3
2a	0-500	0	3
2b	0-500	25	3
2h	250	0-50	3
3a	100-500	10-50	3
4a	250	25	3
5	250-500	10-25	3
6a	250	25	2-6
6b	250	25	2-6
6i	100-250	25	2
Supp. 1d	0	0	2-6
Supp. 4	250	50	3
Supp. 5	250	25	3
Supp. 6	250	25	3

57 **Supplemental Table 1 | Microenvironmental cues utilized in experiments by figure.**
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- 60 **Supplemental Movie 1** | Endothelial sprouts cultured with 100 nM S1P and 25 ng ml⁻¹ PMA in 3 mg ml⁻¹ collagen over 5
61 days.
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- 63 **Supplemental Movie 2** | Endothelial sprouts cultured with 250 nM S1P and 25 ng ml⁻¹ PMA in 3 mg ml⁻¹ collagen over 5
64 days.
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- 66 **Supplemental Movie 3** | Endothelial sprouts cultured with 500 nM S1P and 25 ng ml⁻¹ PMA in 3 mg ml⁻¹ collagen over 5
67 days.
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- 69 **Supplemental Movie 4** | Endothelial sprouts cultured with 250 nM S1P and 10 ng ml⁻¹ PMA in 3 mg ml⁻¹ collagen over 5
70 days.
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- 72 **Supplemental Movie 5** | Endothelial sprouts cultured with 250 nM S1P and 50 ng ml⁻¹ PMA in 3 mg ml⁻¹ collagen over 5
73 days.