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SUPPLEMENTARY INFORMATION

Development of a stack, porous silk scaffold neuroblastoma model for investigating spatial differences in cell and drug responsiveness

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Table S1. Delta CT values for original scaffold holder

Condition	VEGF	CAIX	GLUT1
Monolayer 21% O_2	1.08	8.02	2.02
Single Scaffold 21% O_2	-2.14	-2.12	-0.98
Single Scaffold 1% O_2	-3.32	-2.94	-1.52
Top Scaffold	-3.20	-2.39	-2.55
Middle Scaffold	-2.86	-1.66	-1.75
Bottom Scaffold	-2.40	-1.73	-1.50

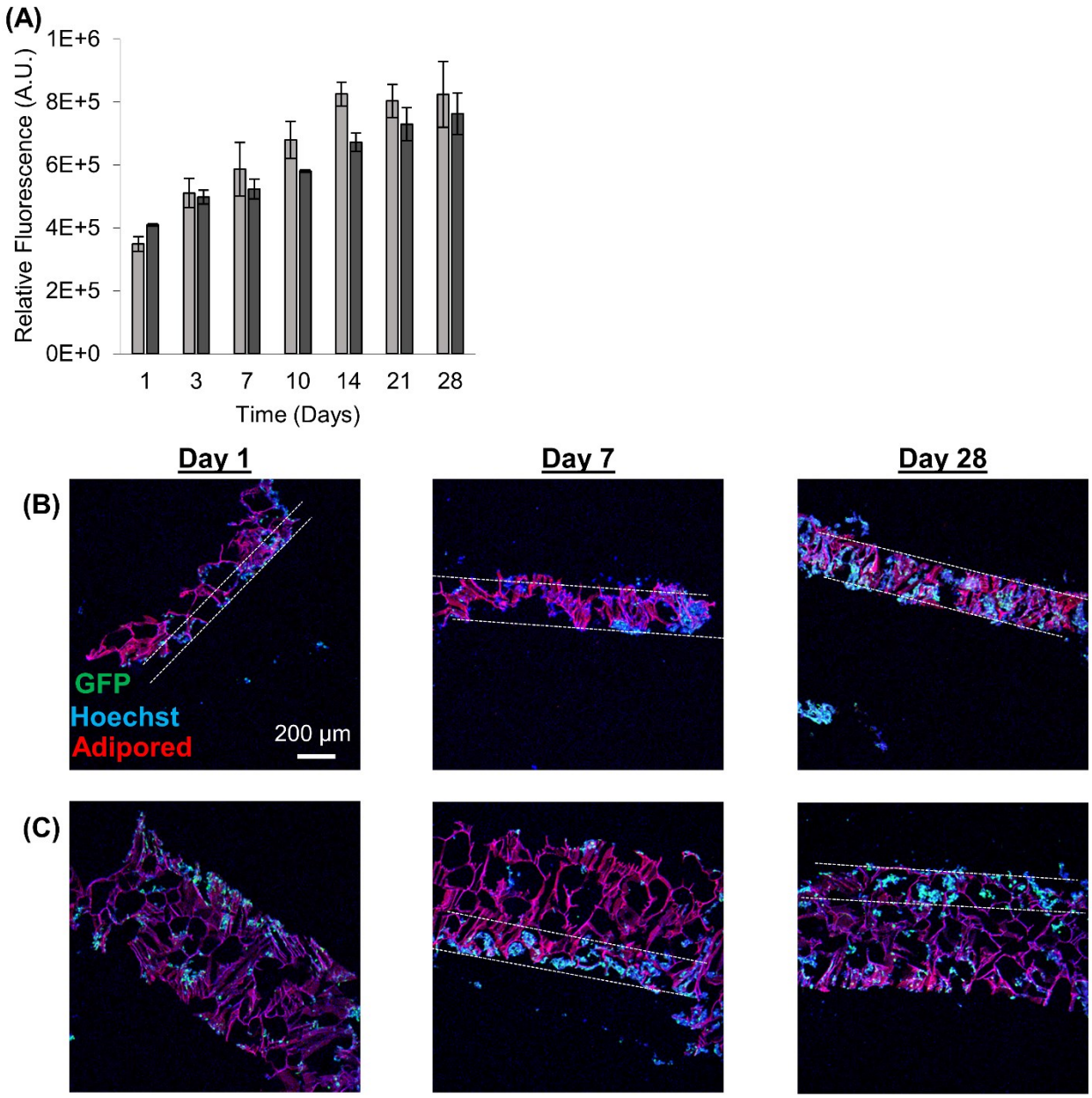


Fig. S1. Impact of scaffold thickness on cell growth (metabolic) and distribution of KELLY NB cells. (A) Impact of scaffold thickness on KELLY NB growth over time (metabolic via Resazurin). Data are presented as mean ± SD of three individual samples. Fluorescent staining shows EGFP-expressing KELLY NB cell distribution over time in (C) 200 µm scaffolds and (D) 600 µm scaffolds. Hoechst 33342 is used to visualize cell nuclei (binds to DNA). AdipoRed (excitation 485 nm; emission 535 nm) is used a s a counterstain to visualize the silk scaffold.

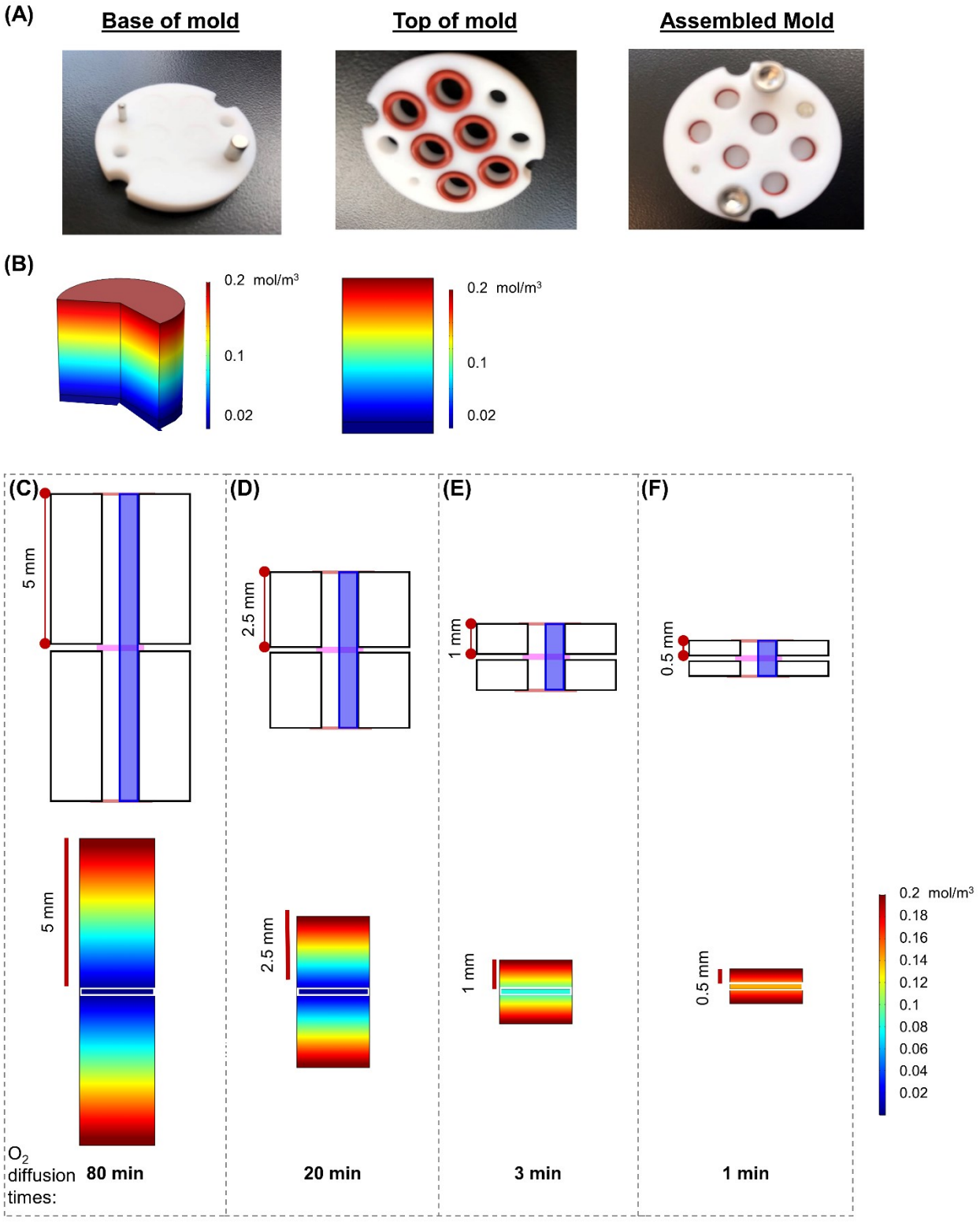


Fig. S2. Original scaffold holder design and its impact on oxygen transport. (A) The original scaffold holder design consisting of two PTFE plates with O-rings to hold the scaffolds in place. (B) COMSOL modeling of the original scaffold holder with a single scaffold at the bottom of the well. Data are presented as mean of a single representative experiment. COMSOL modeling of a

scaffold holder with a media reservoir depth of (C) 5 mm, (D) 2.5 mm, (E) 1 mm, and (F) 0.5 mm. The results of this depth modeling defined that a 0.5 mm depth would oxygen concentrations at ~>15% O_2, or minimal reduction in oxygen concentration throughout the single scaffold thickness. This 0.5 mm depth was used for the final scaffold holder design, thinner depths would result in a PTFE holder with a region thinner than 500 μm, which was deemed to be liable to easily break.

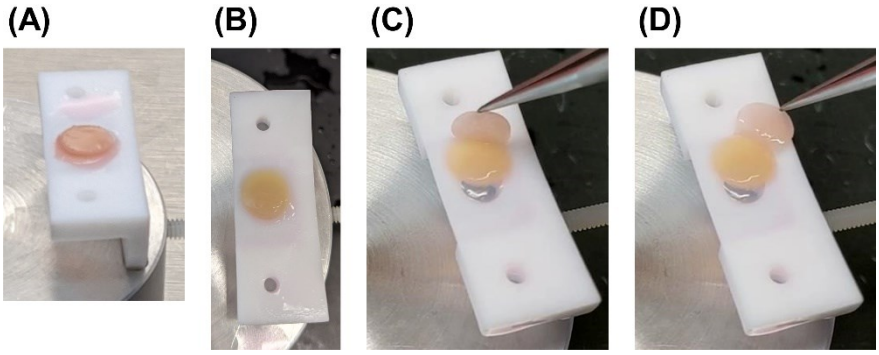


Fig. S3. Images of cell-laden scaffolds within the scaffold holder in an open configuration. (A) Image of quadruple stacked scaffold with SK-N-AS NB cells seeded at time zero of a stacked culture. (A) Image of quadruple stacked scaffold with SK-N-AS NB cells seeded at the end of a stacked culture. (C and D) Images of pealing the scaffold layers apart for later assessment of the cells within the individual layers.

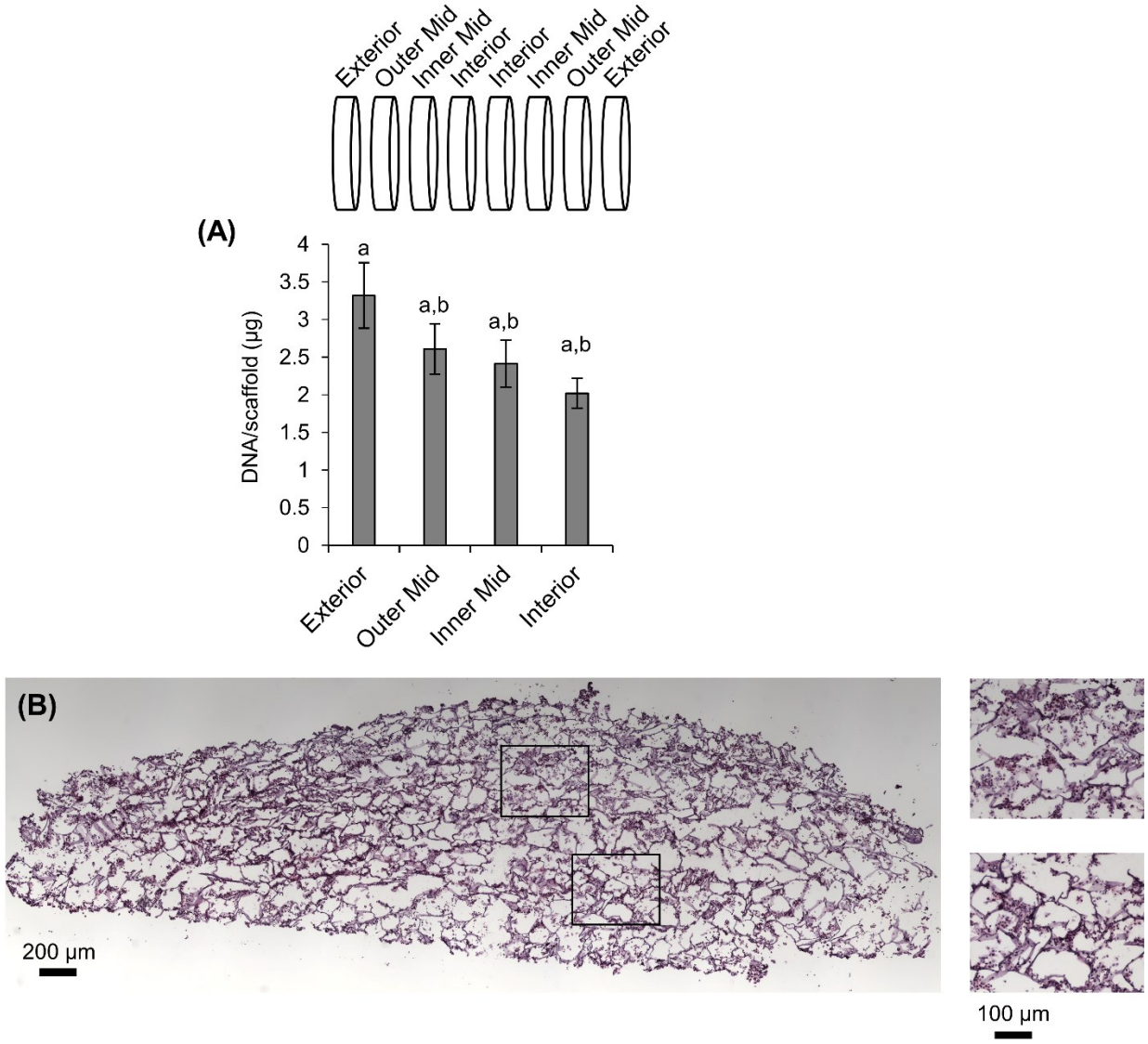
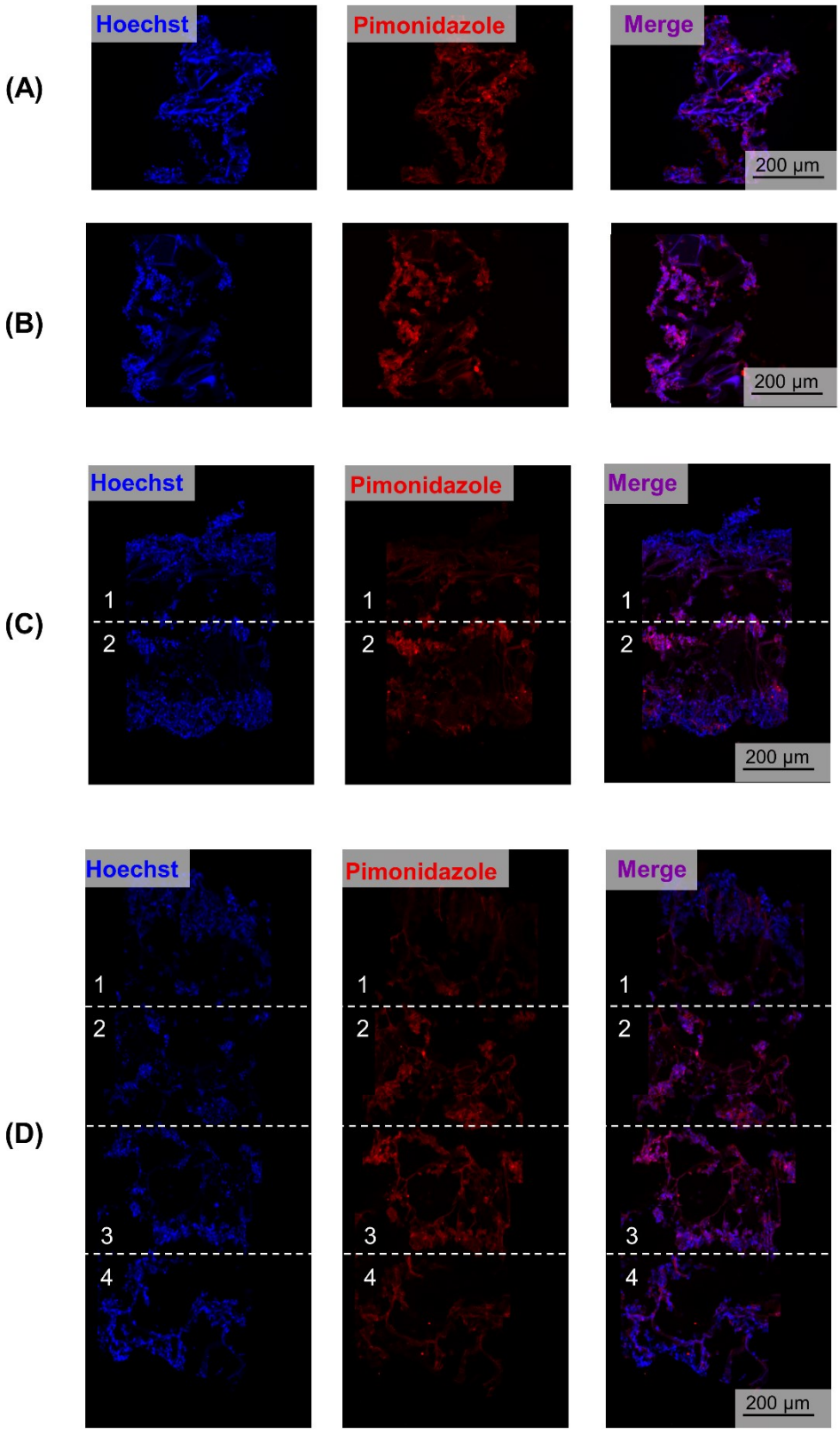
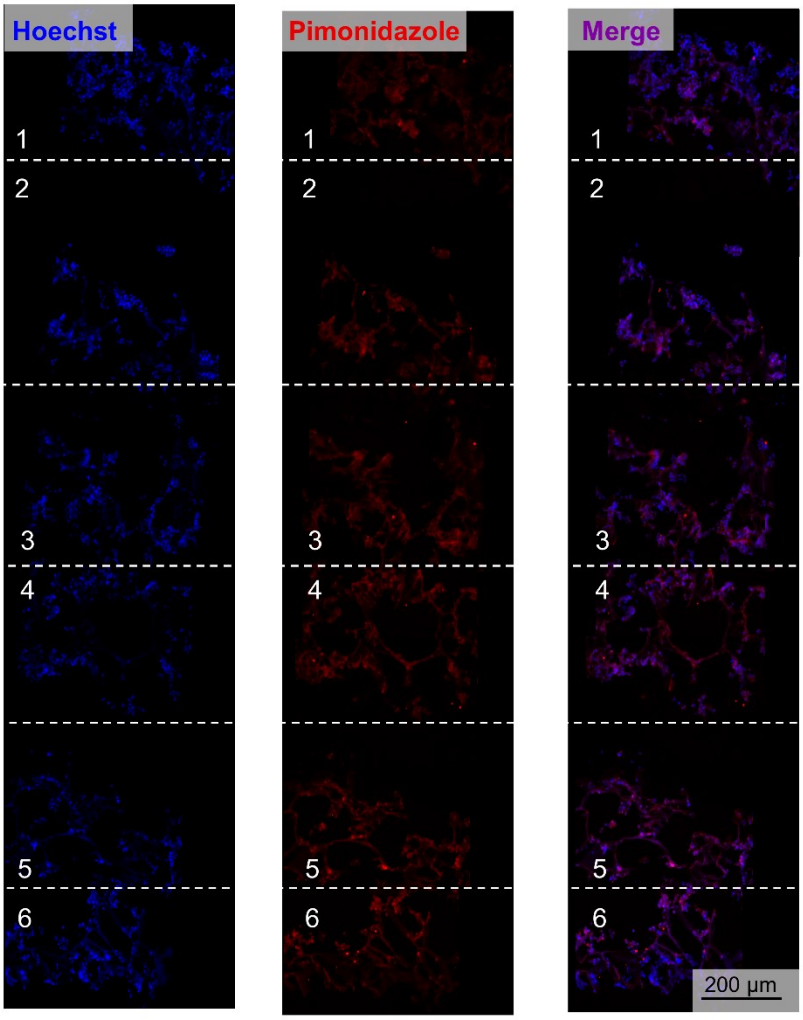


Fig. S4. DNA content and histological imaging of the SK-N-AS NB cells grown in the octuplet stacked scaffold model 3 days post-stacking. (A) Evaluation of dsDNA content of SK-N-AS NB cells grown in the octuplet stacked scaffold model within each individual layer. Data are presented as mean ± SD of three independent experiments. a denotes no statistical significance from single scaffolds grown at ambient oxygen, 'b' denotes no statistical significance from single scaffolds grown at low oxygen. (B) Hematoxylin and eosin staining to visualize SK-N-AS NB cell distribution throughout the octuplet scaffold stack.


(A)
Hoechst
Pimonidazole
Merge
200 μm
(B)
200 μm
(C)
Hoechst
Pimonidazole
Merge
1
2
200 μm
(D)
Hoechst
Pimonidazole
Merge
1
2
3
4
200 μm

(E)



(F)

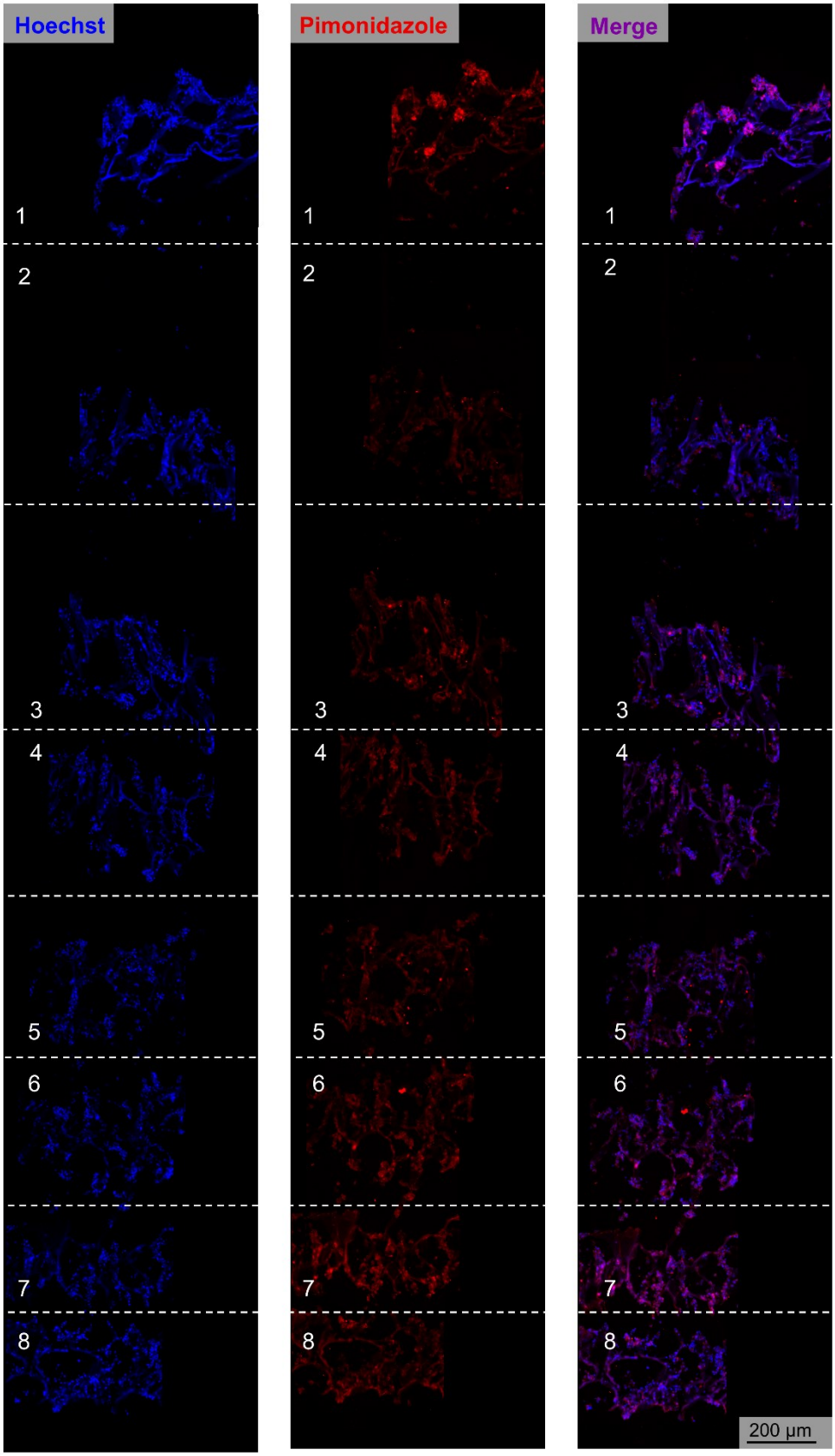


Fig. S5. Pimonidazole (red) and Hoechst (blue) staining of SK-N-AS NB cells in stacked scaffolds to visualize hypoxia within stacks 3 d post stacking. Pimonidazole is converted to a reactive molecule that forms adducts with thiol-containing proteins in hypoxic cells. (A) Single scaffolds grown in ambient oxygen (21%). (B) Single scaffolds grown in low oxygen (1%). (C) Double stacked scaffolds. (D) Quadruple stacked scaffolds. (E) Sextuple stacked scaffolds. (F) Octuple stacked scaffolds.

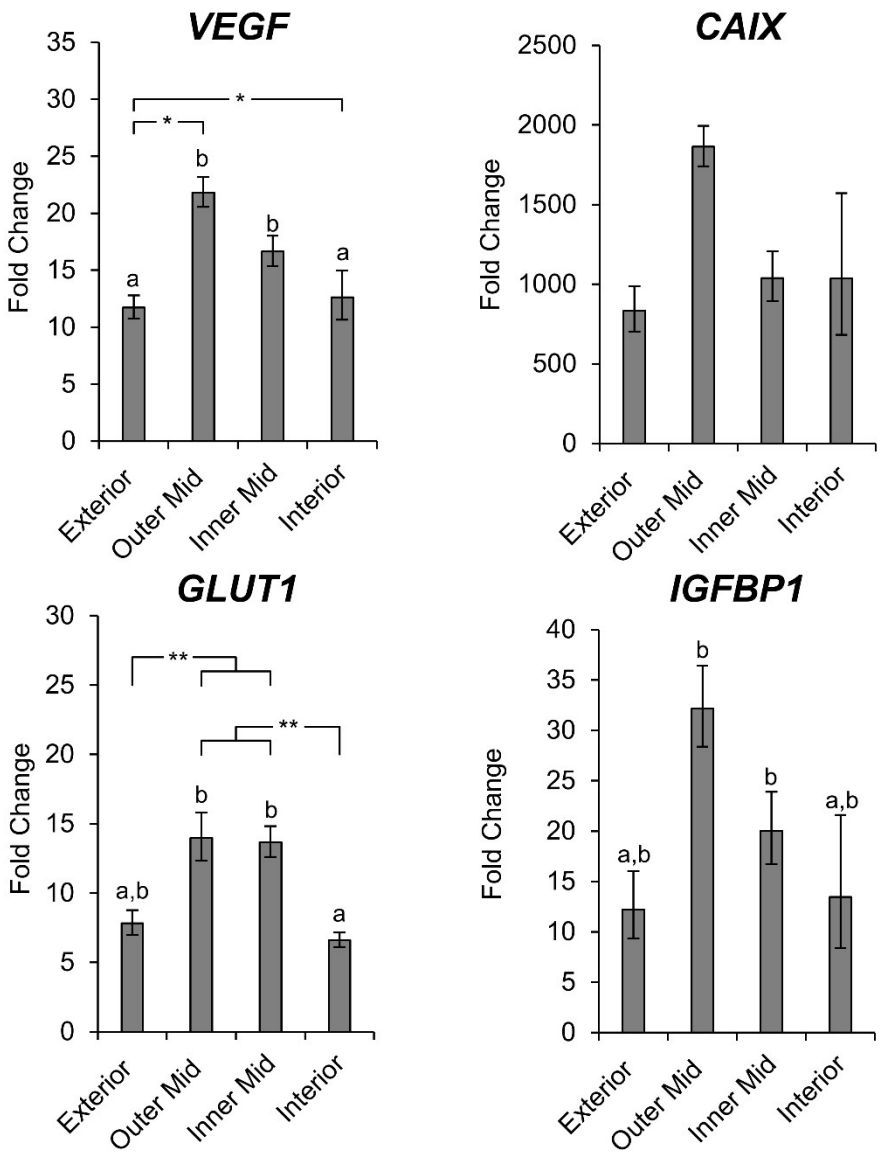


Fig. S6. Evaluation of hypoxia related gene expression of SK-N-AS NB cells cultured within the octuplet stacked scaffold model after 3 days culturing in stacked conditions. Data are presented as mean ± SD of three independent experiments. 'a' denotes no statistical significance from single scaffolds grown at ambient oxygen, 'b' denotes no statistical significance from single scaffolds grown at low oxygen. All gene expression is normalized to a monolayer, ambient oxygen control. Asterisk indicate statistical significance between the groups (*** $p<0.001$, ** $p<0.01$, *$p<0.05$).

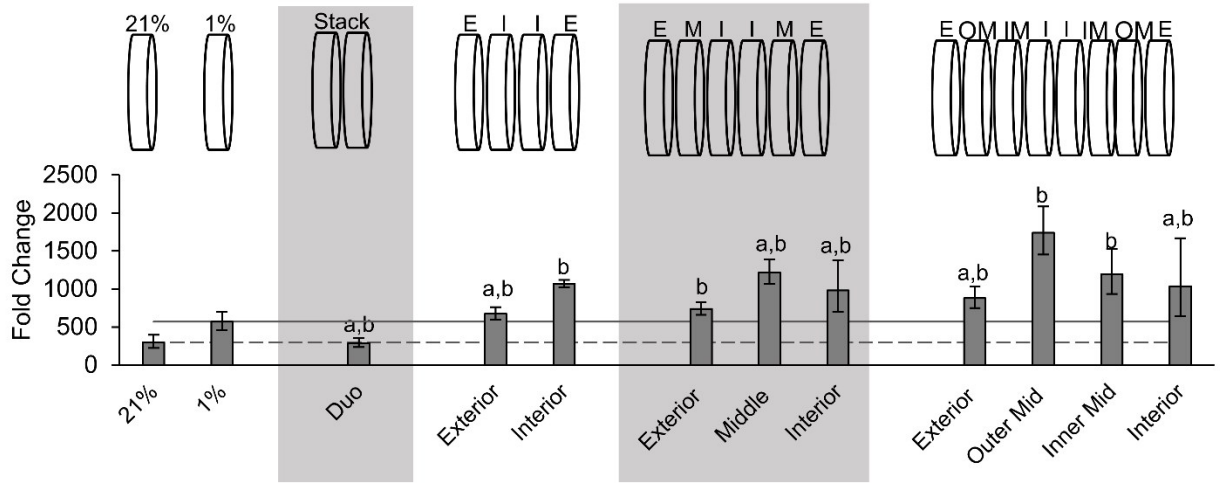


Fig. S7. Evaluation of *MMP9* gene expression of SK-N-AS NB cells cultured within the stacked scaffolds (2, 4, 6, or 8) after 3 days. Gene expression of *MMP9* by SK-N-AS NB cells stacked scaffolds 3 d post stacking. Ambient and low oxygen conditions are represented by 21% (ambient) and 1%, respectively. Representation of each stacking configuration can be found above the graph. “E” corresponds to exterior, “I” corresponds to interior, “M” corresponds to the middle, “OM” corresponds to the outer middle, and “IM” corresponds to the inner middle. Data are presented as mean ± SD of three independent experiments. ‘a’ denotes no statistical significance from single scaffolds grown at ambient oxygen, ‘b’ denotes no statistical significance from single scaffolds grown at low oxygen. All gene expression is normalized to a monolayer, ambient oxygen control. Asterisk indicate statistical significance between the groups (*** $p<0.001$, ** $p<0.01$, **$p<0.05$).

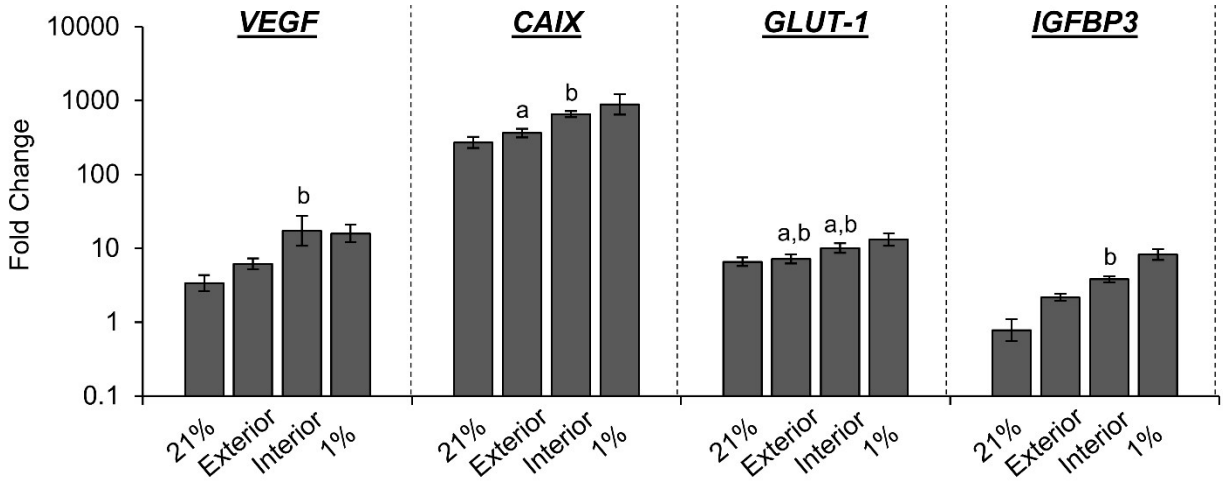


Fig. S8. Evaluation of hypoxia related gene expression of KELLY NB cells cultured in the stacked scaffolds (2, 4, 6, or 8) after 3 days. Ambient and low oxygen conditions are represented by 21% and 1%, respectively. All gene expression is normalized to a monolayer, ambient oxygen control. 'a' denotes no statistical significance from single scaffolds grown at ambient oxygen, 'b' denotes no statistical significance from single scaffolds grown at low oxygen. Data are presented as mean ± SD of three independent experiments.

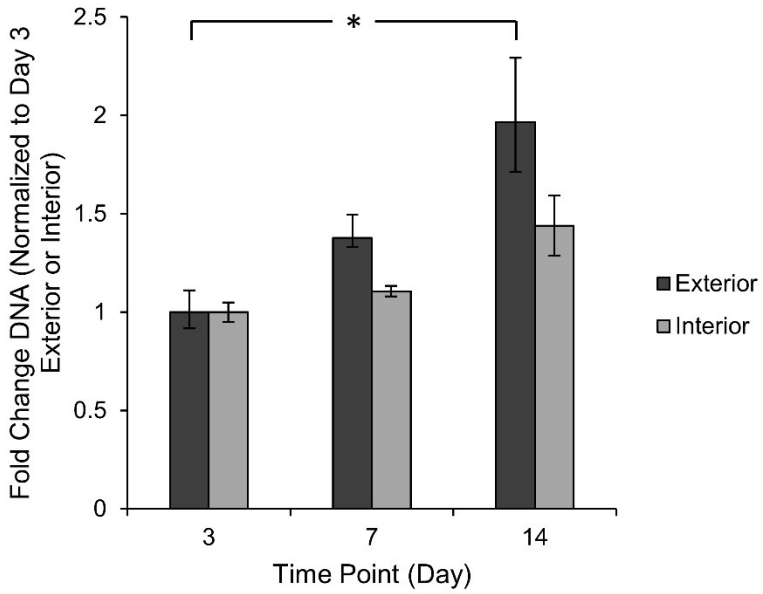


Fig. S9. Evaluation of fold change in dsDNA content of SK-N-AS NB cell seeded quadruple stacked scaffolds over time. Asterisk indicate statistical significance between day 3 and day 14 of the exterior scaffold cultured cells (*$p<0.05$).