



Supporting Information

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A Robust Method for Perfusable Microvascular Network Formation In Vitro

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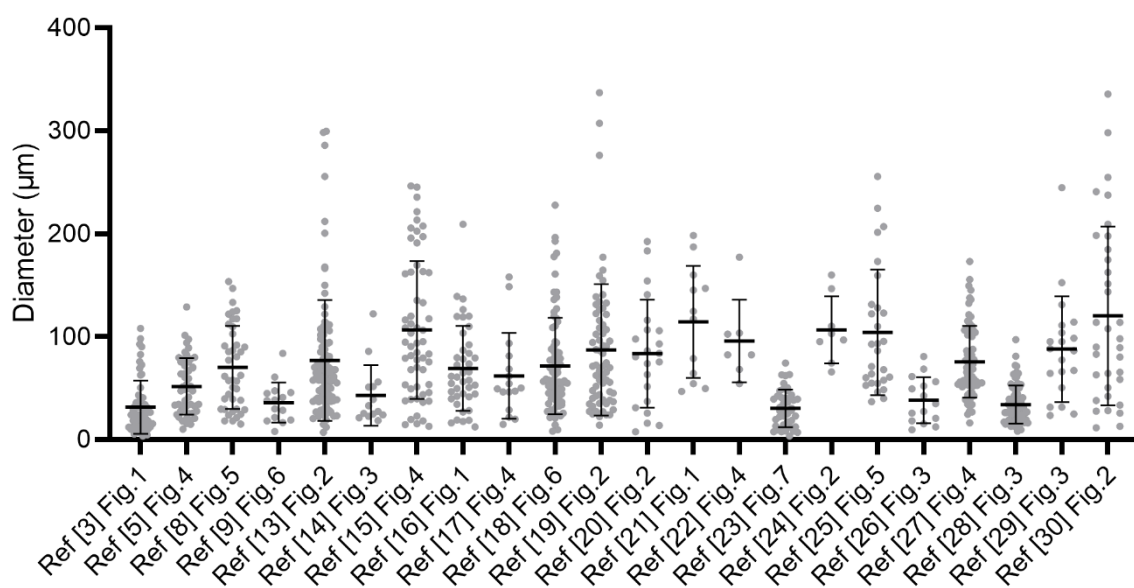
Figure S1

Figure S1. Vessel diameter of perfusable MVNs from published studies. Published 2D MVN figures were used for vessel diameter analysis. Diameters were converted and calculated using scale bar information in the published figures. Method for vessel diameter measurement is provided in Methods section and Figure S4.

Figure S2

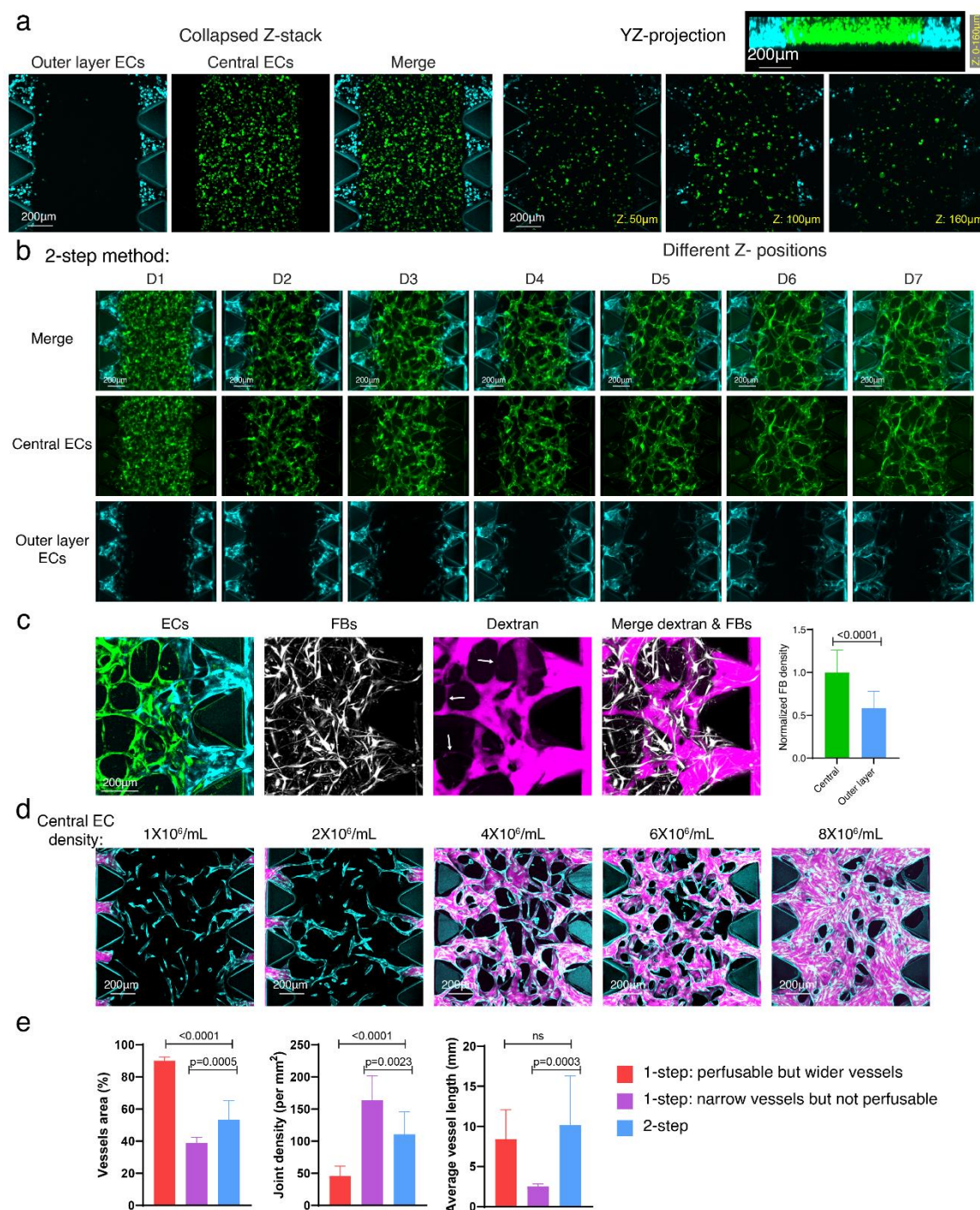


Figure S2. Features of 2-step seeding method produced MVNs. a) Representative images of central and outer layer EC distribution at day 0. Central ECs are uniformly distributed, while outer layer cells are slightly denser at the bottom. Collapsed Z-stack (left), YZ-projection (top right) and different Z-positions (bottom right) are shown. Cyan, ImHUVeCs expressing BFP. Green, ImHUVeCs expressing GFP. b) Representative epifluorescent images showing the 2-step MVN formation process from day 1 to day 7 (outer layer: ImHUVeCs expressing BFP; central: ImHUVeCs expressing GFP with lung FBs). c) Representative images of FB

locations (left) and statistical analysis of normalized FB density (right) in the microfluidic device seeded with 2-step method. Immortalized lung FBs expressing mCherry were used. White arrows point to the perfusable narrow microvessels. Fewer FBs are located at the regions between microposts compared to the central region. Cyan, ImHUVeCs expressing BFP. Green, ImHUVeCs expressing GFP. Magenta, dextran (70 kDa). Bars represent mean \pm S.D. Two-tailed t tests were performed for the statistical comparisons ($n = 2$ devices, 12 ROIs each). d) Confocal images of MVNs made with 2-step seeding method using different density of central ECs. Lung FBs in the central region are $1.5 \times 10^6/\text{mL}$ for all the groups. Lower density ($4\text{-}6 \times 10^6/\text{mL}$) ECs tend to form perfusable narrow MVNs, while too few ECs lead to nonconnected microvessels, although the vessels made of outer layer ECs have open lumens. Cyan, ImHUVeCs expressing BFP. Magenta, dextran (70 kDa). e) Statistical analysis of vessel area percentage (left), joint density (middle), and average vessel length (right) of central MVNs made with 1-step or 2-step seeding method. Bars represent mean \pm S.D. Two-tailed t tests were performed for the statistical comparisons ($n = 3$ devices, 5 ROIs each).

Figure S3

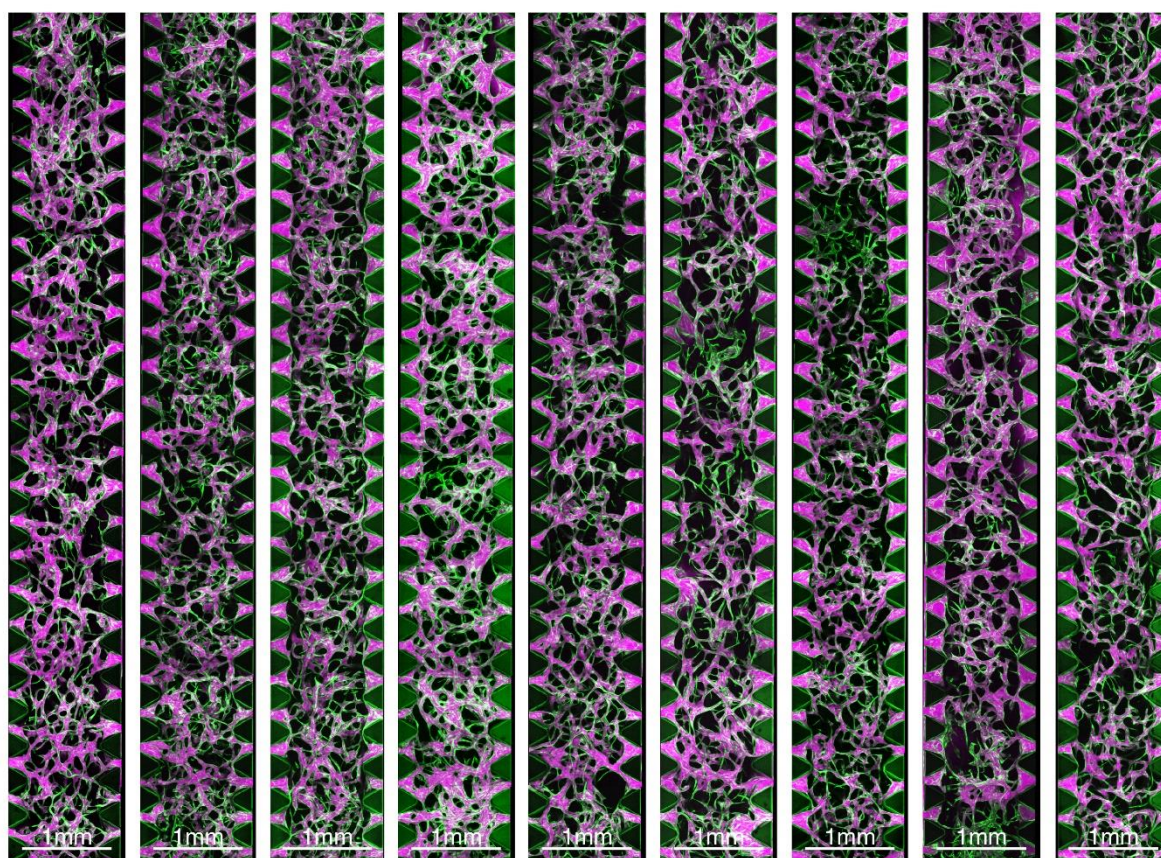


Figure S3. Representative images of 2-step MVNs in the entire device. Nine devices were seeded with ImHUVeCs expressing BFP and lung FBs using 2-step seeding methods, which

indicates a high reproducibility. Opening % is $99.78 \pm 0.67\%$; central perfusable MVN % is $98.14 \pm 3.86\%$ (n = 9 devices). Green, ImHUVeCs. Magenta, dextran (70 kDa).

Figure S4

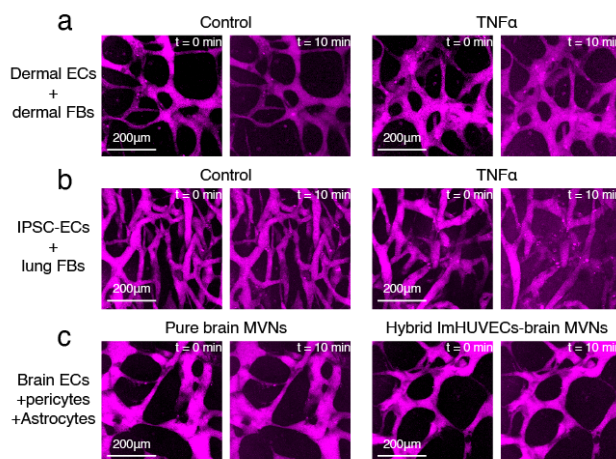


Figure S4. Representative images of permeability tests in 2-step MVNs. a) Representative images of 70 kDa dextran in 2-step dermal MVNs treated with or without TNF α . b) 2-step MVNs formed with iPSC-ECs and lung FBs were treated with or without TNF α . 70 kDa dextran (magenta) was used for permeability analysis. c) Representative images of 10 kDa dextran perfused in pure brain MVNs made with 2-step strategy, or in hybrid brain MVNs (outer layer ECs: ImHUVeCs; central: brain ECs+brain pericytes+astrocytes) over 10 min.

Figure S5

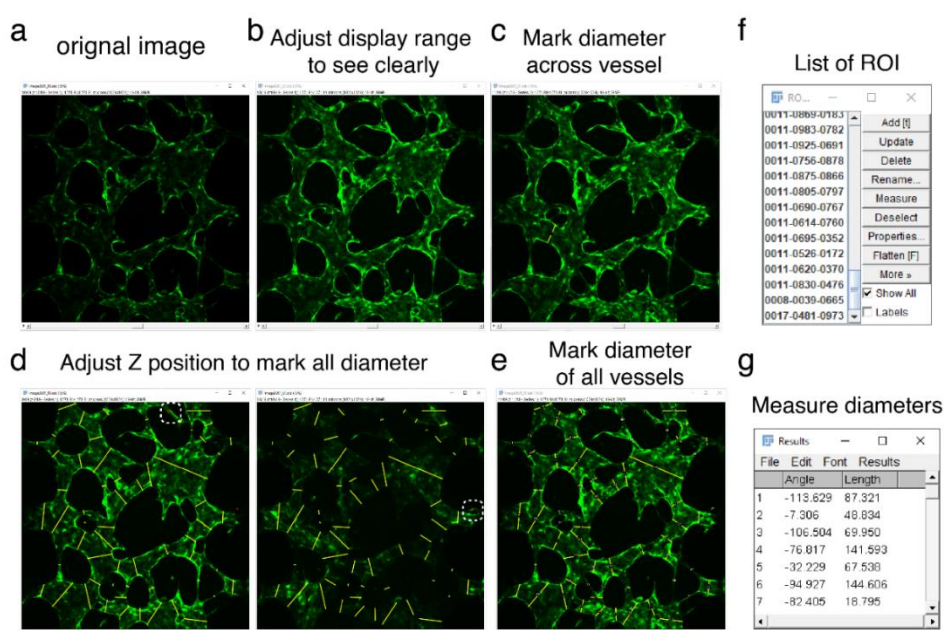


Figure S5. Vessel diameter analysis. a) Load an image in ImageJ. b) adjust display range of the image using brightness/contract function. c) Mark diameter of a lumen using straight ROI tool and add it to ROI manager. Yellow line shows a ROI. Similarly mark other vessel lumens on the same slice. d) adjust Z positions of the slices, and mark other vessel lumens at different Z positions. Red lines in the white dashed squares highlight 2 vessels at different Z positions. e) Mark all the vessel lumens. After labeling all the vessel lumen, f) click the measure button on ROI manager window. g) The results of the vessel diameter would be shown in results window.

Table S1: Experimental parameters in each figure.

Figure title.	1- or 2-step	ECs	Outer layer ECs	Central ECs	Stromal cells	Matrix info	Time window
Figure 1 (b-d)	1-step	ImHUVECs expressing BFP (various concentration)			Lung FBs (various concentration)	Bovine Fibrin 3 mg/mL	Day 7
Figure 2	1-step	ImHUVECs expressing GFP (7×10^6 /mL)			Lung FBs (1×10^6 /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure 3	1-step	ImHUVECs expressing BFP (various concentration)			Lung FBs (various concentration)	Bovine Fibrin 3 mg/mL	Day 7
Figure 4b	1-step wider	ImHUVECs expressing BFP (8×10^6 /mL)			Lung FBs (1×10^6 /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure 4b	1-step narrower	ImHUVECs expressing BFP (5×10^6 /mL)			Lung FBs (1.5×10^6 /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure 4c	2-step		ImHUVECs expressing BFP (10×10^6 /mL)	ImHUVECs expressing GFP (5×10^6 /mL)	Lung FBs (1.5×10^6 /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure 5a	2-step		ImHUVECs expressing BFP (10×10^6 /mL)	ImHUVECs expressing GFP (5×10^6 /mL)	Lung FBs (1.5×10^6 /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure 5b top	1-step	ImHUVECs expressing BFP (8×10^6 /mL)			Lung FBs (1×10^6 /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure 5b bottom Figure 5 d-e	2-step		ImHUVECs expressing BFP (10×10^6 /mL)	ImHUVECs expressing GFP (5×10^6 /mL)	Lung FBs (1.5×10^6 /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure 6a	2-step		ImHUVECs expressing BFP (10×10^6 /mL)	ImHUVECs expressing GFP (5×10^6 /mL)	Patient-derived thyroid FBs (1.5×10^6 /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure 6b	2-step		Brain microvascular ECs expressing GFP (12×10^6 /mL)	Brain microvascular ECs expressing GFP (6×10^6 /mL)	Brain pericytes (1×10^6 /mL) Astrocytes (0.5×10^6 /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure 6 c	2-step		HDBMEC expressing BFP (12×10^6 /mL)	HDBMEC expressing BFP (6×10^6 /mL)	Dermal FBs (1.5×10^6 /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure 6 e	2-step		HDBMEC expressing BFP (10×10^6 /mL)	HDBMEC expressing BFP (5×10^6 /mL)	Dermal FBs (1.5×10^6 /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure 6 f-h	2-step		iPSC-ECs (10×10^6 /mL)	iPSC-ECs (5×10^6 /mL)	Lung FBs (1.5×10^6 /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure 7b	2-step		ImHUVECs expressing BFP (10×10^6 /mL)	Brain microvascular ECs expressing GFP (6×10^6 /mL)	Brain pericytes (1×10^6 /mL) Astrocytes (0.5×10^6 /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure 7d	2-step		ImHUVECs expressing BFP (10×10^6 /mL)	iPSC-ECs (5×10^6 /mL)	Lung FBs (1.5×10^6 /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure 8b (left)	1-step	ImHUVECs expressing GFP (8×10^6 /mL)			Lung FBs (1×10^6 /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure 8b (right)	1-step	ImHUVECs expressing GFP (8×10^6 /mL)			Lung FBs (1×10^6 /mL)	Bovine Fibrin 5 mg/mL	Day 7
Figure 8c	2-step		ImHUVECs expressing GFP (10×10^6 /mL)	ImHUVECs expressing GFP (5×10^6 /mL)	Lung FBs (1.5×10^6 /mL)	Bovine Fibrin Central:5 mg/mL; outer layer: 3 mg/mL	Day 7

Figure 8e	1-step	ImHUVECs expressing GFP (8 x10 ⁶ /mL)			Lung FBs (1 x10 ⁶ /mL)	Bovine Fibrin 5 mg/mL	Day 7
Figure 8b	1-step	ImHUVECs expressing GFP (8 x10 ⁶ /mL)			Lung FBs (1 x10 ⁶ /mL)	90% 6 mg/mL human fibrin mixed with 10% 1.5 mg/mL Alexa488 human fibrin	Day 7
Figure 8f	2-step		ImHUVECs expressing GFP (10 x10 ⁶ /mL)	ImHUVECs expressing GFP (5 x10 ⁶ /mL)	Lung FBs (1.5 x10 ⁶ /mL)	Outer layer: Bovine Fibrin 5 mg/mL; Central: 90% 6 mg/mL human fibrin mixed with 10% 1.5 mg/mL Alexa488 human fibrin	Day 7
Figure S2 a-b	2-step		ImHUVECs expressing BFP (10 x10 ⁶ /mL)	ImHUVECs expressing GFP (5 x10 ⁶ /mL)	Lung FBs (1.5 x10 ⁶ /mL)	Bovine Fibrin 3 mg/mL	Day 0 to Day 7.
Figure S2 c	2-step		ImHUVECs expressing BFP (10 x10 ⁶ /mL)	ImHUVECs expressing GFP (5 x10 ⁶ /mL)	Immortalized lung FBs expressing mCherry (1.5 x10 ⁶ /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure S2 d	2-step		ImHUVECs expressing BFP (10 x10 ⁶ /mL)	ImHUVECs expressing GFP (various concentration)	Lung FBs (1.5 x10 ⁶ /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure S3	2-step		ImHUVECs expressing BFP (10 x10 ⁶ /mL)	ImHUVECs expressing BFP (5 x10 ⁶ /mL)	Lung FBs (1.5 x10 ⁶ /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure S4a	2-step		HDBMEC expressing BFP (10 x10 ⁶ /mL)	HDBMEC expressing BFP (5 x10 ⁶ /mL)	Dermal FBs (1.5 x10 ⁶ /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure S4b	2-step		iPSC-ECs (10 x10 ⁶ /mL)	iPSC-ECs (5 x10 ⁶ /mL)	Lung FBs (1.5 x10 ⁶ /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure S4c left	2-step		Brain microvascular ECs expressing GFP (12 x10 ⁶ /mL)	Brain microvascular ECs expressing GFP (6 x10 ⁶ /mL)	Brain pericytes (1 x10 ⁶ /mL) Astrocytes (0.5 x10 ⁶ /mL)	Bovine Fibrin 3 mg/mL	Day 7
Figure S4c right	2-step		ImHUVECs expressing BFP (10 x10 ⁶ /mL)	Brain microvascular ECs expressing GFP (6 x10 ⁶ /mL)	Brain pericytes (1 x10 ⁶ /mL) Astrocytes (0.5 x10 ⁶ /mL)	Bovine Fibrin 3 mg/mL	Day 7