Stepwise subcutaneous injection as an *in vivo* bioreactor strategy for time-sequenced vascularized skeletal muscle regeneration

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Abstract

Diffusion limits remain a major barrier to engineering thick skeletal muscle with uniform vascularization and mature myofibers. Here, we present a stepwise subcutaneous injection strategy that uses temporally staged delivery of distinct cell populations to better recapitulate the natural sequence of vascular and myogenic development. By tailoring the timing and mixing configurations of vascular-forming and myogenic cells, this platform enables the formation of thick, uniformly vascularized muscle constructs with improved structural maturity. In addition, the injectable and modular design provides a customizable foundation for future adaptation to diverse tissue geometries and regenerative applications.

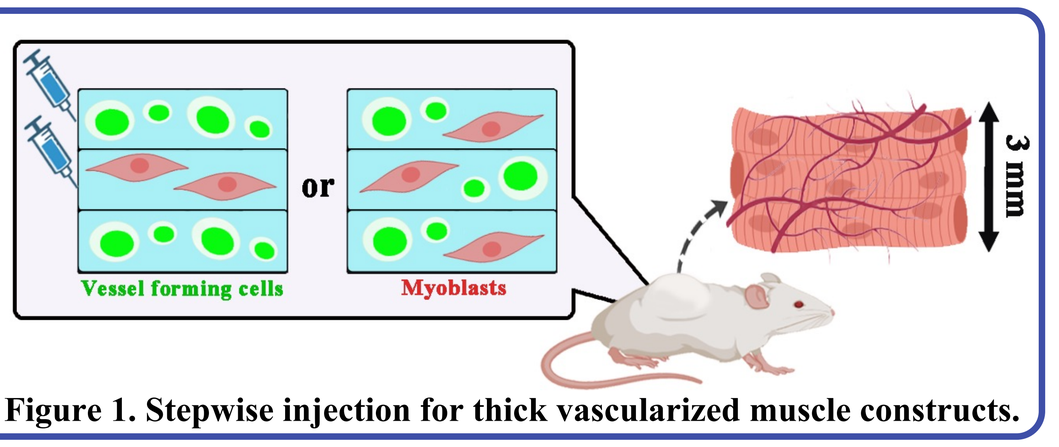


Figure 1. Stepwise injection for thick vascularized muscle constructs.

Results

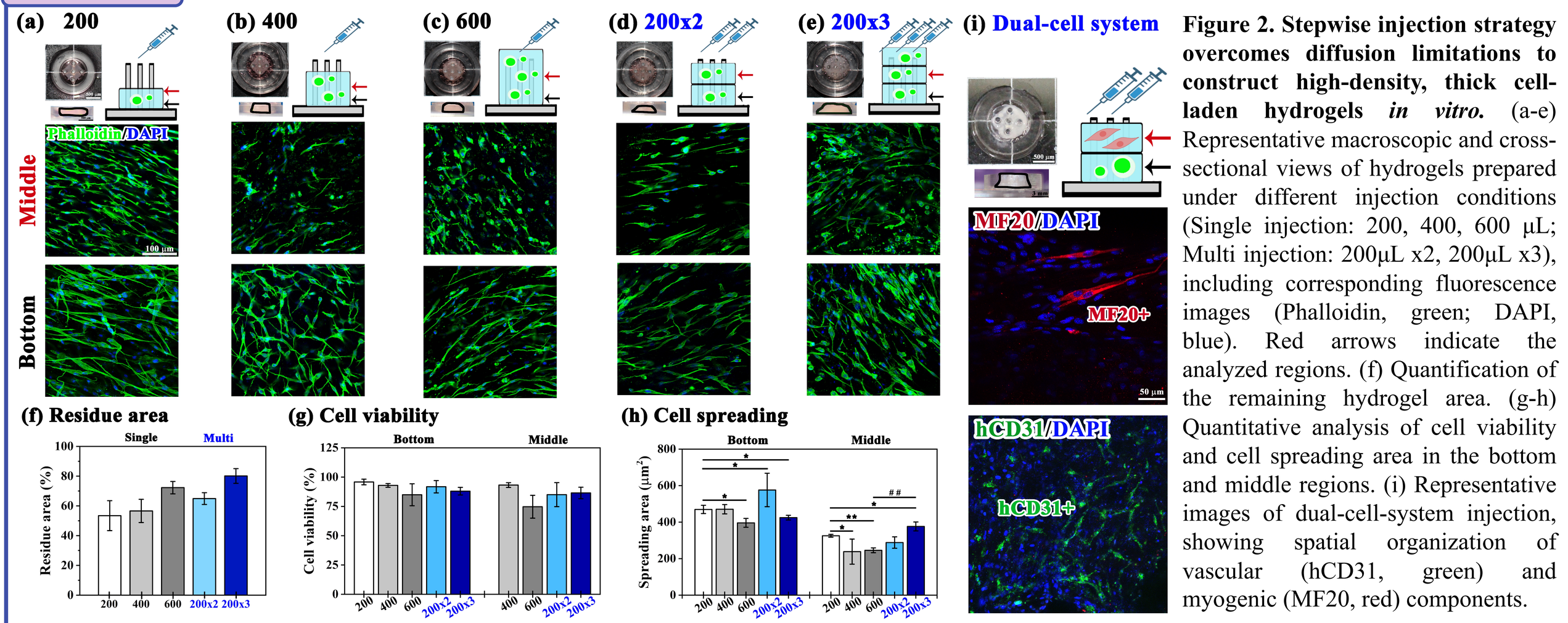


Figure 2. Stepwise injection strategy overcomes diffusion limitations to construct high-density, thick cell-laden hydrogels *in vitro*. (a-e) Representative macroscopic and cross-sectional views of hydrogels prepared under different injection conditions (Single injection: 200, 400, 600 μL; Multi injection: 200μL x2, 200μL x3), including corresponding fluorescence images (Phalloidin, green; DAPI, blue). Red arrows indicate the analyzed regions. (f) Quantification of the remaining hydrogel area. (g-h) Quantitative analysis of cell viability and cell spreading area in the bottom and middle regions. (i) Representative images of dual-cell-system injection, showing spatial organization of vascular (hCD31, green) and myogenic (MF20, red) components.

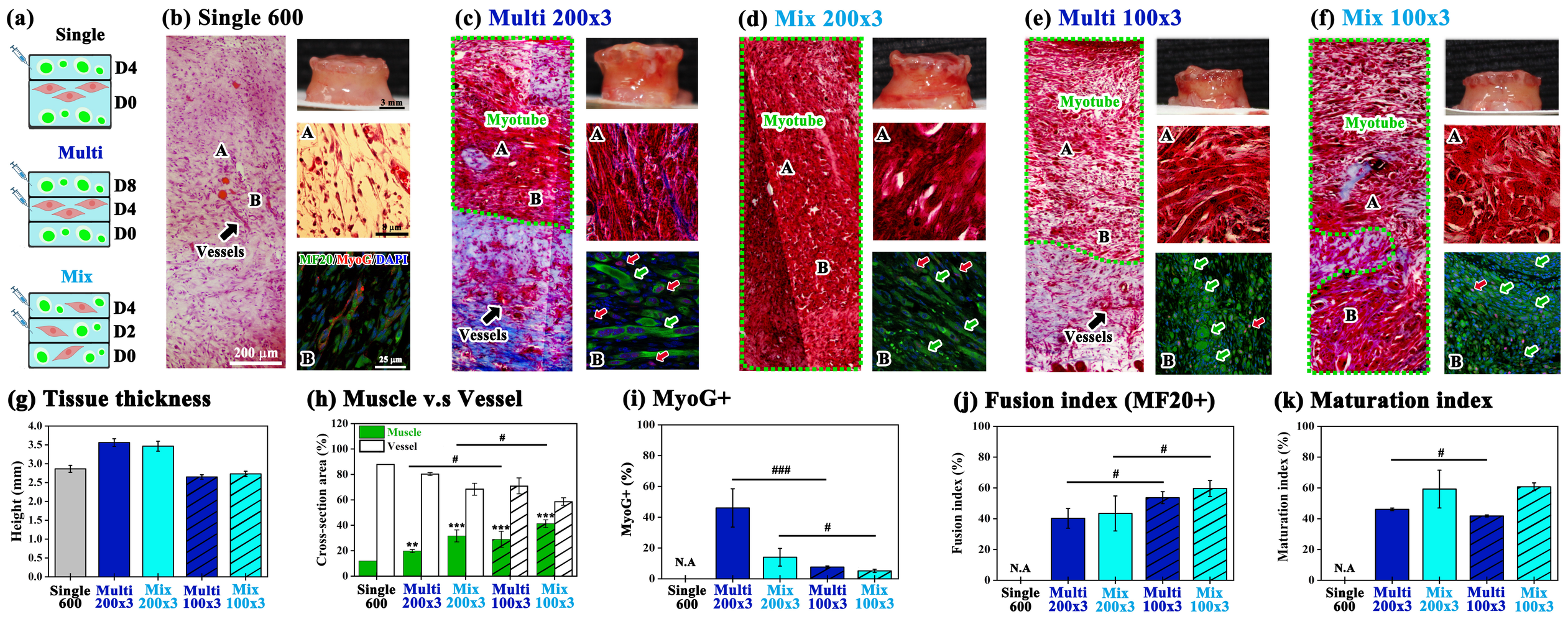


Figure 3. Evaluation of dual-cell-system stepwise injection strategies *in vivo*. (a) Schematic illustration of *in vivo* stepwise injections. (b–f) Representative macroscopic and histological images of constructs generated under different injection conditions (Single, Multi, and Mix injection; 200 μL and 100 μL groups), including immunofluorescence staining. (A-B) and the green dashed-line areas indicate muscle regions, green arrows indicate MF20+ myofibers, and red arrows indicate MyoG+ myoblasts. (g–k) Quantitative analysis of construct height, muscle vs. vessel area ratio, MyoG-positive cells, fusion index, and maturation index. Data are presented as mean ± SD (n = 3); **p < 0.01, ***p < 0.001 vs. Single 600 group; #p < 0.05, ###p <0.001 vs. Multi group.

Conclusion

This study demonstrates that stepwise injection improves tissue development both *in vitro* and *in vivo*. It enhanced cell growth and construct formation *in vitro*, and generated distinct muscle regions *in vivo*, whereas the Single injection produced almost no detectable muscle area. Among the stepwise strategies, Multi favored larger vascular networks, while Mix promoted greater muscle formation and earlier myogenic maturation. With further reduced injection volume, the system produced ~3 mm thick vascularized muscle tissue within 14 days. These findings highlight this dual-cell-system platform as a customizable strategy for vascularized muscle regeneration.

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