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(54) Title: INJECTABLE MICROTISSUE SYSTEMS, DEVICES, AND METHODS

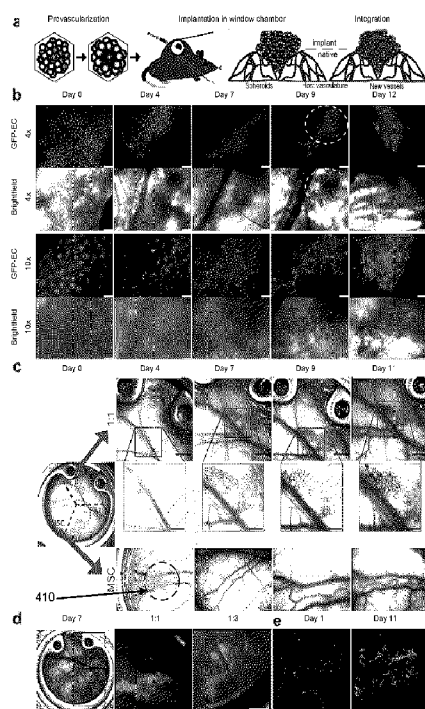


FIG. 18

(57) Abstract: Spheroid microtissues can mimic native tissue-like structure and function, and are thus useful for broad research applications and regenerative medicine. Disclosed embodiments include spheroid production methods that are high-throughput, suitable for efficient production, maintainable over long-term culture, and/or offer repeatable control over size distribution. Embodiments disclosed include spheroids that include blood vessels, which are proven useful for tissue survival and function. Embodiments include spheroids with functional, blood-perfused vascular networks upon injection in vivo. Dissolvable hydrogel microwell arrays enable high throughput parallel formation of spheroids in a single pipetting step and easy retrieval for downstream applications. Dynamic culture conditions stimulated endothelial cell and mesenchymal stem cell cocultures to self-organize and form blood-vessel structures within the spheroids. Vascularized spheroids were injected into mice and rapid perfusion of implant vessels within 4-7 days were observed.





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INJECTABLE MICROTISSUE SYSTEMS, DEVICES, AND METHODS

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0001] This invention was made with government support under R01HL095477 and 341/300-123012 awarded by the National Institutes of Health (NIH). The government has certain rights in the invention.

FIELD

[0002] The present disclosure relates generally to tissue engineering, and, more particularly, to injectable microtissues.

BACKGROUND

[0003] Spheroid microtissues - small aggregates of cells produced in vitro - can mimic the structure and function of native tissues, and are useful for a range of research and clinical applications, including in vitro drug screening and in vivo tissue transplants. Spheroids form when multiple individual cells aggregate and then adhere to each other more strongly than surrounding materials, resulting in formation of a sphere-shaped cluster. Although a wide variety of methods have been developed to produce spheroids, current approaches lack simplicity.

[0004] Challenges in this area include production issues such as size distribution and vascularization after injection or implantation. The hanging-drop method, in which a small droplet of cells placed on the lid of a petri dish and inverted to cause aggregation at the bottom of the drop is commonly used. Seeding cells on non-adhesive 2D cell culture ware and guiding cells into a cluster by centrifugation, a standing ultrasound wave or a magnetic field are also used. Other methods for producing spheroids include seeding cells in spinner cultures. Macroporous carriers and microcarriers such as Cultispher-S with aggregation up to

microspheres in spinner flasks, or coating the surface of polymer microspheres with cells are also known.

[0005] Vascularization, injectability, and other issues are important challenges for regenerative medicine. In vivo, most cells are found no more than 200 μm from the nearest capillary, which reflects the diffusion limit of oxygen. In order to create a larger tissue, a functional vascular network must be included to overcome the mass transfer limitations in the design of tissues engineered in vitro. Known approaches for vascularization include in vitro methods such as stacking endothelial cell layers between other cell sheets, scaffold guided endothelial co-culture, scaffold functionalization, and in vivo systems such as the arteriovenous loop chamber. In vivo approaches may rely on host blood vessel invasion of an avascular implant, which is slow and limited to several tenths of micrometers per day.

BRIEF DESCRIPTION OF DRAWINGS

[0006] Embodiments will hereinafter be described in detail below with reference to the accompanying drawings, wherein like reference numerals represent like elements. The accompanying drawings have not necessarily been drawn to scale. Where applicable, some features may not be illustrated to assist in the description of underlying features.

[0007] Fig. 1 is a timeline diagram illustrating aspects and associated images of spheroid fabrication and delivery, according to one or more embodiments of the disclosed subject matter.

[0008] Fig. 2 is a process flow diagram of a method for spheroid fabrication and delivery, according to one or more embodiments of the disclosed subject matter.

[0009] Fig. 3A is an image of an endothelial cell spheroid in a microwell after 3 days culture in a media without growth factors VEGF and bFGF, according to one or more embodiments of the disclosed subject matter.

[0010] Fig. 3B is an image of an endothelial cell spheroid in a microwell after 3 days culture in a media with growth factor VEGF.

[0011] Fig. 3C is an image of an endothelial cell spheroid in a microwell after 10 days culture in a media without growth factors VEGF and bFGF, according to one or more embodiments of the disclosed subject matter.

[0012] Fig. 3D is an image of an endothelial cell spheroid with endothelial core that is induced to mature and produce a lumen and/or sprout by addition of growth factors added to the media after the endothelial cores have formed, according to one or more embodiments of the disclosed subject matter.

[0013] Fig. 4 are images (bottom two rows at higher magnification) illustrating the kinetics of vascular formation for spheroids at different time points in vivo, according to one or more embodiments of the disclosed subject matter.

[0014] Fig. 5 are stereoscopic images (bottom row at higher magnification) illustrating the progression of injected spheroids into blood vessels in vivo, according to one or more embodiments of the disclosed subject matter.

[0015] Fig. 6 shows images illustrating a newly formed blood vessel in vivo, according to one or more embodiments of the disclosed subject matter.

[0016] Fig. 7 is a graph illustrating the relationship between cell ratios, spheroid size, and various features of the resulting network architecture, according to one or more embodiments of the disclosed subject matter.

[0017] Fig. 8 are images of microwells seeded with different conditions including microwell size and ratio of endothelial cell to mesenchymal stem cell ratio immediately after seeding, according to one or more embodiments of the disclosed subject matter.

[0018] Fig. 9 are images of microwells seeded with different conditions including microwell size and ratio of endothelial cell to mesenchymal stem cell ratio at 3 days of culture without VEGF, according to one or more embodiments of the disclosed subject matter.

[0019] Fig. 10 is a simplified graph illustrating the size of spheroids and radial distribution of endothelial cells and mesenchymal stem cells over time, according to one or more embodiments of the disclosed subject matter.

[0020] Fig. 11 is a graph of spheroid diameter over time for spheroids fabricated according to one or more embodiments of the disclosed subject matter.

[0021] Fig. 12 is a graph of spheroid diameter as a function of the number of cells in spheroids fabricated according to one or more embodiments of the disclosed subject matter.

[0022] Fig. 13 shows a schematic diagram and results of spheroid fabrication method. (a) Schematic diagrams and the corresponding experimental images showing the steps of spheroid fabrication and their final application. First, a co-culture of therapeutic cells and endothelial cells is seeded on the alginate microwells. Second, the cells are cultured in the microwells in maintenance medium without vasculogenic growth factors for 4 days, allowing the cells to self-organize into spheroids with an endothelial core. After the initial 4 days, the spheroids are cultured in the microwells in vasculogenic medium with vasculogenic growth factors for an additional 4 days, allowing the endothelial cores to mature into vessels. Third, the alginate microwells are dissolved with 5% sodium citrate to release the spheroids into suspension. Fourth, the suspension of spheroids may be spun down and assembled into a macro-tissue in vitro to study the vascular formation, or injected into the subdermis of a mouse demonstrate engraftment in vivo. Fifth, the injected spheroids were observed to form fully-functional and perfused microvasculature in vivo.

[0023] Fig. 14 a-f shows a schematic diagram and results of a spheroid fabrication method using (a-b) the same steps as illustrated in Fig. 13 to form 1000, 3000 or 30,000 spheroids depending on the size of the alginate construct; (c) 250,000,000 cells to be seeded on alginate microwells for massive high throughput; (d) Cells seeded in the 60 mm construct with 30,000 wells to create 30,000 spheroids. Scale bar is 1 cm; (e) Collecting the 30,000 mature spheroids will create a macro-tissue with an area of 1 cm² and a height of 1 mm. Scale bar is 1 cm; (f) The spheroids

are closely packed and all with endothelial cores in the macro-tissues. Scale bars are 1 mm and 500 μm in close-up.

[0024] Fig. 15 shows endothelial cores formed by directed self-organization of the cells through timed exposure to growth factors. The first three days, culture in media without growth factors (VEGF and bFGF) induces the endothelial cells to migrate to the spheroid centers and form endothelial cores (top). After endothelial core formation, spheroids continuously cultured in media without growth factors will remain as endothelial cores (top right). Endothelial cores may be induced to mature and produce lumen and sprouts if growth factors are added to the media after the endothelial cores have formed (bottom right). Endothelial cells in spheroids grown in media with growth factors for the first three days migrate to the periphery of the sphere, do not form endothelial cores and cannot be prevascularizes.

[0025] Fig. 16 shows endothelial cores formed by directed self-organization of the cells through timed exposure to growth factors. (a) The method can produce a large array of spheroids of different sizes (formed in microwells with either 100, 200 or 400 μm diameter) and with different endothelial core sizes (formed by different ratios of endothelial cells to mesenchymal stem cells (EC:MSC)). All spheroids in these arrays show the endothelial core formation after culture in maintenance medium, and maturation of these cores after culture in vasculogenic medium. (b) The contraction of the cells into spheroids (upper curve) and formation of endothelial cores (lower curve) as illustrated by the maximum radial distance of MSCs and ECs from the center of the spheroids ($n > 20$) for each day of culture in (a). (b) The method can control the size of spheroids because it allows a specific number of cells to be adjacent. Spheroid diameter over time for spheroids containing different number of cells. c) Spheroid diameter as a function of the number of cells they contain. With the fitted function of $r = (6/\pi v_{\text{cell}} n_{\text{cell}})^{1/3/2}$.

[0026] Fig. 17 shows the production and maturation of spheroids. (a) Early development of spheroid of 1 human ECs to 1 human MSCs in the first 20 hours (between Day 0 and Day 1) in 400 μm microwell. Shown are brightfield, GFP ECs and merge images. (b) In vitro formation of

spheroid and vasculature of human ECs and MSCs under nine conditions (EC:MSC ratio and well size). Overlay of fluorescence: GFP ECs and MSCs. Shown are the spheroids immediately after seeding, after 3 days of culture in maintenance medium, and after 8 days of culture, the last 5 days with vasculogenic factors. (c) Spheroid fusion. Shown are spheroids of 1:1 and 1:3 HUVEC to MSC ratio that were formed and prevascularized in 200 μm microwells for 9 days with last 6 days in vasculogenic medium. To mimic an in vivo environment, which is adhesive for cells, spheroids were placed in collagen-alginate microwell. Displayed are brightfield, GFP-EC and merge images of first 24 h of spheroid fusion. Scale bar = 200 μm .

[0027] Fig. 18 shows the vascular formation of injecting spheroids in vivo. (a) shows the experimental set up. Spheroids were formed and prevascularized in vitro, implanted in a window chamber on a SCID mouse and vascular formation and integration was observed. (b) shows the kinetics of vessel formation; Spheroids at different time points in vivo, cultivated in 200 μm microwell, GFP-Huvec:MSC = 1:1; Prior in vitro cultivation: 3d in maintenance medium followed by 6d in vasculogenic medium; Arrow heads point at blood filled vessels. Dotted line encircles newly formed lumenous vessels. Scale bar = 250 μm . (c) Stereoscopic images at different time points in vivo. Top row shows 1 Huvec . 1 MSC spheroids. Square shows implants. Newly formed vessels are blood filled and functional. Day 7 is the first time blood in vessels was observed. Bar in top row = 1.25 mm. Bar in middle row = 0.5 mm. Bottom row shows pure MSC spheroids. No signs of vascularization were observed. Dotted line encircles implant. Bar in bottom row = 0.5 mm. (d) Day 7, newly formed, blood filled vessels colocalize with GFP signal from implants. Scale bar = 1.25 mm (e) Spheroids in 9 well insert in vivo (Fig. 24f). 1 GFP-Huvec :1 MSC spheroids, formed and prevascularized in 200 μm microwells. Shown are maximum projection images of confocal Z-stack GFP-Huvec images. Z-stack at day 11 = 172 μm in 32 slices. Individual spheroids on day 1 form dense vascular network until day 11. Spatial control in vivo is exerted through 9 well insert. Scale bar = 200 μm .

[0028] Fig. 19 shows the fabrication of alginate-based micro-wells. A) shows six cross-linked alginate microwell constructs in the PDMS mold with the top three constructs removed. B-D) The alginate molds are carefully loosened with a pair of sterile tweezers and removed from the mold holding on only to the “wings” to preserve the microwell structures in the middle. E) The alginate constructs must be flipped to expose the microwells before being placed in the well plate (F).

[0029] Fig. 20 a-d show injecting the spheroids in vivo. (a) Injecting a solution with spheroids (endothelial ratio 1:3 and size 200 μm) into the adipose tissue of the obliques. A small incision along the back was made to ensure the correct position of the injection site. (b) The mice were sacrificed and the entire obliques (white dashed circle) were excised after 18 days of implantation. Black scale bar is 20 mm and the white scale bars are 2.5 mm at the oblique in the dashed circle. (c) Front and back of the extracted obliques. Scale bar 2.5 mm. d) Fluorescent image of the obliques that shows the extent of the tissue formed by the injected spheroids.

[0030] Fig. 21 a-c show an initial phase of spheroid formation and self-sorting. Shown are first 20 h of self-assembly and self-sorting immediately after seeding GFP-HUVEC and MSC cell mixtures in alginate microwells (100, 200, 400 μm). Fluorescent and brightfield images were acquired with a 10x objective. Scale bar = 200 μm . (a) 1 HUVEC : 1 MSC spheroids. (b) 1 HUVEC : 3 MSC spheroids. (c) MSC spheroids.

[0031] Fig. 22 shows spheroid fusion from microtissue to macro-tissue: Spheroids were created in 200 μm sized microwells at 1:1 and 1:3 GFP HUVEC to MSC ratio and cultured for 3 days in maintenance medium and 6 days in a vasculogenic medium to induce prevascularization (see 0 h). For this assay the same vasculogenic medium was used. Prevascularized spheroids were placed in a collagen-alginate microwell. GFP fluorescence and brightfield images were acquired with a 10x objective in time-lapse microscopy over 24 h. Scale bar = 200 μm .

[0032] Fig. 23 a-c illustrates robustness to shear stress: Images of spheroids before (a) and after injection through a 27 gauge needle (b) and a 30 gauge needle (c). Scale bar is 50 μ m. It was determined that spheroids (clusters) remain intact

[0033] Fig. 24 a-f shows window chamber surgery: (a) Dorsal skin was spread out and backside of window chamber was connected; Needles were used to create holes for window chamber; (b) A circle was marked on the skin to indicate the opening for the window chamber and the skin was carefully cut out; (c) Front and back of window chamber were assembled; (d) Nuts were used to lock the window chamber; A custom 3D printed backing was used to hold the skin in place; (e) Closed window chamber in place; Secured with lateral sutures; (f) A custom-made window chamber insert to test up to 9 conditions simultaneously was included.

DETAILED DESCRIPTION

[0034] The disclosed embodiments include methods for producing microtissues with blood-vessel building blocks for rapid vascularization and cell survival upon in vivo implantation. Co-cultures of cells within spheroids may enable cells to self-organize into a bulk tissue. Spheroids may be scaffold-free and may not require post-formation tissue maturation or a potentially toxic cell deposition process

[0035] In embodiments of the disclosed subject matter, spheroid micro-tissues can provide native-like microenvironments for cells to be delivered in vivo. In particular, a high-throughput protocol for manufacturing injectable spheroids is disclosed herein, whereby the resulting micro-tissues may be used to deliver a treatment or therapy to a patient (e.g., human or animal), for example, by providing a mesenchymal stem cell (MSC) therapy.

[0036] In one or more embodiments, each spheroid micro-tissue produced using the disclosed methods has a primitive vasculature. Upon injection into the patient, the primitive vasculature in each micro-tissue can connect with existing vasculature and/or with the primitive vasculature of

other injected micro-tissues to form a complete, perfused vasculature, for example, in as little as four days. Micro-tissues-containing blood vessel units may be engineered with directed cell self-organization using timed exposures to growth factors and injected as treatment of critical limb ischemia, for example.

[0037] In one or more embodiments, a clinically significant number (e.g., on the order of millions) of spheroids may be grown. Each spheroid can have multiple cell types. The structure of spheroids may be engineered to form micro-tissues by directing self-organization of the different cell types using particular patterns of timing for exposure to, and privation from, growth factors. For example, methods may be used to engineer micro-tissues spheroids of mesenchymal stem cells, smooth muscle cells, dermal fibro-blast and adipose cells, each of which contain a unit of blood vessel. Based on the current disclosure, one of ordinary skill in the art may appreciate that different cells and combination of cells are also possible, as well as different growth factors. Accordingly, embodiments of the disclosed subject matter are not limited to the particular cell types, combination of cells, and/or growth factors specifically described herein.

[0038] Referring to Figs. 1-2, an embodiment for forming injectable microtissues is illustrated. At 102, a co-culture of therapeutic cells and endothelial cells may be seeded over a construct with a multitude of microwells (for example, as illustrated in Fig. 1). The construct, or at least surfaces of the microwell in contact with the cell, may be formed of a material that is substantially non-adhesive to the cells within the microwell. For example, the construct may be formed of alginate, agarose, and/or polydimethylsiloxane (PDMS), among other materials. The cells may be seeded on the construct and then be provided with a predefined time to settle into the microwells, with approximately the same number of cells in each well. Cells may tend to adhere to adjacent structures. The present microwell is non-adhesive such that the cells only to adhere to each other. Within an initial culture period (e.g., over first 24 hours), the cells in each microwell adhere to each other and contract to form spheroids. The distribution of the cells

within the spheroid is random, reflecting the random distribution originating when they were initially seeded into the microwells.

[0039] At 104, the self-organization of the different cell types in the spheroid co-cultures may be directed by timed exposure to growth factors. For example, when employing endothelial cells to form blood vessel, the endothelial cells respond to vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) with increased proliferation and migration. If a gradient of these growth factors is present, the endothelial cells will respond with a directed migration towards the higher concentration. Most cell-types will express either one or both of these growth factors in response to hypoxia (less than optimal oxygen). These responses may be used to direct the endothelial cells self-organization into blood vessel units at the center of the spheroids.

[0040] After seeding the cells in non-adherent micro-wells, cell culture media were provided without VEGF or bFGF. After an initial culture period (e.g., on the order of a day), the cells were organized into a spheroid, with the cells in the middle of each spheroid beginning to express VEGF and bFGF, thereby creating a gradient from the center of the spheroid to the exterior thereof. After an intermediate culture period (e.g., at 3-4 days), the endothelial cells were observed to have migrated into the middle of the spheroid forming an endothelial core, i.e., a blood vessel unit. Growth factor was then be introduced to allow the spheroids to further mature.

[0041] For example, the cells may be cultured in the microwells without vasculogenic growth factors for 3 days (i.e., to allow formation of the endothelial core), followed by an additional 7 days of culture with vasculogenic growth factors. The cells self-organized into spheroids with an endothelial core that matured into vessels. Referring to Fig. 3, images illustrating features of the endothelial cores formed by directed self-organization of cells through timed exposure to growth factors are shown. As illustrated in Fig. 3A, the first three days of culture in media without growth factors (VEGF and bFGF) induces the endothelial cells to migrate to the spheroid centers

and to form endothelial cores. In contrast, Fig. 3B illustrates endothelial cells in spheroids grown in media with growth factors for the first three days. In Fig. 3B, the endothelial cells migrated to the periphery of the spheroid instead of the center. After endothelial core formation, spheroids continuously cultured in media without growth factors remain as endothelial cores, as illustrated in Fig. 3C. However, endothelial cores induced to mature and produce lumen and/or sprouts if growth factors were added to the media after the endothelial cores had formed, as illustrated in Fig. 3D.

[0042] Thus, micro-tissues with primitive vasculature may be formed and harvested from the construct with microwells at 106 for subsequent injection at 108. For example, the microwells (e.g., formed of alginate) may be dissolved to release the spheroids into suspension as has been confirmed by experiment. Such a suspension of spheroids may be assembled into a tissue in vitro to study the vascular formation or injected into a patient to effect a treatment. For example, in mouse studies described further below, hundreds of such micro-tissues, each containing a blood vessel unit, were injected into a window chamber. Upon injection, the blood vessel units connect and form a new vascular network with the endothelial core as nodes. The new vascular network connects to the mouse's own vasculature after about seven days and is perfused. In one or more embodiments, timed, consecutive seedings may be used to further direct self-organization.

[0043] Referring to Figs. 3A, 3B, 3C, various images illustrating vascular formation by injected spheroids in vivo are shown. Fig. 4 shows images of spheroids at different time points in vivo. The spheroids were grown in microwells having 200 μ m using Human Umbilical Vein Endothelial Cells (HUVEC) with Green Fluorescent Protein (GFP) in a ratio of 1:1 with MSCs. The cells were cultured in the microwell for three days using a maintenance medium (i.e., without growth factors) followed by six days in a vasculogenic medium. In Fig. 4, arrow blood-filled vessels can be observed. The scale bars represent 250 μ m.

[0044] Fig. 5 shows stereoscopic images of injected microtissues at different time points in vivo.

As may be confirmed from the figures and as observed in the laboratory, newly formed vessels resulting from the injection are blood-filled and functional. The newly formed blood vessels were first observed at day 7 in Fig. 5, and close-ups of the blood vessels are shown in the bottom row. In Fig. 5, the scale bar in the top row of images represents 1.25 mm and the scale bar in the bottom row of images represents 0.5 mm. The dotted oval in Fig. 5 encircles the implant region.

Fig. 6 shows images of the GFP signal from the newly-formed blood-filled vessels at Day 7.

[0045] The resulting vasculature of the micro-tissues both in vitro and in vivo may be controlled.

For example, the size of the spheroids may be adjusted by varying the number of cells seeded into a microwell that eventually organize into a single spheroid and/or by adjusting the size of the microwell. The size of the microwells and the concentration of cells adjusted to determine the amount of cells that will settle in each of the wells. For example, the microwells can each have a diameter less than 500 μ m, for example, between 10 μ m and 500 μ m, such as 100 μ m, 200 μ m, and 400 μ m. Other microwell diameters are also possible according to one or more contemplated embodiments. Moreover, although diameter has been used herein to refer to the size of the microwells, it is contemplated that the microwell may have a shape other than circular, for example, hexagonal as illustrated in Fig. 1, elliptical, rectangular, or other shapes may be employed. Varying the size of spheroids before allowing them to aggregate into tissues can provide control of the resulting vasculature. The disclosed methods are highly scalable. For example, the methods may be used to produce up to half-a-billion cells, which would be enough to form a 10 mm x 10 mm x 1 mm tissue in vitro.

[0046] According to embodiments of the disclosed subject matter, treatments for various vascular issues are be provided. In embodiments, the disclosed methods for making spheroids are followed by injection or implantation to provide a treatment for critical limb ischemia (CLI). Suitable therapeutic cells may be selected for this purpose. A patient may be injected on an outpatient basis with the disclosed engineered microtissues, each of which contains a blood

vessel unit (i.e., a spheroid with an endothelial core). Upon injection, these microtissue units are observed to connect to form a vascular network, which formation occurs faster than the formation of a vascular network by single cell therapies. Multiple injections may be given along the affected limb resulting in a new vascular network that can perfuse the limb and save it from amputation. The therapy may be autologous by using blood vessel units formed from the patient's own cells, for example, harvested with liposuction and injected in-clinic by a clinician.

[0047] Other therapeutic technologies include, but are not limited to, stem cell injections, endothelial progenitor cell injections, endothelial cell injections and growth factor injections. In addition, lymphatic endothelial cells may be used instead of vascular endothelial cells. Thus, a lymph vessel unit may be formed instead of a blood vessel unit. Injection of the lymph vessel units can create a lymphatic network that may relieve lymphedema.

[0048] Moreover, embodiments of the disclosed subject matter may be extended to other cell types to form injectable micro-tissues of different organ tissues, thereby providing an alternative to whole organ transplants.

[0049] In another example, embodiments of the disclosed spheroids may be used to treat subcutaneous adipose tissue loss. Subcutaneous adipose tissue loss is associated with numerous conditions including oncologic resection, congenital birth defects and traumatic injury. The current clinical treatment strategies for adipose tissue reconstruction primarily involve the transplantation of autologous composite tissue flaps or synthetic substitutes. However, the use of autologous tissues is associated with the creation of a donor site defect and potentially, the need for costly and complex surgical procedures. Synthetic implants are associated with immune rejection, implant migration and resorption, and a failure to integrate into the host tissues. Scaffolds for tissue engineering may be differentiated according to whether they are implantable or injectable. Injectable scaffolds may minimize risk of infections and scarring as well as the ability to fill irregularly shaped defect sites, making them particularly applicable for adipose tissue engineering.

[0050] Mesenchymal stem cells (MSCs) are commonly characterized by expression of fibroblastic markers and by their in vitro differentiation potential into, at a minimum, adipocytes, chondrocytes and osteoblasts. Moreover, MSCs display the general features of primary and non-oncogenic (i.e., non-transformed) cell type: serum dependency, limited growth rate and limited life span during in vitro culture. Moreover, other in vivo beneficial effects of MSCs are related to secretion of trophic factors. For example, C-MSCs present antigenic potential, including, but not limited to, expression of vascular endothelial growth factor (VEGF), insulin like growth factor (IGF), platelet derived growth factor (PDGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), transforming growth factor β 1 (TGF β 1), angiopoietin-1 (Ang-1), and stem cell factor (SCF).

[0051] In one or more embodiments, controlling the architecture of the vascular network may be influenced by various factors. By packing larger spheroids a larger distance between the endothelial cores may be achieved versus packing smaller spheroids. The endothelial cores can act as nodes in the vascular network, so that the larger distance between the endothelial cores in larger spheroids can result in longer branches in the resulting fully-formed vascular network. Secondly, micro-vessels may be formed by a single, or several, endothelial cells. Micro-vessel walls are one cell thick, so micro-vessels made of more cells will have larger diameters and/or be wider. All, or a proportion, of the available endothelial cells provided by the spheroids can participate in the forming of the vascular network. Accordingly, the higher ratio of endothelial cells to mesenchymal stem cells may yield wider vessels. Fig. 7 is a schematic illustrating the above described relationships depending on spheroid size and cell ratio.

[0052] An array of different spheroid sizes and cell ratios were tested to demonstrate that ratio of cells in, and size of, the spheroid can determine the architecture of the resulting blood vessel network. In particular, three different microwell sizes, which determine the ultimate size of the spheroid, were tested: 100 μ m, 200 μ m, and 400 μ m. In addition, three different cell ratios were

tested: 3 endothelial cells to 1 mesenchymal stem cell (3:1), 1 endothelial cell to 1 mesenchymal stem cell (1:1), and 1 endothelial cell to 3 mesenchymal stem cells (1:3).

[0053] Figs. 8-9 show the formation of these pre-vascularized spheroids as a three-by-three array for the time period immediately after seeding at day 0 (Fig. 8) and after initial spheroid formation in basal media with distinct endothelial cores at day 3 (Fig. 9). Fig. 10 illustrates the size of the spheroids and radial distribution of endothelial cells and mesenchymal stem cells over time.

[0054] The first time point, illustrated by Fig. 8, is at day 0 right after seeding the co-culture of endothelial and mesenchymal stem cells into the microwells. The second time point, illustrated by Fig. 9, is at day 3 after the cells have been cultured in the microwells allowing them to form spheroids. The first three days, the cells/spheroids are cultured in a basal media without any exogenous growth factors thereby observed to cause the endothelial cells to self-organize and migrate to the center of the spheroid creating an endothelial core within each spheroid. While not wishing to be bound by any particular theory, cells at the center of the spheroid exude endogenous growth factors that guide the endothelial cells on the periphery of the spheroid toward the center. As a result, so the cells in the microwell self-organize to form a spheroid with an endothelial core and a mesenchymal stem cell shell whose thickness may be varied, for example by adjusting the proportion of the MSC and EC. After day 3, when the endothelial cores have formed, the spheroids were further cultured in vascularization media with exogenous growth factors, which was observed to cause the cells in the endothelial core to sprout toward the surface and mature into lumenous vessels.

[0055] Producing the spheroids in non-adhesive microwells, rather than in, for example hanging drops, allows for large numbers of spheroids to be made in relatively small space with very little labor. The disclosed spheroid fabrication procedure yields copious amount of spheroids, all with endothelial cores prompted by culture without exogenous growth factor and with reproducible sizes. Fig. 10 shows the size of the spheroids over time, as well as the intra-spheroid

organization of the endothelial cells and mesenchymal stem cells. There is very little variance in the size of the cells, and the core's position is centered in all the spheroids.

[0056] The diameter of each spheroid is related to the number of cells allocated to each spheroid (i.e., the cells settling in each microwell), as illustrated in Figs. 11-12. The size of the spheroid may thus be controlled by seeding the same number of cells into fewer and larger wells, or by seeding more cells into the same diameter wells (in which case, the depth of the well may be increased in order to hold the added number of cells).

[0057] In addition to the advantages noted above, the use of a non-adhesive material such as alginate for the microwell can have other advantages as well. For example, the alginate polymers may be de-crosslinked so as to dissolve the microwells after the spheroids are fully formed and/or pre-vascularized. Dissolving the alginate microwells leaves the spheroids floating in a solution, which may be gently pipetted into tubes, spun down and resuspended in whichever media is desirable for cell delivery. The dissolvable alginate provides a gentle but effective way of harvesting the spheroids for future therapeutic use.

[0058] The construct can have a large number of microwells, for example, at least 1000 microwells. For example, the construct may be molded using alginate. A master mold for the alginate microwell construct may be fabricated using SU-8 patterned on 3-inch silicon wafers by photolithography. A polydimethylsiloxane (PDMS) replica may be formed from the master mold and made hydrophilic by plasma treatment. The PDMS replica may be submerged in distilled water to retain their hydrophilicity. The PDMS replica can then be autoclaved and cooled prior to casting.

[0059] Cold collagen solutions may be mixed with cold 1.6% w/v alginate in a 1:1 ratio. The resulting collagen-alginate solution may be placed on ice before pipetting the solution into the PDMS mold. Thus, the hydrogels contained a constant amount of alginate (0.8% w/v), which may be selected to be a minimum amount to support molding and handling of microfluidic hydrogels at low collagen density. The top of the molds may be closed with cellulose dialysis

membranes (6000 kD MWCO). The membranes may be flattened, for example, by using the edge of a sterile glass slide. The collagen component may be allowed to gel for 50 min at either room temperature or 37°C before pipetting a 60 mM CaCl₂ HEPES buffer solution on top of the membrane for at least 45 min to crosslink the alginate at room temperature. The hydrogels may be removed from the molds and placed in HEPES saline buffer solution supplemented with 1.8 mM CaCl₂ to prevent leaching of the calcium ions from the hydrogels.

[0060] The GFP-labelled human umbilical vein endothelial cells (GFP-hUVECs) may be cultured in Endothelial Growth Medium 2 at 37°C and 5% CO₂. The hUVECs may be passaged gently using TrypLE and used only until passage P6. The RFP-labelled mouse mesenchymal stem cells (RFP-mMSCs) may be cultured in DMEM with 10% FBS and 1% PenStrep at 37°C and 5% CO₂. The mMSCs can also be passaged gently using TrypLE and used only until passage P8.

[0061] To culture the spheroids, the 1.8 mM CaCl₂ solution that the alginate microwells were stored in may be removed and replaced with enough DMEM media to cover the constructs. The microwell constructs can then be placed in the incubator at 37°C and 5% CO₂ to equilibrate for 20 minutes. Then the media may be removed and the microwell constructs gently dried using surgical spears. The spheroids can then be seeded in the microwells using a positive displacement pipette with 20 µL of the desired cell suspensions. The cells can then be cultured in 0.5 mL of DMEM with 10% fetal bovine serum (FBS), 1% PenStrep and 20 ng/mL ascorbic acid for 3 days with a media change on the second day.

[0062] To assemble the spheroids in vitro, the media may be removed from the microwells, which may subsequently be de-crosslinked with 200 µL of 5% sodium-citrate, thereby releasing the fully-formed spheroids into suspension. The suspension may be gently spun down at 220g for 5 minutes. The supernatant may be removed and the spheroids resuspended in 0.5 mL vascularization media (DMEM) with 10% fetal bovine serum (FBS), 1% PenStrep, 20 ng/mL ascorbic acid, 20 ng/mL vascular endothelial growth factor (VEGF) and 20 ng/mL basic

fibroblast growth factor (bFGF). The spheroids can then be spun down again at 220g for 10 minutes and left in the incubator for an hour to form primary cell-cell adhesions between the spheroids, thereby forming macroscopic aggregates.

[0063] To inject spheroids *in vivo*, the media may be removed from the microwells, which can subsequently be de-crosslinked with 200 μ L of 5% sodium-citrate, thereby releasing the fully-formed spheroids into suspension. The suspension may be gently spun down at 220g for 5 minutes. The supernatant may be removed and the spheroids resuspended in 200 μ L PBS. The resulting solution may be injected into a patient under sterile conditions, e.g., on to a mouse's open window chamber.

[0064] The above method was employed and confirmed in laboratory experiments but the disclosed subject matter is not limited to the specific method described.

[0065] Embodiments of the disclosed subject matter may provide for or include one or more of the following features or advantages:

- Improve cell survival after delivery – by delivering the building blocks of a vascular network together with the therapeutic cells, the perfusion and/or vascularization and/or integration with the existing vascular network of the patient may be enabled.
- Mature tissues – endothelial cells in the injectable micro-tissues have been induced to form mature vessels with appropriate timing of growth factors.
- Improved vascularization – pre-vascularization of the microtissue allows delivery of blood vessels rather than single cells which may or may not assemble into a blood vessel.
- Scaffold-free – structures may be formed without using a scaffold.
- Injectable – microtissues may be created and controlled *ex vivo*.
- Ease of manufacturing – disclosed method is high throughput and less labor intensive than existing methods, and may be automated.
- Self-organization using growth factors – for complex tissue/organ engineering,

relying on the cells' ability to self-organize may avoid or at least reduce the challenge of growing larger functional tissues *in vitro*.

[0066] According to embodiments, a method for making spheroids includes, as illustrated in Fig. 13a was verified in a laboratory. A co-culture containing endothelial cells (of either mouse or human origin) was seeded in alginate micro-wells (see Fig. 19 and associated description for fabrication details). Second, the self-assembly and self-sorting of the endothelial cells into endothelial cores was directed by culturing them in maintenance medium without growth factors and these cores were matured into blood vessel units by culturing in vasculogenic medium. Third, the spheroids were harvested by gently dissolving the alginate microwells to release the spheroids. Fourth, the spheroids may be injected and used as a therapy without disrupting the blood vessel unit structure. Fifth, within 4-7 days the spheroids interconnect to each other and the host to form a fully-functional, perfused vascular network.

[0067] The above method is scalable for high throughput. The same number of steps can take a large number of cells harvested (Fig. 14a) limited only by the number of cells available for seeding (in examples, a quarter of a billion cells). One construct can produce more than 30,000 spheroids within a single 60 mm diameter petri dish (Fig. 14a), as they are seeded into the alginate construct (Fig. 14d).

[0068] The use of alginate has several benefits. It is non-adhesive to cells, and it can also be uncrosslinked with a chelator that does not disrupt cell function. Uncrosslinking of alginate (Fig. 20) leaves the spheroids suspended in a solution, which can be gently pipetted into tubes, centrifuged and resuspended in whichever media is desirable for cell delivery, hence providing a gentle but effective way of harvesting the spheroids for future research or clinical use. This method of releasing the spheroids (compared to flipping the wells or squirting with saline) results in a controlled release of spheroids with high yield under low mechanical stress. Producing the spheroids in non-adhesive alginate allows the fabrication of a copious amount of

spheroids in very little space with very little labor. These features were demonstrated in a laboratory.

[0069] Second, a co-culture was established containing endothelial cells in the alginate microwells. The cells were then in a non-vasculogenic medium (e.g. in a co-culture of ECs and MSCs, an adipogenic medium). These conditions prompted the endothelial cells in these spheroids to self-organize, migrate to the center of the spheroid, and form an endothelial core within 3 days by culturing the spheroids in media without any exogenous growth factors (Fig. 15). After the endothelial cores formed, the spheroids' medium was changed to vasculogenic medium containing exogenous growth factors (VEGF and bFGF) to mature the blood vessel units. In this manner, a pre-vasculature was achieved within a spheroid that would promote vessel formation among the endothelial-cell cores upon implantation – or injection.

[0070] After incubation in a vasculogenic medium for 4 to 11 days, the spheroids were harvested by uncrosslinking the alginate wells. The spheroids could also be dislodged from the microwells with gentle flushing of media or ultrasaline, or by flipping the microwells upside-down in which case the spheroids are deposited in the same hexagonal pattern.

[0071] The spheroids were collected by centrifuging, removing supernatant, and resuspending. For example, the spheroids were collected on day 8 into a macroscopic tissue with surface area of 1 cm² and a height of 1 mm (Fig. 1E). Fluorescence imaging of this macroscopic tissue was performed (Fig. 13f). The images showed a high density of spheroids with endothelial-cell cores (ECs were labelled with GFP) throughout the tissue (containing RFP-labelled MSCs). Finally, the intact spheroids may be injected (e.g. into an animal model, subcutaneously in a window chamber, to observe in vivo assembly or into a collagen or other hydrogel to observe in vitro assembly); when injected in vivo, the endothelial cores in the spheroids connected to form a perfused vascular network.

[0072] The disclosed methods provide a stable culture technique which allows for easy medium change. This was utilized to direct the self-organization of co-culture spheroids and produce a

large number of microtissues with blood vessel units, with high control over the size of the spheroid and size of the blood vessel unit. The self-organization of mouse endothelial cells in the spheroids was directed through the absence of exogenous growth factors (bFGF and VEGF). When co-cultures were first seeded and contracted into spheroids, the RFP-MSCs and the GFP-ECs were randomly distributed (Fig. 15a). When the co-culture spheroids were cultured without growth factors for 3 days, the endothelial cells migrated to the center of the spheroid and formed an endothelial core (Fig. 15a, top row). By contrast, this self-organization into endothelial cores in spheroids that were cultured in media with exogenous growth factors was not observed (Fig. 15a, bottom row). After the endothelial cores formed, the spheroids were cultured in vasculogenic media with exogenous growth factors. The exogenous growth factors caused the ECs in the core to sprout towards the surface and mature into vessels (day 8 or 10).

[0073] The methods can produce different spheroid sizes and mesenchymal-stem-cell-to-endothelial-cell ratios (Fig. 16a). Three different spheroid sizes were produced by cultivation in three different micro-well sizes (100, 200, and 400) resulting in spheroids with diameters of approximately 40, 80, and 160 μm , respectively. Experiments produced results for three different endothelial cell ratios: 3 endothelial cells to 1 mesenchymal stem cells (3:1), 1 endothelial cells to 1 mesenchymal stem cells (1:1), and 1 endothelial cells to 3 mesenchymal stem cells (1:3). The endothelial core formation is consistent for all of these ratios. Formation of these prevascularized spheroids as a three-by-three array for three key time points: 1) immediately after seeding at day 0; 2) after initial spheroid formation in basal media with distinct endothelial cores at day 3, after which the spheroids were cultured in a vasculogenic medium which ; 3) at day 10 after 7 days of subsequent pre-vascularization by culture in vasculogenic medium with added exogenous growth factors (VEGF and bFGF) (Fig. 2B) that caused the cells in the endothelial core to mature into vessels and sprout towards the surface.

[0074] The methods allow for generation of primitive vascular architecture with high throughput and reproducibility of size. The formation of the EC-core and the size of the core and spheroid

were highly reproducible (within 90%), as there was little variance over 50 spheroids in the distance of the most distal MSC to the center (Fig. 16b). Over time, the cells in the well contracted into a spheroid, while the distribution of endothelial cells showed migration of the ECs towards the core (Fig. 16b). In addition, the size of the spheroids could be controlled from 40 to 90 μm by controlling the seeding cell concentration and size of wells, such that the well size was big enough to contain the number of cells at initial seeding but small enough to ensure cell-cell contract to form a single spheroid rather than multiple spheroids. It was found that the size of the fully contracted spheroids (at day 2 and after) correlates to the number of cells in the spheroid (Fig. 16c). The diameter of the spheroids' cross-sections related to the number of cells in the spheroid and the cells typical volume, consistent with the relationship $d = (6/\pi v_{\text{cell}} n_{\text{cell}})^{1/3}$ (Fig. 16c). All spheroids self-assembled in the beginning of the in vitro cultivation period, with only minor changes in shape occurring after day 3. Spheroids contracted over the course of in vitro cultivation. This compaction was more pronounced for larger sizes. Smaller spheroids (100 μm) almost did not change their size. All spheroids were stable during the entire in vitro cultivation period and were uniform in shape.

[0075] Referring now to Fig. 17, which relates to HUVECs, human adipose-derived MSCs, to analyze the process of self-assembly and self-sorting, 1 EC : 1 MSC cell suspension was seeded on non-adherent 400 μm alginate microwell (Fig. 17a). Immediately after placement in the recesses of the alginate mold, the cells were observed to start to migrate towards each other and cluster up. After 20 h, spheroids were formed.

[0076] EC/MSC self-sorting was analyzed. Initially the co-cultured cells were a randomly distributed layer of ECs and MSCs that then self-assembled and also self-sorted. Interestingly ECs first migrated to each other (see Fig. 17a, 5h GFP image) and then migrated as larger EC units to form a central EC core covered in MSCs. Centrally placed ECs even migrated away from the center of the spheroid in order to cluster up with other ECs and afterwards returned to

the center in a larger EC unit. Self-assembly and self-sorting were observed happening simultaneously.

[0077] To examine fully the self-assembly and self-sorting mechanisms of the spheroids over the whole in vitro cultivation period, and also investigate the effects of in vitro prevascularization, time-lapse microscopy was applied to image the spheroids over the course of 8 days (Fig. 17b). Cell suspensions of different ratios (1:1, 1:3, MSC only) were placed on non-adherent alginate constructs patterned with micromolds of different size (100, 200, 400 μm). Cells settled within the cavities and as mentioned above, self-sorting and also self-assembly happened immediately after seeding. Self-sorting happened until day 3. ECs migrated to the center of the spheroid forming an endothelial core surrounded by MSCs. Under vasculogenic stimulation, these ECs started to spread out from the endothelial core and formed sprouts and vessel-like structures throughout the entire spheroid as can be seen in the GFP-fluorescent images. Vessel-like structures spread throughout the entire co-cultured spheroid creating an early stage of a vessel network. This was observed to be more pronounced in larger co-cultured spheroids.

[0078] To examine the ability of the spheroids to form a macro-tissue in vitro, namely, creating a larger tissue from smaller tissues units, prevascularized and self-assembled spheroids were harvested from their initial alginate microwells and placed in a collagen-alginate microwell to observe their assembly (Fig. 17C). Over the course of the experiment, the outer border of the fused tissue became smoother and more compact. Spheroids attached to each other and contracted to form a larger spheroid. The coherently amalgamated macro-tissue was uniform in shape, had no distinguishable units and was stable throughout the entire experiment.

Furthermore, in this experiment the ability to fuse the individual preformed vascular-like structures was examined. While initially forming an endothelial core and sprouting under vasculogenic stimuli, the already formed vessel-like structures connected to create one extensive endothelial cell network within the fused tissue. The individual networks became connected rapidly and spanned the whole macro-tissue. ECs maintained their ability to change architecture

until the end of this experiment, which was observed by their continuous rearrangement within the macro-tissue.

[0079] Referring now to Fig. 18, to improve graft acceptance, SCID mice have been used to study the integration of a HUVEC/MSC xenograft. Self-assembled in 200 μ m alginate microwells and prevascularized HUVEC/MSC spheroids were implanted. The spheroids were implanted by injection. Once the skin on one side of the window chamber was removed, the fascia on the remaining side was lifted up to allow for injection through the fascia and into the space between the fascia and the subdermis. The spheroids created with the disclosed methods can be delivered by injection, as they can withstand the shear stress of injection through syringes (See Fig. 24). The range of shear to which spheroids attended injection at rates of 0.1 to 2 mL/min through 27ga (0.27 mm) and 30ga (0.159mm). The disclosed embodiments may be limited to a spheroid (cluster) integrity of toughness that can survive this level of shear.

[0080] Day 0 shows post-operative images of the implanted micro-tissues (Fig. 18b). Similarly to in vitro experiments (Fig. 17c), the ECs started to sprout and connect to their adjacent spheroids. The endothelial cores of spheroids, which were not placed adjacent, did not form connections. A primitive vascular network formed at day 4. Between day 4 and day 7 this network matured, formed lumen, connected to the host vasculature, and became perfused, as indicated by the read arrowheads at day 7. A thin endothelial cell layer is seen in the fluorescent images enclosing the lumina. The lumina were blood-filled (as can be seen from Figs. 18b and 18b with the co-localization of the lumina of the GFP-ECs). The dashed line (indicated at 410) points out blood-filled vessels. Stereoscopic images taken immediately after implantation until day 11 demonstrated the kinetics of vascular network formation (Fig. 18c). Day 0 gives an overview of the implant placement with the three types of spheroids; 1 EC : 1 MSC, 1 EC : 3 MSC, and MSC only. The vascular structure for 1 EC : 1 MSC and MSC only are shown for day 4, 7, 9, and 11. Until day 4 newly formed red blood cell filled vessels are hardly seen. Between day 4 and day 7 vessel formation intensifies massively in the pre-vascularized spheroids, the implanted

vasculature becomes perfused and functional. After day 7 the blood filling in the vessels becomes more prominent and intense. The vascularization is only seen in pre-vascularized spheroids. Spheroids contain MSCs-only remained unvascularized throughout the extent of the experiment (28 days).

[0081] One can determine that the origin of the newly formed vessels are the endothelial cores in the implanted spheroids, as these ECs lining the newly formed vessels are GFP labelled (Fig. 18b and 18d) and co-localize with the new vessels in the grayscale images (Fig. 18b). It is inferred from this observation that a rudimentary vessel is formed during in vitro cultivation and this is identified as a lumen structure, although such a structure has not been consistently observed through direct imaging.

[0082] Maximum projection of confocal images taken of the GFP-labeled endothelial cells of EC/MSC spheroids in a spatially defined window chamber insert on day 1 and 11, also show that the endothelial cells in the prevascularized spheroids interconnected to form a vascular network whose spatial dimensions were controlled.

[0083] The embodiments employing microwells can achieve high throughput. To make a comparison, several iterations of the hanging drops have been developed in an attempt to semi-automate the production or at least make it less labor intensive (Perfecta3D 96 well hanging drop plates or Trevigen's 96 well spheroid formation plate), but even with these developments, the scalability is still severely limited by the number of wells - 96 or 384. Using the smallest constructs illustrated in Fig. 1c which produces 1000 spheroids, one can produce 24,000 spheroids rather than 96 spheroids in the same area and with fewer steps than these hanging drop iterations. That is a 60-250 fold increase in throughput.

[0084] As presently disclosed, a spheroid method embodiments are high throughput (and simple, so it may be automated). The spheroid method may be relevant for both 1) therapy production and 2) various research purposes, such as developmental biology. For example, developmental biologists use stem cell spheroids to check pathways, differentiation protocols, etc. and this

would speed up their screening/characterization process. Their focus is usually organogenesis – which all happens in the vicinity of blood vessels. An interaction, which is completely neglected in current published studies.

[0085] As presently disclosed, a high throughput approach to spheroid production is minimally labor intensive and will make it possible to produce enough spheroids that they could constitute an actual treatment.

[0086] It has been demonstrated that it is possible to direct the EC's self-organization by co-culturing the spheroids with MSCs in non-vasculogenic medium without growth factors. It is believed that at the initial formation of the spheroid on day 1 the cells at the center of the spheroid exude endogenous growth factors that guide the endothelial cells on the spheroid's periphery to the center, so the cells self-organize to a spheroid with an endothelial core and a shell of mesenchymal stem cells. This shell provides the 3D structure within which the endothelial core can form and the polarity necessary for correct lumen formation. In addition to providing therapeutic cells, the mesenchymal shell also apparently protects the endothelial structure from shear stress during injections through 25 to 30 gage needles (Supplementary Fig.5). Known spheroids may be torn apart by the shear stress attending injection. The mesenchymal stem cells in the shell may serve two additional purposes: (1) they can act as an immune suppressive shield and (2) may also provide additional angiogenic signaling. The shell can also be formed with other cell types such as fibroblasts or fat cells.

[0087] As presently disclosed, an injectable prevascularized spheroid can have many medical applications. They may be used to deliver therapeutic cells (in the cases presented here MSCs can be delivered as a treatment of Crohn's disease and MSCs differentiated into brown adipose tissue could be used for facial reconstruction or as a treatment for obesity and diabetes).

[0088] A hindrance to known cell therapies may be the low survival rate of the injected therapeutic cells. Using disclosed methods to produce prevascularized spheroids of therapeutic cells may increase their survival and treatment potential since the injected micro-tissues would

vascularize quicker. The disclosed injectable prevascularized spheroids connect to form functional micro-vasculature within 4 days, and may also be used to treat ischemia and non-healing wounds by directly delivering new micro-vasculature to the affected tissue with injections.

[0089] The method does not require any initial scaffold to construct spheroids, as any extra cellular matrix will be endogenously produced by the therapeutic and endothelial cells. Scaffold free approaches may be easier to get into clinical trials and through regulatory approval.

[0090] In embodiments, the cells are seeded onto a non-adhesive hydrogel, preferably alginate or other material to which the cells do not adhere and further preferably a material that can release the cellular spheroids after a period of in vitro cultivation. In an example, an alginate construct with 379 to 30,000 microwells is used. The cells settle into these microwells, and the alginate provides no adherence structure for the cells. Further the cells have been demonstrated to adhere strongly to each other forming spherical cell aggregates over the initial 24 hours.

[0091] The alginate microwells may be cast from hydrophilic PDMS molds. In examples, master molds were fabricated in SU-8 (SU-8 3050, Microchem, Newton, MA) on 3-inch Si wafers (Silicon Sense, Nashua, NH) by photolithography as described before 34 to cast polydimethylsiloxane (PDMS, Sylgard 184, Essex Brownell, Fort Wayne, IN) replicas from the masters. The PDMS molds were made hydrophilic by plasma treatment, and submerged them in distilled water to retain their hydrophilicity. The PDMS molds were autoclaved prior to casting alginate.

[0092] A 2% w/v alginate (Pronova UltraPure MVG, NovaMatrix, Drammen, Norway) was prepared and autoclaved in HEPES saline buffer solution (Ultrasaline A, Lonza, Basel, Switzerland). The alginate was pipetted into the PDMS molds. Positive-displacement pipettes were used for accurate pipetting of viscous alginate solutions and to avoid bubbles. The top of the molds were closed with cellulose dialysis membranes (6000 D MWCO), and flattened the membranes using the edge of a sterile glass slide. A 60 mM CaCl₂ HEPES buffer solution was

pipetted on top of the membrane for at least 60 min to crosslink the alginate at room temperature. The hydrogels were removed from the molds using sterilized tools, and placed the hydrogels in HEPES saline buffer solution (Ultrasaline A, Lonza, Basel, Switzerland) supplemented with 1.8 mM CaCl_2 (to prevent leaching of the calcium ions from the hydrogels). The alginate hydrogels were transferred into sterile culture ware, such as 24-well plates (Fisher Scientific, Fair Lawn, NJ) with the open micro wells facing up and stored them at 4°C until further use.

[0093] GFP-labelled human umbilical vein endothelial cells (GFP-hUVECs) (Angioproteomie, MA, USA) were cultured in Endothelial Growth Medium 2 (PromoCell, Heidelberg, Germany). Adipose derived human mesenchymal stem cells (hMSCs) (Promocell, Heidelberg, Germany) were cultured in Mesenchymal Stem Cell Growth Medium (Promocell, Heidelberg, Germany). The RFP-labelled mouse mesenchymal stem cells (RFP-mMSCs) (Cyagen, CA, USA) were cultured in DMEM with 10% FBS and 1% PenStrep (all from LifeTechnologies). All cells were gently passaged at 80-90% confluency using TrypLE (LifeTechnologies) and used only until passage P6 and mMSC until P8. Cells were cultured in 37°C and 5% CO_2 -balanced, humidified atmosphere.

[0094] HUVECs and MSCs were harvested from 2D cell culture, counted and desired cell ratios of HUVECs to MSCs were prepared: MSC only, 1 HUVEC to 3 MSC, 1 HUVEC to 1 MSC and 3 HUVEC to 1 MSC.

[0095] Then the 1.8 mM CaCl_2 solution that the alginate microwells were stored in was removed, and replaced with DMEM (ATCC, Manassas, VA). The microwells were then placed in the incubator at 37°C and 5% CO_2 to equilibrate for at least 20 minutes. Then DMEM was removed and the microwell constructs gently dried using surgical spears (Braintree Scientific, Braintree, MA) leaving the microwell features covered.

[0096] Cell suspensions were then pipetted on to alginate molds of 100, 200 and 400 μm microwell size using a positive displacement pipette. Cells were left to settle to the bottom of the microwells for 20 minutes and the culture wells were then filled up with culture medium.

[0097] The first 3-4 days after seeding, the cells were cultured in maintenance medium:

Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% PenStrep (all from LifeTechnologies), with 50 $\mu\text{g}/\text{mL}$ Sodium L-ascorbate (Sigma-Aldrich). For Figs. 17 and 18, the maintenance medium also included 20 mM Hepes (Fisher Scientific, Fair Lawn, NJ), 1 μM Insulin (LifeTechnologies, Carlsbad, CA), 250 nM T3, 1 μM dexamethasone, 0.5 mM IBMX, 50 μM Indomethacine, 1 μM Rosiglitazone and 1 μM CL316243 (all from Sigma, St. Louis, MO). After the first 3-4 days, the media was changed from maintenance medium to vasculogenic medium: Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% PenStrep (all from LifeTechnologies), with 50 $\mu\text{g}/\text{mL}$ Sodium L-ascorbate (Sigma-Aldrich), 40 ng/mL bFGF and 40 ng/mL VEGF recombinant human protein (both from LifeTechnologies). For Figs. 17 and 18, the vasculogenic media also included 20 mM Hepes, 1 μM Insulin, and 250 nM T3. The spheroids were cultured in vasculogenic medium up to day 11. Spheroid media was changed every other day.

[0098] To test the ability of spheroids to assemble in vitro and fuse to a larger tissue, 200 μm sized spheroids of pure MSCs, 1 EC : 3 MSC and 1 EC : 1 MSC ratio were placed in a 400 μm sized microwell composed of 3.5% collagen and 1% alginate. These spheroids had previously been prevascularized as described above. Spheroid fusion was conducted in vasculogenic medium and observed for 24 h.

[0099] Window chamber surgeries were conducted. In brief, a titanium window chamber (APJ Trading, Ventura, CA) was surgically implanted midline on the dorsum of male SCID mice (strain: ICRSC-M-M, 5-6 weeks of age, Taconic, Hudson, NY) after hair removal and ethanol and iodine disinfection. To reduce variability between mice prevascularized and non-prevascularized spheroids were implanted in individual compartments of the same window

chamber. Spheroids were delivered by injection and pipetting underneath the fascia of connective tissue to the subcutaneous adipose tissue. Window chambers were closed with a circular glass cover slip and a retaining ring (APJ Trading, Ventura, CA). A custom-made 3D printed window chamber backing was attached to reduce skin movement in the window chamber. In a subset of experiments, a custom-made ultem plastic 9 well array was screwed onto the front frame of the window chamber to allow for high throughput in vivo testing. Here, spheroids were placed into one of the 9 wells.

[0100] Animals were housed aseptically in frog cages to allow for enough clearance for the window chamber while still permitting easy access to standard laboratory chow (Irradiated globe rodent diet, Fisher Scientific, Fair Lawn, NJ) and drinking water ad libitum. Follow up buprenorphine administration (0.1mg/kg bodyweight) for pain management was given subcutaneously every 6-12 hours after surgery for the next 2 days post-OP. CO₂ euthanasia and cervical dislocation were performed after 30 days or earlier if necessary.

[0101] The animal procedures were approved and carried out in accordance to local regulations and authorities. The surgeries were conducted in aseptic technique.

[0102] A Leica DMI 6000B inverted microscope with 4x and 10x objectives, equipped with a motorized stage (Leica Microsystems, Bannockburn, IL) and a QImaging Retiga 2000R monochrome camera (QImaging, Surrey BC, Canada) was used to acquire fluorescence and brightfield images (Fig. 17 and Fig. 18). Leica LAS X software was used for image acquisition. Cropping, color adjustments and contrast enhancements of images as well as Z-stack projections were performed in ImageJ image analysis software. For time lapse imaging of spheroid formation and spheroid fusion an environmental chamber was used to maintain 37°C and 5% CO₂. Images were acquired every 30min. Confocal images of the window chamber were taken using a Leica SP5 confocal system with a 10.0x 0.30 N.A. objective. Due to the stressfulness of the imaging procedure, in vivo images were acquired every 2-3 days. Fluorescent and grayscale overview images of the window chamber (Fig. 4 d) were acquired with a CRi Maestro 2 in vivo

Imaging System. To be able to image the window chamber mice were anesthetized with isoflurane.

[0103] To observe individual spheroids precisely (Figs. 13-16), Stacks of confocal images (1024x1024 pixels, 41 images with a z- spacing of 0.25 microns) were taken using a Leica SP5 confocal microscope, with a 100x 1.43 N.A. oil immersion objective (Leica Microsystems) at a resolution of 0.132 $\mu\text{m}/\text{pixel}$ (image stacks were thus 135 mm * 135 mm * 10 mm). The differential interference contrast (DIC) images as well as the RFP- and GFP-signal were simultaneously collected.

[0104] To collect spheroids the alginate hydrogel was uncrosslinked. For this, the culture medium of the spheroids was replaced with 5%w/v sodium citrate solution for approximately 20 min. This chelator liquefied the alginate, and allowed for resuspension of the spheroids in a desired medium. Spheroids were then centrifuged at 300 rpm for 5 minutes or as specified and the spheroid pellet carefully collected for further use.

[0105] The media was gently removed from the microwells. The alginate microwells were uncrosslinked with 200 μl of 5% sodium-citrate, releasing the fully-formed spheroids into suspension. The suspension was gently spun down at 220 g for 5 minutes. The supernatant was removed and the spheroids resuspended in 0.5 ml vascularization media (DMEM with 10% fetal bovine serum (FBS), 1% PenStrep, 20 ng/mL ascorbic acid, 20 ng/mL vascular endothelial growth factor (VEGF) and 20 ng/mL basic fibroblast growth factor (bFGF). The spheroids were then spun down again at 220 g for 10 minutes and left in the incubator for an hour to form primary cell-cell adhesions between the spheroids to form macroscopic aggregates. The aggregates were gently tapped free of the tube bottom and the whole tube content (tissue-aggregate and vascularization media) was up-ended and transferred into an imaging well. The tissue-aggregates were cultured for an additional 10 days in vitro with a vascularization media change every other day.

- [0106] Spheroids were collected as described above. The suspension was gently spun down at 220 g for 5 minutes. The supernatant was removed and the spheroids resuspended in 200 μ l PBS. This solution was injected on to a mouse's open window chamber under sterile conditions and closed.
- [0107] In vitro created, prevascularized and non-prevascularized spheroids were implanted in a window chamber to allow for continuous in vivo monitoring of the vascularization and integration process.
- [0108] All tools, surgical instruments and window chambers were autoclaved. Surgical procedures were done in a laminar flow hood in aseptic technique to reduce risk of contamination and infection. Surgeons were wearing an apron (Kimberly-Clark isolation gown, Fisher Scientific, Fair Lawn, NJ), a hair net (Fisherbrand bouffant caps, Fisher Scientific, Fair Lawn, N), and a surgical facemask (Kimberly-Clark classic surgical facemask, Fisher Scientific, Fair Lawn, NJ). Because mice can lose a significant amount of body temperature during surgery, a 42°C warm water heating pad (heated hard pad, Gaymar warm water pump, both from Braintree Scientific, Braintree, MA) was used.
- [0109] Animal cages were disinfected with MB10 (Quip Laboratories) and opened in a laminar flow hood. Bodyweight was measured. For anesthesia Ketamine/Xylazine was injected intraperitoneally using a concentration of 80-100 mg/kg ketamine and 5-10 mg/kg xylazine (both from Butler Schein, Melville, NY). For maintenance of anesthesia an additional dose of 30% of the original dose of ketamine was given. To monitor the depth of anesthesia toes were pinched every 5 minutes. The mouse eyes were covered with eye gel (Fisher Scientific, Fair Lawn, NJ) to avoid damage. Hair removal at the surgical site was then done with an electrical animal hair trimmer (Braintree Scientific, Braintree, MA). The mouse was then placed on an animal restraint plate (Small animal surgery board, Braintree, Scientific, Braintree, MA). Sterile gloves (Fisher Scientific, Fair Lawn, NJ) were put on. The surgical site on the mouse's dorsum was disinfected twice with scrubbing solution (Betadine surgical scrubbing solution, Fisher

Scientific, Fair Lawn, NJ) followed by cleaning with 70% ethanol. Then buprenorphine (between the scapulae, 0.1mg/kg body weight) and lidocaine (at the surgical site, 5mg/kg body weight) (both from Butler Schein, Melville, NY) were injected subcutaneously. Mouse disinfection was then finished with the application of another round of scrubbing solution and ethanol and completed with application of prepping solution (Povidone iodine prepping solution, Fisher Scientific, Fair Lawn, NJ) at the surgical site. Sterile gloves were changed and marked the completion of the animal preparation.

[0110] The mouse was placed parallel underneath the bar (custom made in lab) of the animal restraint plate. Three sutures (4.0 non-absorbable nylon suture, Fisher Scientific, Fair Lawn, NJ) were placed to the front, middle and end of the back to spread out the skin of the dorsum and guided over the metal bar of the animal restraint plate. The middle suture was used as guidance to connect the backside of the window chamber (Window chamber kit, small, APJ Trading, Ventura, CA) to the skin. Sharp 18G hypodermic needles (Fisher Scientific, Fair Lawn, NJ) were inserted in blunt 15G dispensing needles (McMaster-Carr, Atlanta, GA). The 18G needles were used to penetrate the spread out skin where holes were needed for window chamber attachment and to push the blunt needles through the skin. Once holes were made, the 18G needles were removed comparable to “mandrin techniques” and only the 15G blunt needles were left in place. A piece of PDMS was used to facilitate the piercing of the skin (Fig. 24a). The light of a led lamp (Fisher Scientific, Fair Lawn, NJ) was used to indicate the inner opening of the window chamber on the mouse and show major vessels under the skin. This shadow was encircled with a marking pen (Fisher Scientific, Fair Lawn, NJ) and the skin area was then disinfected as mentioned above. Next a subcutaneous lidocaine injection at the surgical site followed. A new set of sterile gloves was put on. The circular marked skin was then cut out with surgical scissors and Adson tissue forceps (both from Fisher Scientific, Fair Lawn, NJ) (Fig. 24b). Next the screws of the assembled front part of the window chamber were inserted into the blunt needles. Pushing together the front and back parts of the window chamber assembled it

(Fig. 24c). Each screw was locked with a nut. All initial sutures were removed after secure window chamber frame placement. Next the site of implantation was prepared. The fascia of the subcutaneous tissue was carefully excised avoiding damage to the host tissue and vasculature. A magnifying camera and monitor were used to increase accuracy. Last remaining layers of fascia were lifted and prior collected spheroids were injected underneath. The window chamber was separated in up to 3 compartments to inject 3 different conditions simultaneously. To avoid air bubble entrapment in the window chamber, which could decrease imaging quality, the window chamber was filled up with Ultrasaline A (Lonza, Basel, Switzerland). The window chamber was closed by layering a glass slide on top and securing it with the retaining ring of the window chamber kit. A custom made, 3D printed holder was screwed on the window chamber and held in place with additional nuts (Fig. 24d) ensuring a tight seal of the window chamber to the skin, . Sutures were placed in lateral holes of the window chamber frame to secure placement (Fig. 24e). As described above, to reduce variability between mice 3 conditions were injected into one window chamber. This concept of simultaneous implantation was extended and in a different set of experiments a 9 well ultem plastic array was milled, and inserted into the window chamber (Fig. 24f). This insert only changed the final steps of the window chamber protocol. Here, fascia was completely removed and spheroids were directly pipetted into one of the 9 wells of the insert.

[0111] In one or more first embodiments, an implantable microtissue comprises a spheroid. The spheroid may comprise a plurality of cells of a first cell type and a plurality of cells of a second cell type. The first cell type may be endothelial cells and can form a lumen in an interior region of the spheroid.

[0112] In the first embodiments or any other embodiment, the endothelial cells are vascular endothelial cells and the lumen forms a blood vessel unit.

[0113] In the first embodiments or any other embodiment, the endothelial cells are lymphatic endothelia cells and the lumen forms a lymph vessel unit.

[0114] In the first embodiments or any other embodiment, the first cell type is different from the second cell type.

[0115] In the first embodiments or any other embodiment, the second cell type is one of mesenchymal stem cells, smooth muscle cells, dermal fibroblasts, and adipose cells.

[0116] In the first embodiments or any other embodiment, the spheroid has a diameter less than 200 μ m.

[0117] In the first embodiments or any other embodiment, the spheroid is constructed to be injected presumably in a solution with other spheroids.

[0118] In the first embodiments or any other embodiment, the lumen is formed at or near a center of the spheroid.

[0119] In one or more second embodiments, a method of forming microtissue for injection can comprise depositing cells of a first and second type into a microwell of a construct. The construct or surfaces of the microwell can comprise a material that is non-adherent with respect to the deposited cells. The method can further comprise, during a first time period, culturing the deposited cells in a culture media without one or more growth factors so as to form a spheroid. Optionally, the method can further comprise, during a second time period after the first time period, further culturing the spheroid with one or more growth factors to form the microtissue.

[0120] In the second embodiments or any other embodiment, the first time period is less than 4 days and the second time period is at least 6 days.

[0121] In the second embodiments or any other embodiment, the first cell type is endothelial cells and the second cell type is different than the first cell type.

[0122] In the second embodiments or any other embodiment, the second cell type is at least one of mesenchymal stem cells, smooth muscle cells, dermal fibroblasts, and adipose cells.

[0123] In the second embodiments or any other embodiment, the culturing during the first time period is such that a gradient of growth factor from the interior to the exterior of the spheroid is

formed by the cells, and the endothelial cells migrate toward the center of the spheroid as a result of the gradient.

[0124] In the second embodiments or any other embodiment, the culturing during the first time period is such that a lumen is formed by the endothelial cells in an interior region of the spheroid.

[0125] In the second embodiments or any other embodiment, the culturing during the second time period is such that the endothelial cells sprout toward the exterior of the spheroid and mature into lumenous vessels.

[0126] In the second embodiments or any other embodiment, the method further comprises harvesting the microtissue from the construct.

[0127] In the second embodiments or any other embodiment, the harvesting comprises dissolving the construct and recovering the microtissue in solution.

[0128] In the second embodiments or any other embodiment, the construct is formed of alginate, agarose, or PDMS.

[0129] In the second embodiments or any other embodiment, the microwell has a diameter or cross-sectional width of less than 500 μ m.

[0130] In the second embodiments or any other embodiment, the microwell has a diameter or cross-sectional width of 400 μ m or less, of 200 μ m or less, or of 100 μ m or less.

[0131] In the second embodiments or any other embodiment, the one or more growth factors include one of vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF).

[0132] In the second embodiments or any other embodiment, the culturing during the first time period is such that there is a non-homogeneous distribution of cells of the first and second type in the spheroid.

[0133] In the second embodiments or any other embodiment, the construct includes a plurality of microwells and the depositing and the culturing during the first and second time periods are performed for the plurality of microwells at the same time.

[0134] In the second embodiments or any other embodiment, the construct includes at least 1000 microwells.

[0135] In one or more third embodiments, a method of treating a patient can comprise forming a plurality of microtissues. Each microtissue can comprise a pre-vascularized spheroid. Each spheroid can comprise multiple cells with at least an endothelial cell core region. The method can further comprise injecting the plurality of microtissues into a patient such that a new vascular network is formed therein with the endothelial cell core regions of the spheroids as nodes.

[0136] In the third embodiments or any other embodiment, the new vascular network connects to the existing vasculature of the patient.

[0137] In the third embodiments or any other embodiment, the connection to the existing vasculature occurs within seven days after the injecting.

[0138] In the third embodiments or any other embodiment, the injecting is effective to treat a condition of ischemia.

[0139] In the third embodiments or any other embodiment, the injecting is performed at multiple locations spaced apart along a limb or other anatomical feature of the patient.

[0140] In the third embodiments or any other embodiment, the multiple cells comprise at least one of mesenchymal stem cells, smooth muscle cells, dermal fibroblasts, and adipose cells.

[0141] In this application, unless specifically stated otherwise, the use of the singular includes the plural and the use of “or” means “and/or.” Furthermore, use of the terms “including” or “having,” as well as other forms, such as “includes,” “included,” “has,” or “had” is not limiting. Any range described herein will be understood to include the endpoints and all values between

the endpoints. Moreover, any use of terms such as “near,” “approximate,” or the like, include the recited limitation as well as values within 10% of the recited limitation.

[0142] According to fourth embodiments, the disclosed subject matter includes a biological tissue product prepared by a process. the process includes co-culturing therapeutic and endothelial cells in a cluster in maintenance medium depleted in vasculogenic growth factors and for a period of time that together are effective to cause the endothelial cells to concentrate in respective central regions of the cluster. continuing the co-culturing for a period of time effective to cause the concentration of endothelial cells to form a lumen in the central region. integrating the cluster in a living host or combining with other like clusters to form a biological tissue in vitro.

[0143] In further fourth embodiments, the co-culturing is performed in a hydrogel well of an array of hydrogel wells. In further fourth embodiments, the process includes dissolving the hydrogel to release the cluster. In further fourth embodiments, the maintenance medium includes no vasculogenic growth factor. In further fourth embodiments, the integrating or combining includes exposing the cluster to vasculogenic growth factors. In further fourth embodiments, the integrating or combining includes exposing the cluster to vasculogenic growth factors. In further fourth embodiments, the exposing is effective to cause endothelial cells of the lumen to connect with external endothelial cells to form interconnecting vessels. In further fourth embodiments, the integrating includes injecting the cluster through a cannula into a living organism. In further fourth embodiments, the integrating or combining includes combining the cluster with other like clusters to form a biological tissue in vitro and further culturing a resulting combination. In further fourth embodiments, the further culturing includes exposing the resulting combination to a vasculogenic growth factor. In further fourth embodiments, the therapeutic cells include mesenchymal stem cells. In further fourth embodiments, the product cluster can be injected through a cannula without disintegration.

[0144] According to fifth embodiments, the disclosed subject matter includes a method for producing a biological tissue product. The method includes co-culturing therapeutic and endothelial cells in a cluster in maintenance medium depleted in vasculogenic growth factors and for a period of time that together are effective to cause the endothelial cells to concentrate in respective central regions of the cluster. The method further includes continuing the co-culturing for a period of time effective to cause the concentration of endothelial cells to form a lumen in the central region and integrating the cluster in a living host or combining with other like clusters to form a biological tissue in vitro.

[0145] In further fifth embodiments, the co-culturing is performed in a hydrogel well of an array of hydrogel wells. In further fifth embodiments, the process includes dissolving the hydrogel to release the cluster. In further fifth embodiments, the maintenance medium includes no vasculogenic growth factor. In further fifth embodiments, the integrating or combining includes exposing the cluster to vasculogenic growth factors. In further fifth embodiments, the integrating or combining includes exposing the cluster to vasculogenic growth factors. In further fifth embodiments, the exposing is effective to cause endothelial cells of the lumen to connect with external endothelial cells to form interconnecting vessels. In further fifth embodiments, the integrating includes injecting the cluster through a cannula into a living organism. In further fifth embodiments, the integrating or combining includes combining the cluster with other like clusters to form a biological tissue in vitro and further culturing a resulting combination. In further fifth embodiments, wherein the further culturing includes exposing the resulting combination to a vasculogenic growth factor. In further fifth embodiments, the therapeutic cells include mesenchymal stem cells. In further fifth embodiments, the product cluster can be injected through a cannula without disintegration.

[0146] According to sixth embodiments, the disclosed subject matter includes a method of forming microtissue for injection. The method includes depositing cells of a first and second type into a microwell of a construct, the construct or surfaces of the microwell including a

material to which the deposited cells are non-adherent. During a first time period, the deposited cells are cultured in a culture media depleted in growth factors, the level of growth factors and length of the first time period being such that a spheroid with a concentration the endothelial cells is formed by the end of the first time period. During a second time period after the first time period, the spheroid is further cultured with one or more growth factors to form the microtissue. In further sixth embodiments, the first time period is less than 4 days and the second time period is at least 6 days. In further sixth embodiments, the first cell type is endothelial cells and the second cell type is different than the first cell type. In further sixth embodiments, the second cell type is at least one of mesenchymal stem cells, smooth muscle cells, dermal fibroblasts, and adipose cells. In further sixth embodiments, the culturing during the first time period is such that a gradient of growth factor from the interior to the exterior of the spheroid is formed by the cells, and the endothelial cells migrate toward the center of the spheroid as a result of the gradient. In further sixth embodiments, the culturing during the first time period is such that a lumen is formed by the endothelial cells in an interior region of the spheroid. In further sixth embodiments, the second time period is such that the endothelial cells sprout toward the exterior of the spheroid and mature into lumenous vessels. Further sixth embodiments include harvesting the microtissue from the construct. In further sixth embodiments, the harvesting comprises dissolving the construct and recovering the microtissue in solution. In further sixth embodiments, the construct is formed of alginate, agarose, or PDMS. In further sixth embodiments, the microwell has a diameter or cross-sectional width of less than 500 μ m. In further sixth embodiments, the microwell has a diameter or cross-sectional width of 400 μ m or less, of 200 μ m or less, or of 100 μ m or less. In further sixth embodiments, the one or more growth factors include one of vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF). The method of claim 56, wherein the culturing during the first time period is such that there is a non-homogeneous distribution of cells of the first and second type in the spheroid. In further sixth embodiments, the construct includes a plurality of

microwells and the depositing and the culturing during the first and second time periods are performed for the plurality of microwells at the same time. In further sixth embodiments, the construct includes at least 1000 microwells.

[0147] According to seventh embodiments, the disclosed subject matter includes a method of forming a biological tissue. The method includes co-culturing endothelial and therapeutic cells to form spheroids each with a size of less than 1000 microns to form a region concentrated in endothelial cells surrounded by a region depleted in endothelial cells. The method includes ex vivo, placing multiple spheroids resulting from the co-culturing in mutual contact under conditions and for a time that permits signaling between the regions concentrated in endothelial cells to form lumens between the spheroids to form a larger vascularized construct. According to seventh embodiments the placing for a time includes exposing the multiple spheroids to vasculogenic growth factors.

[0148] According to eighth embodiments, the disclosed subject matter includes an injectable microtissue. The microtissue includes a plurality of spheroids, each with a plurality of cells of a first cell type and a plurality of cells of a second cell type, the first cell type being endothelial cells that are concentrated in an interior region of the each spheroid. Each of the plurality of spheroids resides in a microwell formed in a soluble matrix and isolated from others of the plurality of spheroids.

[0149] In further eighth embodiments, the endothelial cells concentrated in an interior region define a lumen-shaped structure. In further eighth embodiments, the plurality of cells of the second type form a durable shell that can withstand the shear strain and stress of injection through a 27ga (0.27 mm) or 30ga (0.159mm) cannula at rates of 0.1 to 2 mL/min. In further eighth embodiments, the endothelial cells are vascular endothelial cells and the lumen-shaped structure is a blood vessel precursor. In further eighth embodiments, the endothelial cells are lymphatic endothelial cells and the lumen-shaped structure forms a lymph vessel precursor. In further eighth embodiments, the second cell type is one of mesenchymal stem cells, smooth

muscle cells, dermal fibroblasts, and adipose cells. In further eighth embodiments, the majority of the spheroids have diameters less than 200 μ m. In further eighth embodiments, the spheroid have a structural integrity capable of withstanding the shear strain of injection through a cannula into a host without disintegration in a solution with other spheroids. In embodiments, the shear is that associated injection through a 27ga (0.27 mm) or 30ga (0.159mm) cannula at rates of 0.1 to 2 mL/min. In further eighth embodiments, the lumens are located at or near the center of a respective one of the plurality of spheroids. In further eighth embodiments, the soluble matrix includes a hydrogel. In further eighth embodiments, the soluble matrix includes alginate.

[0150] According to ninth embodiments, the disclosed subject matter includes an injectable microtissue including a plurality of spheroids, each having a plurality of cells of a first cell type and a plurality of cells of a second cell type. The first cell type is endothelial and they are concentrated in an interior region of the each spheroid. The spheroids have diameters of less than 200 μ m.

[0151] In further ninth embodiments, the plurality of spheroids is free of vascular interconnections therebetween. In further ninth embodiments, the plurality of spheroids is suspended in an injectable blood-normal medicament. In further ninth embodiments, the endothelial cells concentrated in an interior region define a lumen-shaped structure. In further ninth embodiments, the plurality of cells of the second type form a durable shell that that can withstand the shear strain and stress of injection through a 27ga (0.27 mm) or 30ga (0.159mm) cannula at rates of 0.1 to 2 mL/min. In further ninth embodiments, the endothelial cells are vascular endothelial cells and the lumen-shaped structure is a blood vessel precursor. In further ninth embodiments, the endothelial cells are lymphatic endothelial cells and the lumen-shaped structure forms a lymph vessel precursor. In further ninth embodiments, the second cell type is one of mesenchymal stem cells, smooth muscle cells, dermal fibroblasts, and adipose cells. In further ninth embodiments, the majority of the spheroids have diameters less than 200 μ m. In further ninth embodiments, the spheroid have a structural integrity capable of withstanding the

shear strain and stress of injection through a 27ga (0.27 mm) or 30ga (0.159mm) cannula at rates of 0.1 to 2 mL/min disintegration in a solution with other spheroids. The microtissue of claim 85, wherein the lumens are located at or near the center of a respective one of the plurality of spheroids.

[0152] According to tenth embodiment, the disclosed subject matter includes an array of microtissue spheroids. The array includes a plurality of spheroids, each comprising a plurality of cells of a first cell type and a plurality of cells of a second cell type, the first cell type being endothelial cells that are concentrated in an interior region of the each spheroid. Each of the plurality of spheroids being isolated from others in a respective well of an array of microwells in a substrate.

[0153] In further tenth embodiments, the endothelial cells concentrated in an interior region define a lumen-shaped structure. In further tenth embodiments, the plurality of cells of the second type form a durable shell that can withstand the shear strain and stress of injection through a 27ga (0.27 mm) or 30ga (0.159mm) cannula at rates of 0.1 to 2 mL/min. In further tenth embodiments, the endothelial cells are vascular endothelial cells and the lumen-shaped structure is a blood vessel precursor. In further tenth embodiments, endothelial cells are lymphatic endothelial cells and the lumen-shaped structure forms a lymph vessel precursor. In further tenth embodiments, the second cell type is one of mesenchymal stem cells, smooth muscle cells, dermal fibroblasts, and adipose cells. In further tenth embodiments, the spheroids have diameters less than 200 μ m. In further tenth embodiments, the spheroid have a structural integrity capable of withstanding the shear strain and stress of injection through a 27ga (0.27 mm) or 30ga (0.159mm) cannula at rates of 0.1 to 2 mL/min without disintegration in a solution with other spheroids. In further tenth embodiments, the lumens are located at or near the center of a respective one of the plurality of spheroids.

[0154] According to eleventh embodiments, the disclosed subject matter includes an injectable microtissue. The microtissue includes a plurality of spheroids, each comprising a plurality of

cells of a first cell type and a plurality of cells of a second cell type, the first cell type being endothelial cells that are concentrated in an interior region of the each spheroid. The spheroids having diameters of less than 200 μ m and each being vascularly isolated including vascularly disconnected from the others of the spheroids and vascularly disconnected from an external host.

[0155] In further eleventh embodiments, the plurality of spheroids is free of vascular interconnections therebetween. In further eleventh embodiments, wherein the plurality of spheroids is suspended in an injectable blood-normal medicament. In further eleventh embodiments, the endothelial cells concentrated in an interior region define a lumen-shaped structure. In further eleventh embodiments, plurality of cells of the second type form a durable shell that can withstand the shear strain and stress of injection through a 27ga (0.27 mm) or 30ga (0.159mm) cannula at rates of 0.1 to 2 mL/min. In further eleventh embodiments, the endothelial cells are vascular endothelial cells and the lumen-shaped structure is a blood vessel precursor. In further eleventh embodiments, the endothelial cells are lymphatic endothelial cells and the lumen-shaped structure forms a lymph vessel precursor. In further eleventh embodiments, the second cell type is one of mesenchymal stem cells, smooth muscle cells, dermal fibroblasts, and adipose cells. In further eleventh embodiments, the majority of the spheroids have diameters less than 200 μ m. In further eleventh embodiments, the spheroid have a structural integrity capable of withstanding the shear strain and stress of injection through a 27ga (0.27 mm) or 30ga (0.159mm) cannula at rates of 0.1 to 2 mL/min without disintegration in a solution with other spheroids. The microtissue of claim 105, wherein the lumens are located at or near the center of a respective one of the plurality of spheroids.

[0156] According to twelfth embodiments, the disclosed subject matter includes a method of growing a tissue structure in a living host. The method includes co-culturing cells of multiple types in respective microwells, including controlling a composition of a nutrient medium such that the cells in each microwell organizes into a respective cluster responsively to growth factors, that naturally arise from the cells, into spheroids with a first cell type concentrated in the

center and depleted at a periphery of the respective cluster. the co-culturing including culturing for a period of days. The method further includes releasing the clusters from the microwells and combining in a solution. injecting the solution, containing the clusters, into a living host.

[0157] In further twelfth embodiments, the releasing includes dissolving a matrix in which the microwells are formed. In further twelfth embodiments, the first cell type is endothelial. In further twelfth embodiments, the multiple types include stem type cells. In further twelfth embodiments, the multiple types include therapeutic cells. In further twelfth embodiments, the co-cultivating includes controlling a composition of a nutrient medium at a first time to include no vasculogenic growth factors and at a second time, controlling a composition of a nutrient medium to include vasculogenic growth factors, wherein the method includes exposing the clusters to the resulting mediums during the respective first and second times.

[0158] According to thirteenth embodiments, the disclosed subject matter includes a method of forming a tissue construct. The method includes creating multiple tissue clusters each with a mixture of endothelial and therapeutic cells organized such that when the tissue clusters are placed together in a culturing medium, the therapeutic cells separate from the endothelial cells, the latter being concentrated in respective nodes at the center of each cluster. The method further includes placing the tissue clusters together in a vasculogenic medium to induce the formation of vascular tissue connections between the endothelial cell nodes. The method further includes culturing for a time sufficient to generate vascular branches interconnecting a substantial number of the nodes, whereby a vascularized tissue construct is formed. The method further includes implanting the vascularized tissue construct in a living host.

[0159] In further thirteenth embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 1:1 through 1:2. In further thirteenth embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 1:1 through 1:3. In further thirteenth embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 1:1 through 2:3. In further thirteenth embodiments, the ratio of

endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 1:2 through 2:1. In further thirteenth embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 2:3 through 3:1. In further thirteenth embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is about 1:1. In further thirteenth embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is about 1:2. In further thirteenth embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is about 1:3.

[0160] According to fourteenth embodiments, the disclosed subject matter includes a method of forming a tissue construct. The method includes creating multiple tissue clusters each with a mixture of endothelial and therapeutic cells organized such that when the tissue clusters are placed together in a culturing medium, the therapeutic cells separate from the endothelial cells, the latter being concentrated in respective nodes at the center of each cluster. The method further includes the creating including culturing tissue clusters in a medium having a first concentration of vasculogenic growth factors for a first time and, after the first time, culturing the tissue clusters in a medium having a second concentration of vasculogenic growth factors for a second time, where the second concentration is greater than the first. The method further includes implanting or injecting the clusters in a living animal.

[0161] In further fourteenth embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 1:1 through 1:2. In further fourteenth embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 1:1 through 1:3. In further fourteenth embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 1:1 through 2:3. In further fourteenth embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 1:2 through 2:1. In further fourteenth embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 2:3 through 3:1. In further fourteenth embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is about 1:1. In further fourteenth

embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is about 1:2.

In further fourteenth embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is about 1:3.

[0162] In any of the foregoing embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), may be in the range of 1:1 through 1:2.

[0163] In any of the foregoing embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), may be in the range of 1:1 through 1:3.

[0164] In any of the foregoing embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), may be in the range of 1:1 through 2:3.

[0165] In any of the foregoing embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), may be in the range of 1:2 through 2:1.

[0166] In any of the foregoing embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), may be in the range of 2:3 through 3:1.

[0167] In any of the foregoing embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), may be about 1:1.

[0168] In any of the foregoing embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), may be about 1:2.

[0169] In any of the foregoing embodiments, the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), may be about 1:3.

[0170] In any of the foregoing embodiments, the therapeutic cells may include stem cells. In any of the foregoing method embodiments, the therapeutic cells may be cells selected responsively to a predefined disease or pathology of a host. In any of the foregoing methods in which spheroids or clusters (the terms being used interchangeably herein) are injected in a suspension thereof, the suspending fluid may include a buffered saline. In any of the foregoing methods in which spheroids or clusters (the terms being used interchangeably herein) are injected in a suspension thereof, the suspending fluid may include a phosphate-buffered saline.

[0171] According to fifteenth embodiments, the disclosed subject matter includes a method of creating a therapeutic tissue for injection. The method includes, in vitro, in 200 micron diameter or less wells, culturing endothelial cells and therapeutic cells forming cell clusters in a medium depleted in vasculogenic growth factor for a first period of time to induce a sorting of the endothelial cells in which the endothelial cells concentrate in a region near a center of the each cell cluster. The method further includes after the first period of time, further culturing in a medium with an increased concentration of vasculogenic growth factor for a second period of time to induce the formation of branching structures extending from the regions, the branching structures comprising the endothelial cells. The method further includes removing the clusters from the wells after at least the first period of time and adding the clusters to an injectable solution to form a suspension.

[0172] In further fifteenth embodiments, the solution is a buffered saline. Further fifteenth embodiments may include centrifuging or otherwise mechanically concentrating the clusters in a medium containing the clusters to produce a supernatant, the adding including adding the supernatant to the injectable solution. In further fifteenth embodiments, the removing the clusters from the wells after at least the first period of time is removing the clusters from the wells after the second period of time. In further fifteenth embodiments, the therapeutic cells include stem cells. In further fifteenth embodiments, the ratio of therapeutic cells to endothelial cells is greater than unity. In further fifteenth embodiments, the ratio of therapeutic cells to endothelial cells is greater than 1.5. In further fifteenth embodiments, the ratio of therapeutic cells to endothelial cells is greater than 2. In further fifteenth embodiments, the ratio of therapeutic cells to endothelial cells is 3. In further fifteenth embodiments, the first period of time is between 2 and 5 days. In further fifteenth embodiments, the sum of the first and second periods of time is less than 6 days. In further fifteenth embodiments, the sum of the first and second periods of time is less than 7 days. In further fifteenth embodiments, the sum of the first and second periods of time is less than 8 days. In further fifteenth embodiments, the sum of the

first and second periods of time is between 6 and 12 days. In further fifteenth embodiments, the first time period is less than 4 days and the second time period is at least 6 days.

[0173] According to sixteenth embodiments, the disclosed subject matter includes a method of creating a therapeutic tissue for implantation. The method includes, in vitro and in 200 micron diameter or less wells, culturing endothelial cells and therapeutic cells forming cell clusters in a medium depleted in vasculogenic growth factor for a first period of time to induce a sorting of the endothelial cells in which the endothelial cells concentrate in a region near a center of the each cell cluster. After the first period of time, the cell clusters are further cultured in a medium with an increased concentration of vasculogenic growth factor for a second period of time to induce the formation of branching structures extending from the regions, the branching structures comprising the endothelial cells. The method further includes removing the clusters from the wells after at least the first period of time and combining the clusters and further culturing to cause fusion and vascular interconnection between the clusters prior to implantation in a living host.

[0174] Further sixteenth embodiments may include centrifuging or otherwise mechanically concentrating the clusters in a medium containing the clusters to produce a supernatant, the adding including adding the supernatant to a culture medium to perform the further culturing.

[0175] The removing the clusters from the wells after at least the first period of time may be removing the clusters from the wells after the second period of time. The therapeutic cells may include stem cells. The ratio of therapeutic cells to endothelial cells may be greater than unity.

[0176] The ratio of therapeutic cells to endothelial cells may be greater than 1.5.

[0177] The ratio of therapeutic cells to endothelial cells may be greater than 2.

[0178] The ratio of therapeutic cells to endothelial cells may be 3.

[0179] The first period of time may be between 2 and 5 days.

[0180] The sum of the first and second periods of time may be less than 6 days.

[0181] The sum of the first and second periods of time may be less than 7 days. The sum of the first and second periods of time may be less than 8 days. The sum of the first and second periods of time may be between 6 and 12 days. The first time period may be less than 4 days and the second time period, at least 6 days.

[0182] In further embodiments, the disclosed subject matter includes a method, microtissue, or product according to any of the method embodiments, including claims, where each includes all combinations or subcombinations of the limitations of their respective dependent claims not otherwise explicitly claimed.

[0183] In further embodiments, the disclosed subject matter includes a therapeutic method employing any of the products, microtissues, or methods (including the claims), in which the therapeutic cells are previously harvested from a host and then implanted and injected in the same host.

[0184] The foregoing descriptions apply, in some cases, to examples generated in a laboratory, but these examples may be extended to production methods. For example, where quantities and methods apply to the laboratory examples, they should not be understood as limiting. In addition, although specific chemicals and materials have been disclosed herein, other chemicals and materials may also be employed according to one or more contemplated embodiments.

[0185] Although in the embodiments, endothelial cells are combined with therapeutic cells, the cells chosen may have further functions and be of different types than these. For example endothelial cells may be combined with cells that provide other functions such as diagnostic or labeling function. Any type of cell that may form a tissue structure and which may be vascularized using the methods of the disclosed subject matter may be used. These cell types may include differentiated cells rather than the stem cells employed in many of the embodiments. The methods may include a step of exposing undifferentiated cell types to signaling the induces differentiation prior to combining with endothelial cells to form spheroids.

Also, any of the embodiments can be modified to include more than one type of cell in addition to the type of cell used to form blood vessels (e.g., endothelial).

[0186] The angiogenic growth factors described herein may be replaced or modified by the addition of one or more additional factors including any of Angiogenin; Angiopoietin-1; Angiopoietin-2; Del-1; Fibroblast growth factors: acidic (aFGF) and basic (bFGF); Follistatin; Granulocyte colony-stimulating factor (G-CSF); Hepatocyte growth factor (HGF) /scatter factor (SF); Interleukin-8 (IL-8); Leptin; Midkine; Placental growth factor; Platelet-derived endothelial cell growth factor (PD-ECGF); Platelet-derived growth factor-BB (PDGF-BB); Pleiotrophin (PTN); Progranulin; Proliferin; Transforming growth factor-alpha (TGF-alpha); Transforming growth factor-beta (TGF-beta); Tumor necrosis factor-alpha (TNF-alpha); Vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF); Phorbol esters.

[0187] Conditions that may be treated using therapeutic angiogenesis include;; Chronic wounds (e.g. diabetic lower extremity ulcers, venous leg ulcerations, pressure ulcers, arterial ulcers); Peripheral arterial disease, critical limb ischemia; Ischemic heart disease; Brain ischemia/stroke; Intestinal ischemia;

[0188] Conditions that may be treated (or modeled for diagnostics, research, drug screening etc.) with vascularized engineered tissues include;; Liver disease; Soft tissue damage due disease or injury; Muscle loss; Bone damage due to disease or injury; Diabetes; Kidney failure; Pulmonary disease; Genetic diseases causing tissue dysfunction; Cancer; Heart disease.

[0189] The endothelial cells described in the embodiments may of various types and sources including: Umbilical vein endothelial cells; Arterial endothelial cells; Venous endothelial cells; Microvascular endothelial cells (e.g. from dermis, fat tissue, brain, retina).

[0190] Stem and progenitor cell sources may be used to derive endothelial cells. Examples of such sources include: Embryonic stem cells; Induced pluripotent stem cells; Mesenchymal stem cells (e.g. bone marrow or adipose tissue); Endothelial progenitor cells (blood and bone marrow).

[0191] Additional cell types that may be included in spheroids or cell clusters according to any of the embodiments include: Stem and progenitor cells; Embryonic stem cells; Induced pluripotent stem cells; Mesenchymal stem cells (e.g. bone marrow or adipose tissue)

[0192] Tissue-specific cells may be differentiated from stem/progenitor cells or from primary cultures at any stage in the disclosed embodiments, including:; Fibroblasts; Dermal papilla cells; Keratinocytes; melanocytes; Neurons; Kidney cells; Pancreas cells (islet cells); Hepatocytes; Cardiomyocytes; Skeletal muscle; Smooth muscle; Intestinal epithelial and submucosal cells; Adipocytes; ; Cancer cells;; Glioblastoma or other brain tumors; Prostate cancer; Cervical cancer; Breast cancer; Liver cancer; Pancreatic cancer; Skin cancer; Bladder cancer; Colon cancer; Ovarian cancer; Testicular cancer.

[0193] Although in the embodiments, spheroids (equivalently, clusters) are formed as a first primitive structure for tissue formation, the methods of the disclosed embodiments may be applied to other types of primitive structures. For example, a sheet of cells containing a mix of endothelial and other cells types may be cultured. The tendency of the endothelial cells to concentrate may cause clusters of endothelial cells to arise due to endothelial self-organizing potential combined with initial variations in the initial concentration of the endothelial cells.

[0194] Although in embodiments, the spheroids are cultured in the presence of one or more vasculogenic growth factors and then injected or implanted, these embodiments may be modified to culture in the absence of vasculogenic growth factors (or sufficiently depleted of such factors) to cause endothelial cells to concentrate in the center of the spheroids and then immediately inject or implant the spheroids, thereby relying on naturally-occurring vasculogenic signaling to promote the vascular integration of the spheroids. In further embodiments, the implantation or injection may include vasculogenic growth factors in a suspending aqueous solution carrying the spheroids.

[0195] While a lumen or vascular precursor has been inferred from observation, it is not evident merely from direct observation that a lumen-shaped structure is formed in vitro in the methods

disclosed. However, further development in the presence of vasculogenic growth factors that goes beyond the concentration of endothelial after cultivation without vasculogenic growth factors provides indirect evidence that a lumen structure or vascular precursor forms. The evidence arises from a distinct region of primarily endothelial cells with a largely a defined boundary and sometimes a degree of greater transparency in the center as well as an oblong shape. The evidence also arises from the observation that observable lumens arise from the region of endothelial cell concentration in the spheroids very rapidly (as reported with regard to the various embodiments) combined with the fact that the lumens that are directly observed connect the regions where the endothelial concentration has been directly observed.

[0196] Embodiments of the disclosed subject matter can provide treatment for various vascular issues. For example, the disclosed methods can provide a treatment for critical limb ischemia (CLI). A patient may be injected on an outpatient basis with engineered microtissues, each of which contains a blood vessel unit (i.e., a spheroid with an endothelial core). Upon injection, these microtissue units are observed to connect to form a vascular network, which formation occurs faster than the formation of a vascular network by single cell therapies. Multiple injections may be given along the affected limb resulting in a new vascular network that can perfuse the limb and save it from amputation. The therapy may be autologous by using blood vessel units formed from the patient's own cells, for example, harvested with liposuction and injected in-clinic by a clinician.

[0197] In any of the embodiments, according to additional embodiments, the culturing in the presence of vasculogenic growth factors may be performed in vitro either before or after releasing from the microwell matrix. In alternative embodiments culturing in the presence of vasculogenic growth factors may be performed initially for a period of time before releasing from the microwells and performed for a further period of time after release in a single vessel, for example in a fluidized bed bioreactor.

[0198] Features of the disclosed embodiments may be combined, rearranged, omitted, etc., within the scope of the invention to produce additional embodiments. Furthermore, certain features may sometimes be used to advantage without a corresponding use of other features.

[0199] Throughout the specification and claims, there are embodiments in which cell clusters or spheroids are limited to 200 microns. In extensions of the disclosed subject matter any of the embodiments may be expanded to a higher size threshold, for example 300 micron. According to known principles, the size may be extended to up to 400 microns according to further embodiments.

[0200] In any of the embodiments identifying microwells, the materials of which the microwells are made may include any type of material that is suitable including other plastics, acrylics, other hydrogels.

[0201] Throughout the specification, all references to “embodiments” refer severally to any of the described devices, methods, articles, systems, apparatuses, etc. whether they are described in the specification or defined by the claims. For example, statements stating that “Any of the embodiments may be modified by the inclusion of X,” means any of the claims may be so modified and such modified claims form part of the original disclosure.

[0202] It is thus apparent that there is provided in accordance with the present disclosure, system, methods, and devices for injectable microtissues. Many alternatives, modifications, and variations are enabled by the present disclosure. While specific embodiments have been shown and described in detail to illustrate the application of the principles of the present invention, it will be understood that the invention may be embodied otherwise without departing from such principles. Accordingly, Applicants intend to embrace all such alternatives, modifications, equivalents, and variations that are within the spirit and scope of the present invention.

CLAIMS

1. An injectable microtissue comprising:

a plurality of spheroids, each comprising a plurality of cells of a first cell type and a plurality of cells of a second cell type, the first cell type being endothelial cells that are concentrated in an interior region of the each spheroid;

the plurality of spheroids being free of vascular interconnections therebetween and suspended in an injectable blood-normal medicament.
2. The microtissue of claim 1, wherein the endothelial cells concentrated in an interior region define a distinct structure with discernable boundaries and composed principally of said endothelial cells.
3. The microtissue of claim 1, wherein the plurality of cells of the second type form a durable shell that can withstand the shear strain and stress of injection through a 27ga (0.27 mm) or 30ga (0.159mm) cannula at rates of 0.1 to 2 mL/min.
4. The microtissue of claim 2, wherein the endothelial cells are vascular endothelial cells and the distinct structure is a blood vessel precursor.
5. The microtissue of claim 2, wherein the endothelial cells are lymphatic endothelial cells and the distinct structure is a lymph vessel precursor.
6. The microtissue of claim 4, wherein the second cell type is one of mesenchymal stem cells, smooth muscle cells, dermal fibroblasts, and adipose cells.
7. The microtissue of claim 1, wherein the majority of the spheroids have diameters less than 200 μ m.
8. The microtissue of claim 1, wherein the spheroids have a structural integrity capable of withstanding the shear strain and stress of injection through a 27ga (0.27 mm) or 30ga (0.159mm) cannula at rates of 0.1 to 2 mL/min without disintegration in a solution with other spheroids.
9. The microtissue of claim 1, wherein the distinct structure is located at or near the

center of a respective one of said plurality of spheroids.

10. A method of forming microtissue for injection, the method comprising:

depositing cells of a first and second type into a microwell of a construct, the construct or surfaces of the microwell comprising a material that is non-adherent with respect to the deposited cells;

during a first time period, culturing the deposited cells in a culture media without one or more growth factors thereby forming a spheroid; and

during a second time period after the first time period, further culturing the spheroid with one or more growth factors to form the microtissue.

11. The method of claim 10, wherein the first time period is less than 4 days and the second time period is at least 6 days.

12. The method of claim 10, wherein the first cell type is endothelial cells and the second cell type is different than the first cell type.

13. The method of claim 12, wherein the second cell type is at least one of mesenchymal stem cells, smooth muscle cells, dermal fibroblasts, and adipose cells.

14. The method of claim 12, wherein the culturing during the first time period is such that a gradient of growth factor from the interior to the exterior of the spheroid is formed by the cells, and the endothelial cells migrate toward the center of the spheroid as a result of the gradient.

15. The method of claim 14, wherein the culturing during the first time period is such that a distinct structure is formed by the endothelial cells in an interior region of the spheroid.

16. The method of claim 15, wherein the culturing during the second time period is such that the endothelial cells sprout from the distinct structure toward the exterior of the spheroid and mature into lumenous vessels.

17. The method of claim 10, further comprising harvesting the microtissue from the construct.

18. The method of claim 17, wherein the harvesting comprises dissolving the construct and

recovering the microtissue in solution.

19. The method of claim 10, wherein the construct is formed of alginate, agarose, or PDMS.

20. The method of claim 10, wherein the microwell has a diameter or cross-sectional width of less than 1000 μ m.

21. The method of claim 20, wherein the microwell has a diameter or cross-sectional width of 400 μ m or less, of 200 μ m or less, or of 100 μ m or less.

22. The method of claim 10, wherein the one or more growth factors include one or both of vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF).

23. The method of claim 10, wherein the culturing during the first time period is such that there is a non-homogeneous distribution of cells of the first and second type in the spheroid.

24. The method of claim 10, wherein the construct includes a plurality of microwells and the depositing and the culturing during the first and second time periods are performed for the plurality of microwells at the same time.

25. The method of claim 24, wherein the construct includes at least 1000 microwells.

26. A method of treating a patient, the method comprising:

forming a plurality of microtissues, each microtissue comprising a pre-vascularized spheroid, each spheroid comprising multiple cells with at least an endothelial cell core region; and

injecting the plurality of microtissues into a patient such that a new vascular network is formed therein with the endothelial cell core regions of the spheroids as nodes.

27. The method of claim 26, wherein the new vascular network connects to the existing vasculature of the patient.

28. The method of claim 27, wherein the connection to the existing vasculature occurs within seven days after the injecting.

29. The method of claim 26, wherein the injecting is effective to treat a condition of

ischemia.

30. The method of claim 26, wherein the injecting is performed at multiple locations spaced apart along a limb or other anatomical feature of the patient.

31. The method of claim 26, wherein the multiple cells comprise at least one of mesenchymal stem cells, smooth muscle cells, dermal fibroblasts, and adipose cells.

32. A biological tissue product prepared by a process comprising:

co-culturing therapeutic and endothelial cells in a cluster in maintenance medium depleted in vasculogenic growth factors and for a period of time that together are effective to cause the endothelial cells to concentrate in respective central regions of the cluster;

continuing the co-culturing for a period of time effective to cause the concentration of endothelial cells to form a distinct structure in said central region;

integrating said cluster in a living host or combining with other like clusters to form a biological tissue in vitro.

33. The product of claim 32, wherein the co-culturing is performed in a hydrogel well of an array of hydrogel wells.

34. The product of claim 33, wherein the process includes dissolving the hydrogel to release the cluster.

35. The product of claim 32, wherein the maintenance medium includes no vasculogenic growth factor.

36. The product of claim 35, wherein the integrating or combining includes exposing the cluster to vasculogenic growth factors.

37. The product of claim 32, wherein the integrating or combining includes exposing the cluster to vasculogenic growth factors.

38. The product of claim 36, wherein the exposing is effective to cause endothelial cells of said distinct structure to connect with external endothelial cells to form interconnecting vessels.

39. The product of claim 32, wherein the integrating includes injecting the cluster through

a cannula into a living organism.

40. The product of claim 32, wherein the integrating or combining includes combining said cluster with other like clusters to form a biological tissue in vitro and further culturing a resulting combination.

41. The product of claim 40, wherein the further culturing includes exposing the resulting combination to a vasculogenic growth factor.

42. The product of claim 32, wherein the therapeutic cells include mesenchymal stem cells.

43. The product of claim 32, wherein the product cluster can be injected through a cannula without disintegration.

44. A method for producing a biological tissue product, comprising:

co-culturing therapeutic and endothelial cells in a cluster in maintenance medium depleted in vasculogenic growth factors and for a period of time that together are effective to cause the endothelial cells to concentrate in respective central regions of the cluster;

continuing the co-culturing for a period of time effective to cause the concentration of endothelial cells to form a distinct structure principally of said endothelial cells in said central region;

integrating said cluster in a living host or combining with other like clusters to form a biological tissue in vitro.

45. The product of claim 44, wherein the co-culturing is performed in a hydrogel well of an array of hydrogel wells.

46. The product of claim 45, wherein the process includes dissolving the hydrogel to release the cluster.

47. The product of claim 44, wherein the maintenance medium includes no vasculogenic growth factor.

48. The product of claim 47, wherein the integrating or combining includes exposing the cluster to vasculogenic growth factors.

49. The product of claim 44, wherein the integrating or combining includes exposing the cluster to vasculogenic growth factors.

50. The product of claim 49, wherein the exposing is effective to cause endothelial cells of said distinct structure to connect with external endothelial cells to form interconnecting vessels.

51. The product of claim 44, wherein the integrating includes injecting the cluster through a cannula into a living organism.

52. The product of claim 44, wherein the integrating or combining includes combining said cluster with other like clusters to form a biological tissue in vitro and further culturing a resulting combination.

53. The product of claim 52, wherein the further culturing includes exposing the resulting combination to a vasculogenic growth factor.

54. The product of claim 44, wherein the therapeutic cells include mesenchymal stem cells.

55. The product of claim 44, wherein the product cluster can be injected through a cannula without disintegration.

56. A method of forming microtissue for injection, the method comprising:

depositing cells of a first and second type into a microwell of a construct, the construct or surfaces of the microwell including a material to which the deposited cells are non-adherent;

during a first time period, culturing the deposited cells in a culture media depleted in growth factors, the level of growth factors and length of the first time period being such that a spheroid with a concentration said endothelial cells is formed by the end of the first time period; and

during a second time period after the first time period, further culturing the spheroid with one or more growth factors to form the microtissue.

57. The method of claim 56, wherein the first time period is less than 4 days and the second time period is at least 6 days.

58. The method of claim 56, wherein the first cell type is endothelial cells and the second

cell type is different than the first cell type.

59. The method of claim 58, wherein the second cell type is at least one of mesenchymal stem cells, smooth muscle cells, dermal fibroblasts, and adipose cells.

60. The method of claim 58, wherein the culturing during the first time period is such that a gradient of growth factor from the interior to the exterior of the spheroid is formed by the cells, and the endothelial cells migrate toward the center of the spheroid as a result of the gradient.

61. The method of claim 60, wherein the culturing during the first time period is such that a distinguishable lumen shaped structure is formed by the endothelial cells in an interior region of the spheroid.

62. The method of claim 61, wherein the culturing during the second time period is such that the endothelial cells sprout toward the exterior of the spheroid and mature into lumenous vessels.

63. The method of claim 56, further comprising harvesting the microtissue from the construct.

64. The method of claim 63, wherein the harvesting comprises dissolving the construct and recovering the microtissue in solution.

65. The method of claim 56, wherein the construct is formed of alginate, agarose, or PDMS.

66. The method of claim 56, wherein the microwell has a diameter or cross-sectional width of less than 500 μ m.

67. The method of claim 66, wherein the microwell has a diameter or cross-sectional width of 400 μ m or less, of 200 μ m or less, or of 100 μ m or less.

68. The method of claim 56, wherein the one or more growth factors include one of vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF).

69. The method of claim 56, wherein the culturing during the first time period is such that there is a non-homogeneous distribution of cells of the first and second type in the spheroid.

70. The method of claim 56, wherein the construct includes a plurality of microwells and the depositing and the culturing during the first and second time periods are performed for the plurality of microwells at the same time.

71. The method of claim 70, wherein the construct includes at least 1000 microwells.

72. A method of forming a biological tissue, comprising:

co-culturing endothelial and therapeutic cells to form spheroids each with a size of less than 1000 microns to form a region concentrated in endothelial cells surrounded by a region depleted in endothelial cells;

ex vivo, placing multiple spheroids resulting from said co-culturing in mutual contact under conditions and for a time that permits signaling between the regions concentrated in endothelial cells to form lumens between the spheroids to form a larger vascularized construct.

73. The method of claim 72, wherein the placing for a time includes exposing the multiple spheroids to vasculogenic growth factors.

74. An injectable microtissue comprising:

a plurality of spheroids, each comprising a plurality of cells of a first cell type and a plurality of cells of a second cell type, the first cell type being endothelial cells that are concentrated in an interior region of the each spheroid;

each of the plurality of spheroids residing in a microwell formed in a soluble matrix and isolated from others of the plurality of spheroids.

75. The microtissue of claim 74, wherein the endothelial cells concentrated in an interior region define a distinct structure composed principally of said endothelial cells.

76. The microtissue of claim 74, wherein the plurality of cells of the second type form a durable shell that can withstand the shear strain and stress of injection through a 27ga (0.27 mm) or 30ga (0.159mm) cannula at rates of 0.1 to 2 mL/min with disintegration.

77. The microtissue of claim 75, wherein the endothelial cells are vascular endothelial cells and the distinct structure is a blood vessel precursor.

78. The microtissue of claim 75, wherein the endothelial cells are lymphatic endothelial cells and the distinct structure forms a lymph vessel precursor.

79. The microtissue of claim 77, wherein the second cell type is one of mesenchymal stem cells, smooth muscle cells, dermal fibroblasts, and adipose cells.

80. The microtissue of claim 74, wherein the majority of the spheroids have diameters less than 200 μ m.

81. The microtissue of claim 74, wherein the spheroid have a structural integrity capable of withstanding the shear strain and stress of injection through a 27ga (0.27 mm) or 30ga (0.159mm) cannula at rates of 0.1 to 2 mL/min without disintegration in a solution with other spheroids.

82. The microtissue of claim 75, wherein the distinct structure is located at or near the center of a respective one of said plurality of spheroids.

83. The microtissue of claim 74, wherein the soluble matrix includes a hydrogel.

84. The microtissue of claim 74, wherein the soluble matrix includes alginate.

85. An injectable microtissue comprising:

a plurality of spheroids, each comprising a plurality of cells of a first cell type and a plurality of cells of a second cell type, the first cell type being endothelial cells that are concentrated in an interior region of the each spheroid;

the spheroids having diameters of less than 200 μ m.

86. The microtissue of claim 85, wherein the plurality of spheroids is free of vascular interconnections therebetween.

87. The microtissue of claim 85, wherein the plurality of spheroids is suspended in an injectable blood-normal medicament.

88. The microtissue of claim 85, wherein the endothelial cells concentrated in an interior region define a distinct structure composed principally of said endothelial cells.

89. The microtissue of claim 85, wherein the plurality of cells of the second type form a

durable shell that can withstand the shear strain and stress of injection through a 27ga (0.27 mm) or 30ga (0.159mm) cannula at rates of 0.1 to 2 mL/min without disintegration.

90. The microtissue of claim 88, wherein the endothelial cells are vascular endothelial cells and the distinct structure is a blood vessel precursor.

91. The microtissue of claim 88, wherein the endothelial cells are lymphatic endothelial cells and the distinct structure forms a lymph vessel precursor.

92. The microtissue of claim 91, wherein the second cell type is one of mesenchymal stem cells, smooth muscle cells, dermal fibroblasts, and adipose cells.

93. The microtissue of claim 85, wherein the majority of the spheroids have diameters less than 200 μ m.

94. The microtissue of claim 85, wherein the spheroid have a structural integrity capable of withstanding the shear strain and stress of injection through a 27ga (0.27 mm) or 30ga (0.159mm) cannula at rates of 0.1 to 2 mL/min without disintegration in a solution with other spheroids.

95. The microtissue of claim 88, wherein the distinct structures are located at or near the center of a respective one of said plurality of spheroids.

96. An array of microtissue spheroids, comprising:

a plurality of spheroids, each comprising a plurality of cells of a first cell type and a plurality of cells of a second cell type, the first cell type being endothelial cells that are concentrated in an interior region of the each spheroid;

each of the plurality of spheroids being isolated from others in a respective well of an array of microwells in a substrate.

97. The microtissue of claim 96, wherein the endothelial cells concentrated in an interior region define a lumen-shaped structure.

98. The microtissue of claim 96, wherein the plurality of cells of the second type form a durable shell that can withstand the shear strain and stress of injection through a 27ga (0.27 mm)

or 30ga (0.159mm) cannula at rates of 0.1 to 2 mL/min without disintegration.

99. The microtissue of claim 97, wherein the endothelial cells are vascular endothelial cells and the lumen-shaped structure is a blood vessel precursor.

100. The microtissue of claim 97, wherein the endothelial cells are lymphatic endothelial cells and the lumen-shaped structure forms a lymph vessel precursor.

101. The microtissue of claim 100, wherein the second cell type is one of mesenchymal stem cells, smooth muscle cells, dermal fibroblasts, and adipose cells.

102. The microtissue of claim 96, wherein the spheroids have diameters less than 200 μ m.

103. The microtissue of claim 96, wherein the spheroid have a structural integrity capable of withstanding the shear strain and stress of injection through a 27ga (0.27 mm) or 30ga (0.159mm) cannula at rates of 0.1 to 2 mL/min without disintegration in a solution with other spheroids.

104. The microtissue of claim 96, wherein the lumen-shaped structures are located at or near the center of a respective one of said plurality of spheroids.

105. An injectable microtissue comprising:

a plurality of spheroids, each comprising a plurality of cells of a first cell type and a plurality of cells of a second cell type, the first cell type being endothelial cells that are concentrated in an interior region of the each spheroid;

the spheroids having diameters of less than 200 μ m and each being vascularly isolated including vascularly disconnected from the others of the spheroids and vascularly disconnected from an external host.

106. The microtissue of claim 105, wherein the plurality of spheroids is free of vascular interconnections therebetween.

107. The microtissue of claim 105, wherein the plurality of spheroids is suspended in an injectable blood-normal medicament.

108. The microtissue of claim 105, wherein the endothelial cells concentrated in an

interior region define a distinct structure.

109. The microtissue of claim 105, wherein the plurality of cells of the second type form a durable shell that can withstand the shear strain and stress of injection through a 27ga (0.27 mm) or 30ga (0.159mm) cannula at rates of 0.1 to 2 mL/min without disintegration.

110. The microtissue of claim 108, wherein the endothelial cells are vascular endothelial cells and the distinct structure is a blood vessel precursor.

111. The microtissue of claim 108, wherein the endothelial cells are lymphatic endothelial cells and the distinct structure forms a lymph vessel precursor.

112. The microtissue of claim 111, wherein the second cell type is one of mesenchymal stem cells, smooth muscle cells, dermal fibroblasts, and adipose cells.

113. The microtissue of claim 105, wherein the majority of the spheroids have diameters less than 200 μ m.

114. The microtissue of claim 105, wherein the spheroid have a structural integrity capable of withstanding the shear strain and stress of injection through a 27ga (0.27 mm) or 30ga (0.159mm) cannula at rates of 0.1 to 2 mL/min without disintegration in a solution with other spheroids.

115. The microtissue of claim 105, wherein the distinct structures are located at or near the center of a respective one of said plurality of spheroids.

116. A method of growing a tissue structure in a living host, comprising:
co-culturing cells of multiple types in respective microwells, including controlling a composition of a nutrient medium such that the cells in each microwell organizes into a respective cluster responsively to growth factors, that naturally arise from the cells, into spheroids with a first cell type concentrated in the center and depleted at a periphery of the respective cluster;

the co-culturing including culturing for a period of days;

releasing the clusters from the microwells and combining in a solution;

injecting the solution, containing the clusters, into a living host.

117. The method of claim 116, wherein the releasing includes dissolving a matrix in which the microwells are formed.

118. The method of claim 116, wherein the first cell type is endothelial.

119. The method of claim 116, wherein the multiple types include stem type cells.

120. The method of claim 116, wherein the multiple types include therapeutic cells.

121. The method of claim 116, wherein the co-cultivating includes controlling a composition of a nutrient medium at a first time to include no vasculogenic growth factors and at a second time, controlling a composition of a nutrient medium to include vasculogenic growth factors, wherein the method includes exposing the clusters to the resulting mediums during the respective first and second times.

122. A method of forming a tissue construct, comprising:

creating multiple tissue clusters each with a mixture of endothelial and therapeutic cells organized such that when the tissue clusters are placed together in a culturing medium, the therapeutic cells separate from the endothelial cells, the latter being concentrated in respective nodes at the center of each cluster;

placing the tissue clusters together in a vasculogenic medium to induce the formation of vascular tissue connections between the endothelial cell nodes;

culturing for a time sufficient to generate vascular branches interconnecting a substantial number of the nodes, whereby a vascularized tissue construct is formed;

implanting the vascularized tissue construct in a living host.

123. The method of claim 122, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 1:1 through 1:2.

124. The method of claim 122, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 1:1 through 1:3.

125. The method of claim 122, wherein the ratio of endothelial cells (EC) to therapeutic

cells (TC), (EC:TC), is in the range of 1:1 through 2:3.

126. The method of claim 122, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 1:2 through 2:1.

127. The method of claim 122, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 2:3 through 3:1.

128. The method of claim 122, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is about 1:1.

129. The method of claim 122, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is about 1:2.

130. The method of claim 122, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is about 1:3.

131. A method of forming a tissue construct, comprising:

creating multiple tissue clusters each with a mixture of endothelial and therapeutic cells organized such that when the tissue clusters are placed together in a culturing medium, the therapeutic cells separate from the endothelial cells, the latter being concentrated in respective nodes at the center of each cluster;

the creating including culturing tissue clusters in a medium having a first concentration of vasculogenic growth factors for a first time and, after the first time, culturing the tissue clusters in a medium having a second concentration of vasculogenic growth factors for a second time, where the second concentration is greater than the first;

implanting or injecting the clusters in a living animal.

132. The method of claim 131, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 1:1 through 1:2.

133. The method of claim 131, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 1:1 through 1:3.

134. The method of claim 131, wherein the ratio of endothelial cells (EC) to therapeutic

cells (TC), (EC:TC), is in the range of 1:1 through 2:3.

135. The method of claim 131, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 1:2 through 2:1.

136. The method of claim 131, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 2:3 through 3:1.

137. The method of claim 131, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is about 1:1.

138. The method of claim 131, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is about 1:2.

139. The method of claim 131, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is about 1:3.

140. The method, product, or microtissue of any of the foregoing claims, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 1:1 through 1:2.

141. The method, product, or microtissue of any of the foregoing claims, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 1:1 through 1:3.

142. The method, product, or microtissue of any of the foregoing claims, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 1:1 through 2:3.

143. The method, product, or microtissue of any of the foregoing claims, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 1:2 through 2:1.

144. The method, product, or microtissue of any of the foregoing claims, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is in the range of 2:3 through 3:1.

145. The method, product, or microtissue of any of the foregoing claims, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is about 1:1.

146. The method, product, or microtissue of any of the foregoing claims, wherein the ratio of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is about 1:2.

147. The method, product, or microtissue of any of the foregoing claims, wherein the ratio

of endothelial cells (EC) to therapeutic cells (TC), (EC:TC), is about 1:3.

148. The method of any of the foregoing claims, wherein said therapeutic cells include stem cells.

149. The method of any of the foregoing claims, wherein said therapeutic cells are cells selected responsively to a predefined disease or pathology of a host.

150. A method as in any of the foregoing methods in which spheroids or clusters (the terms being used interchangeably herein) are injected in a suspension thereof, wherein the suspending fluid includes a buffered saline.

151. A method as in any of the foregoing methods in which spheroids or clusters (the terms being used interchangeably herein) are injected in a suspension thereof, wherein the suspending fluid includes a phosphate-buffered saline.

152. A method of creating a therapeutic tissue for injection, comprising:

in vitro, in 200 micron diameter or less wells, culturing endothelial cells and therapeutic cells forming cell clusters in a medium depleted in vasculogenic growth factor for a first period of time to induce a sorting of the endothelial cells in which the endothelial cells concentrate in a region near a center of the each cell cluster;

after the first period of time, further culturing the cell clusters in a medium with an increased concentration of vasculogenic growth factor for a second period of time to induce the formation of branching structures extending from the regions, the branching structures comprising the endothelial cells;

removing the clusters from the wells after at least the first period of time; and

adding the clusters to an injectable solution to form a suspension.

153. The method of claim 152, wherein the solution is a buffered saline.

154. The method of claim 152, further comprising centrifuging or otherwise mechanically concentrating the clusters in a medium containing the clusters to produce a supernatant, the adding including adding the supernatant to said injectable solution.

155. The method of claim 152, wherein the removing the clusters from the wells after at least the first period of time is removing the clusters from the wells after the second period of time.

156. The method of claim 152, wherein the therapeutic cells include stem cells.

157. The method of claim 152, wherein the ratio of therapeutic cells to endothelial cells is greater than unity.

158. The method of claim 152, wherein the ratio of therapeutic cells to endothelial cells is greater than 1.5.

159. The method of claim 152, wherein the ratio of therapeutic cells to endothelial cells is greater than 2.

160. The method of claim 152, wherein the ratio of therapeutic cells to endothelial cells is 3.

161. The method of claim 152, wherein the first period of time is between 2 and 5 days.

162. The method of claim 152, wherein the sum of the first and second periods of time is less than 6 days.

163. The method of claim 152, wherein the sum of the first and second periods of time is less than 7 days.

164. The method of claim 152, wherein the sum of the first and second periods of time is less than 8 days.

165. The method of claim 152, wherein the sum of the first and second periods of time is between 6 and 12 days.

166. The method of claim 152, wherein the first time period is less than 4 days and the second time period is at least 6 days.

167. A method of creating a therapeutic tissue for implantation, comprising:
in vitro, in 200 micron diameter or less wells, culturing endothelial cells and therapeutic cells forming cell clusters in a medium depleted in vasculogenic growth factor for a first period

of time to induce a sorting of the endothelial cells in which the endothelial cells concentrate in a region near a center of the each cell cluster;

after the first period of time, further culturing cell clusters in a medium with an increased concentration of vasculogenic growth factor for a second period of time to induce the formation of branching structures extending from the regions, the branching structures comprising the endothelial cells;

removing the clusters from the wells after at least the first period of time; and

combining the clusters and further culturing a resulting combination to cause fusion and vascular interconnection between the clusters prior to implantation in a living host.

168. The method of claim 167, further comprising centrifuging or otherwise mechanically concentrating the clusters in a medium containing the clusters to produce a supernatant, the adding including adding the supernatant to a culture medium to perform said further culturing.

169. The method of claim 167, wherein the removing the clusters from the wells after at least the first period of time is removing the clusters from the wells after the second period of time.

170. The method of claim 167, wherein the therapeutic cells include stem cells.

171. The method of claim 167, wherein the ratio of therapeutic cells to endothelial cells is greater than unity.

172. The method of claim 167, wherein the ratio of therapeutic cells to endothelial cells is greater than 1.5.

173. The method of claim 167, wherein the ratio of therapeutic cells to endothelial cells is greater than 2.

174. The method of claim 167, wherein the ratio of therapeutic cells to endothelial cells is 3.

175. The method of claim 167, wherein the first period of time is between 2 and 5 days.

176. The method of claim 167, wherein the sum of the first and second periods of time is

less than 6 days.

177. The method of claim 167, wherein the sum of the first and second periods of time is less than 7 days.

178. The method of claim 167, wherein the sum of the first and second periods of time is less than 8 days.

179. The method of claim 167, wherein the sum of the first and second periods of time is between 6 and 12 days.

180. The method of claim 167, wherein the first time period is less than 4 days and the second time period is at least 6 days.

181. A method, microtissue, or product according to any of the preceding independent claims where each includes all combinations or subcombinations of the limitations of their respective dependent claims not otherwise explicitly claimed.

182. A therapeutic method employing any of the foregoing products, microtissues, or methods, in which the therapeutic cells and/or endothelial cells are previously harvested from a host and then implanted and injected in the same host.

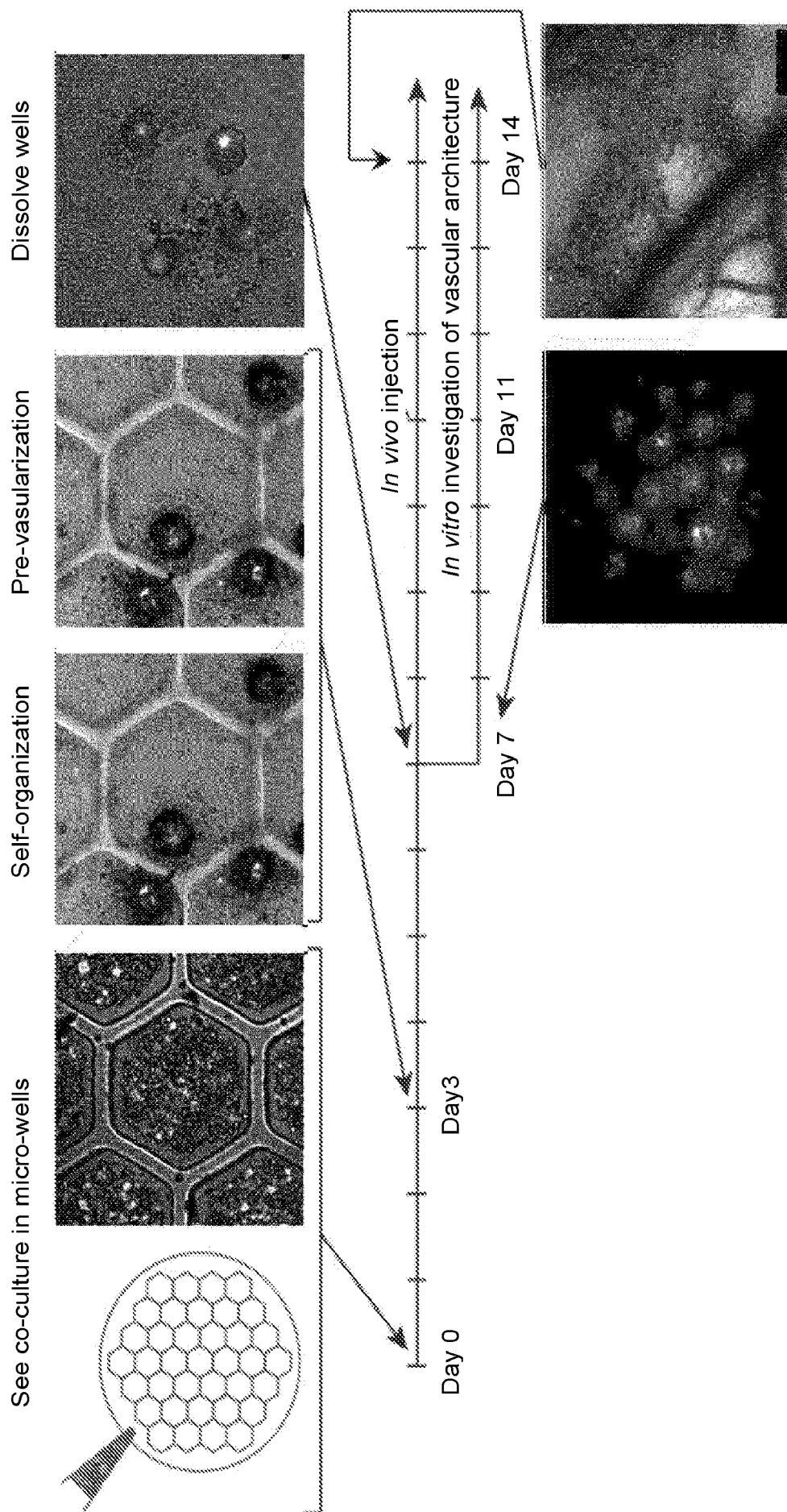
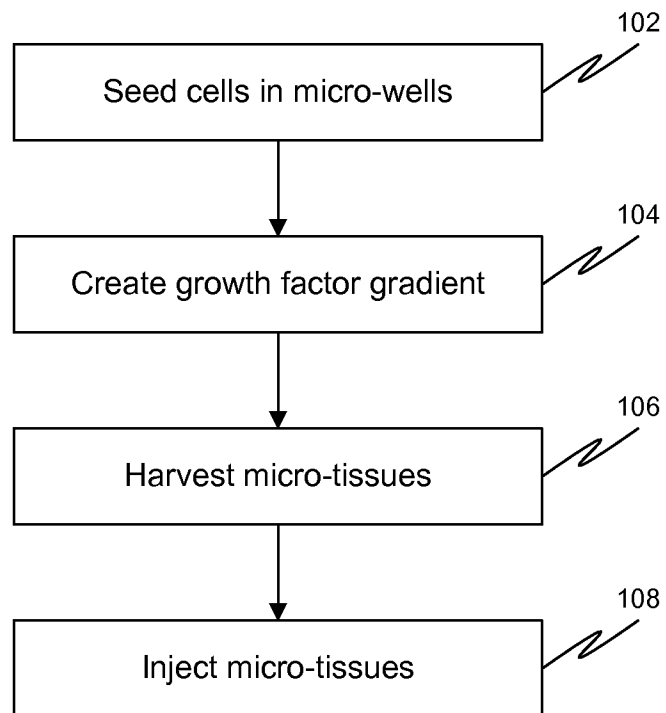


FIG. 1

**FIG. 2**

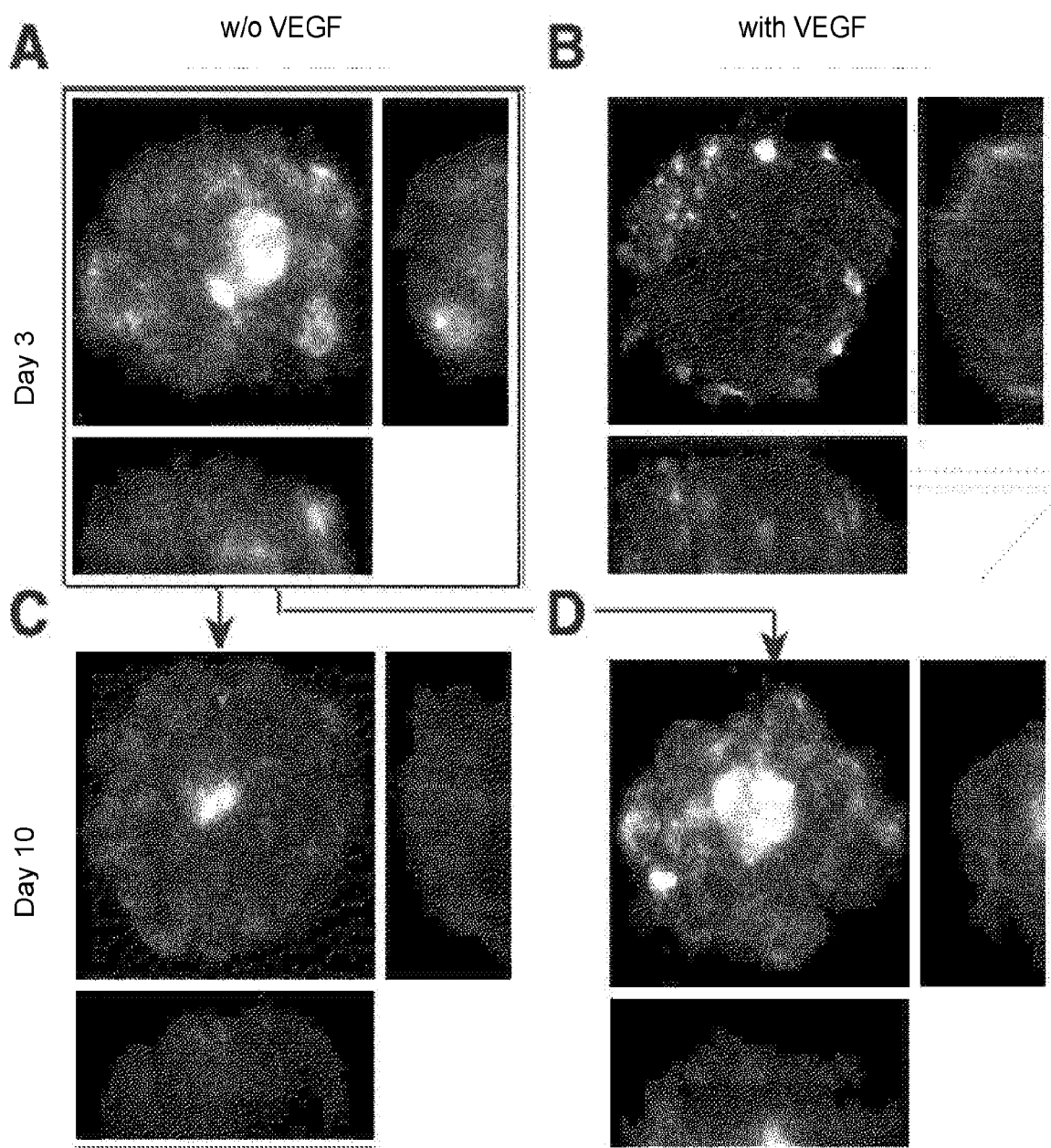


FIG. 3

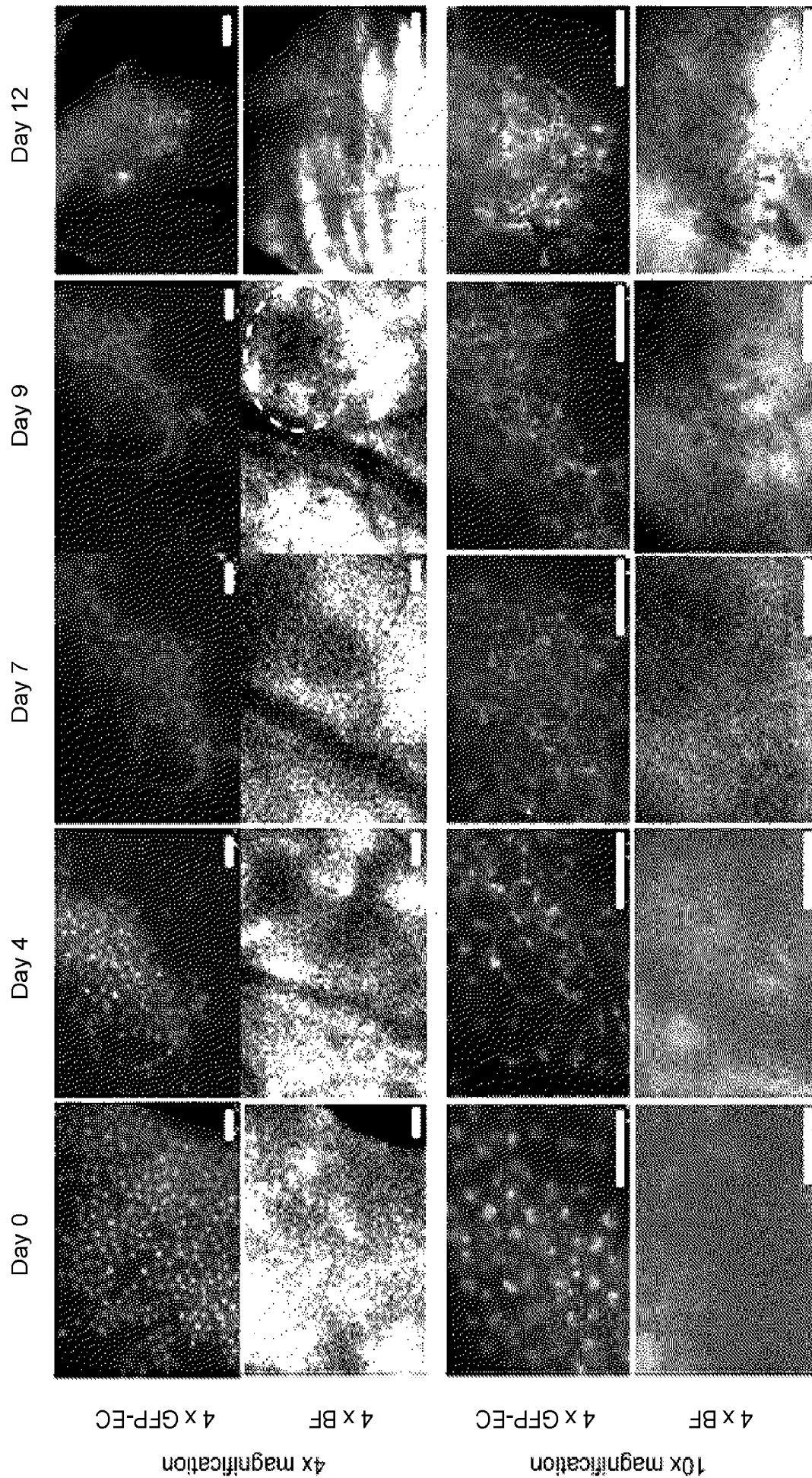


FIG. 4

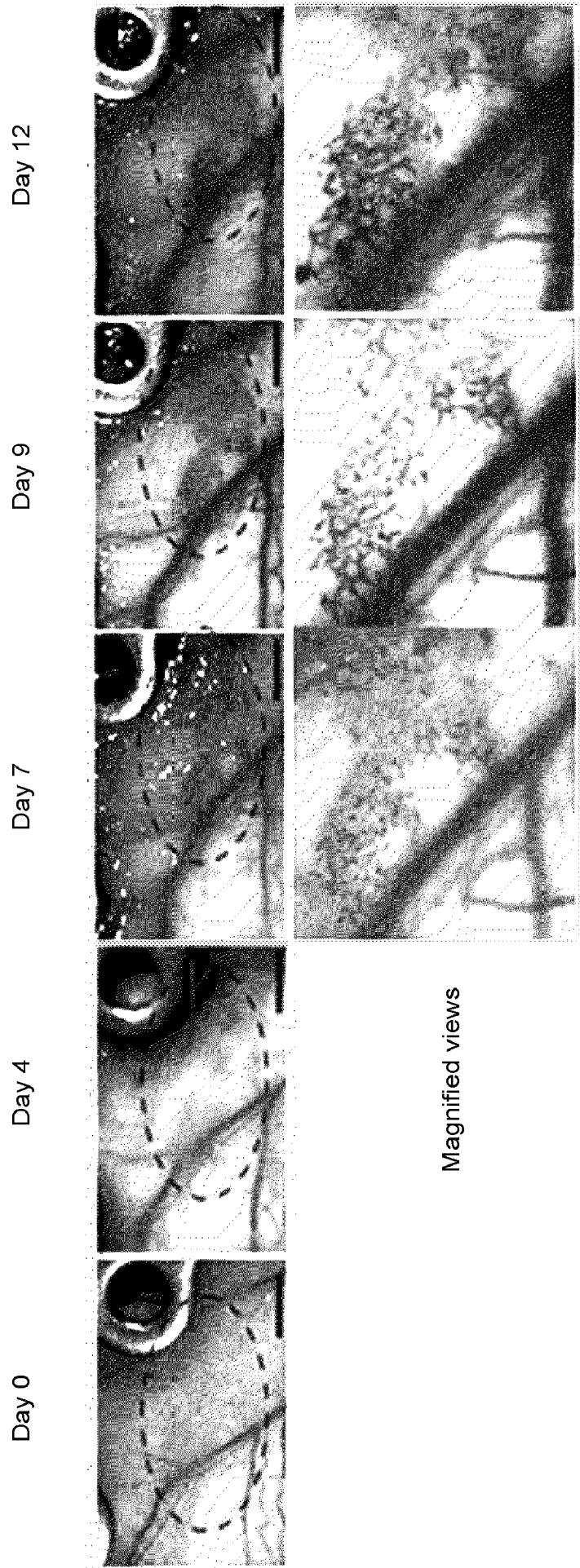


FIG. 5

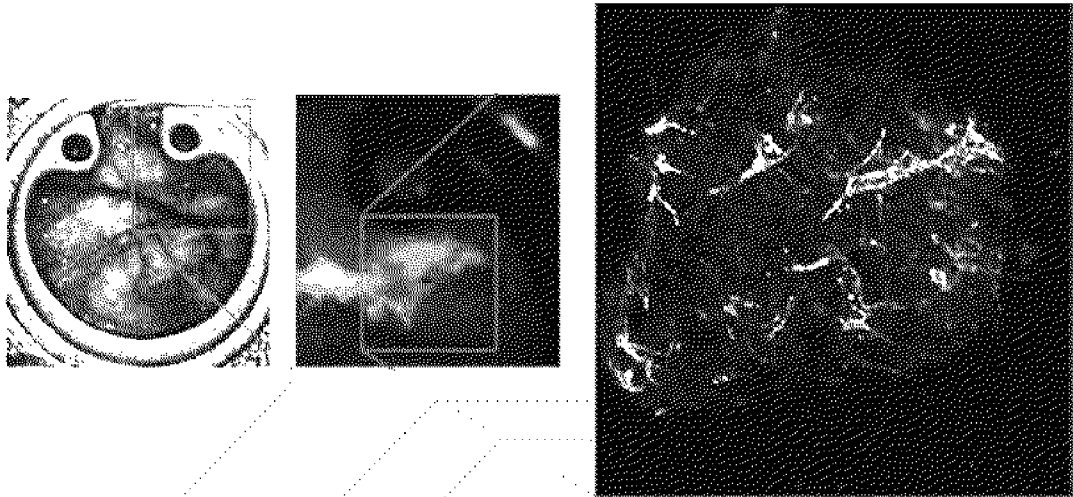


FIG. 6

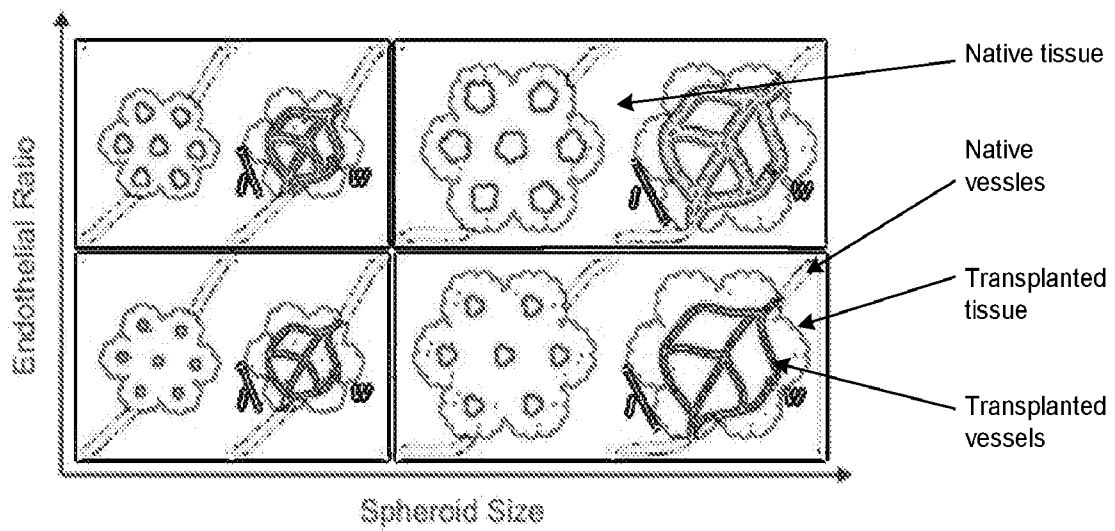
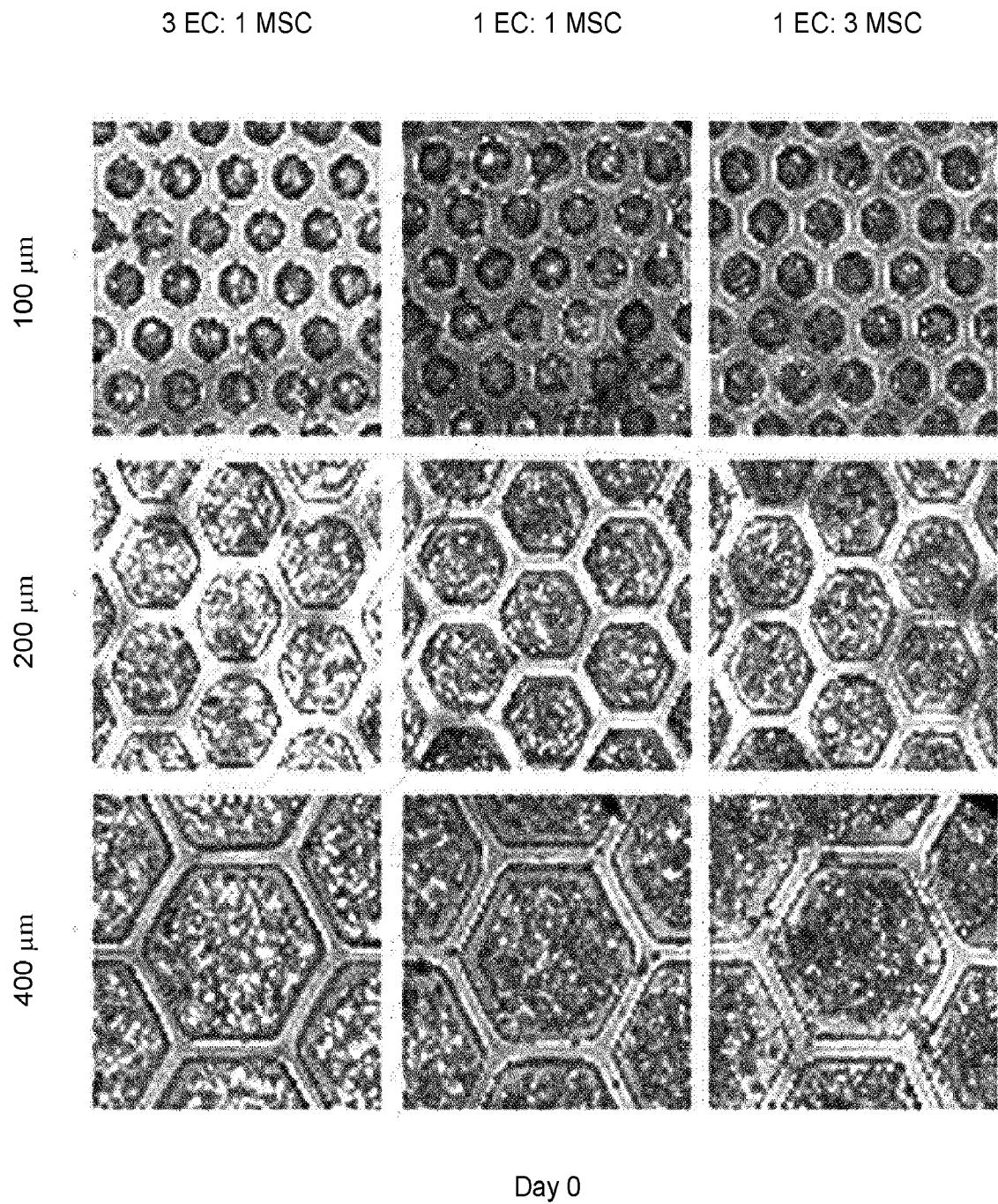
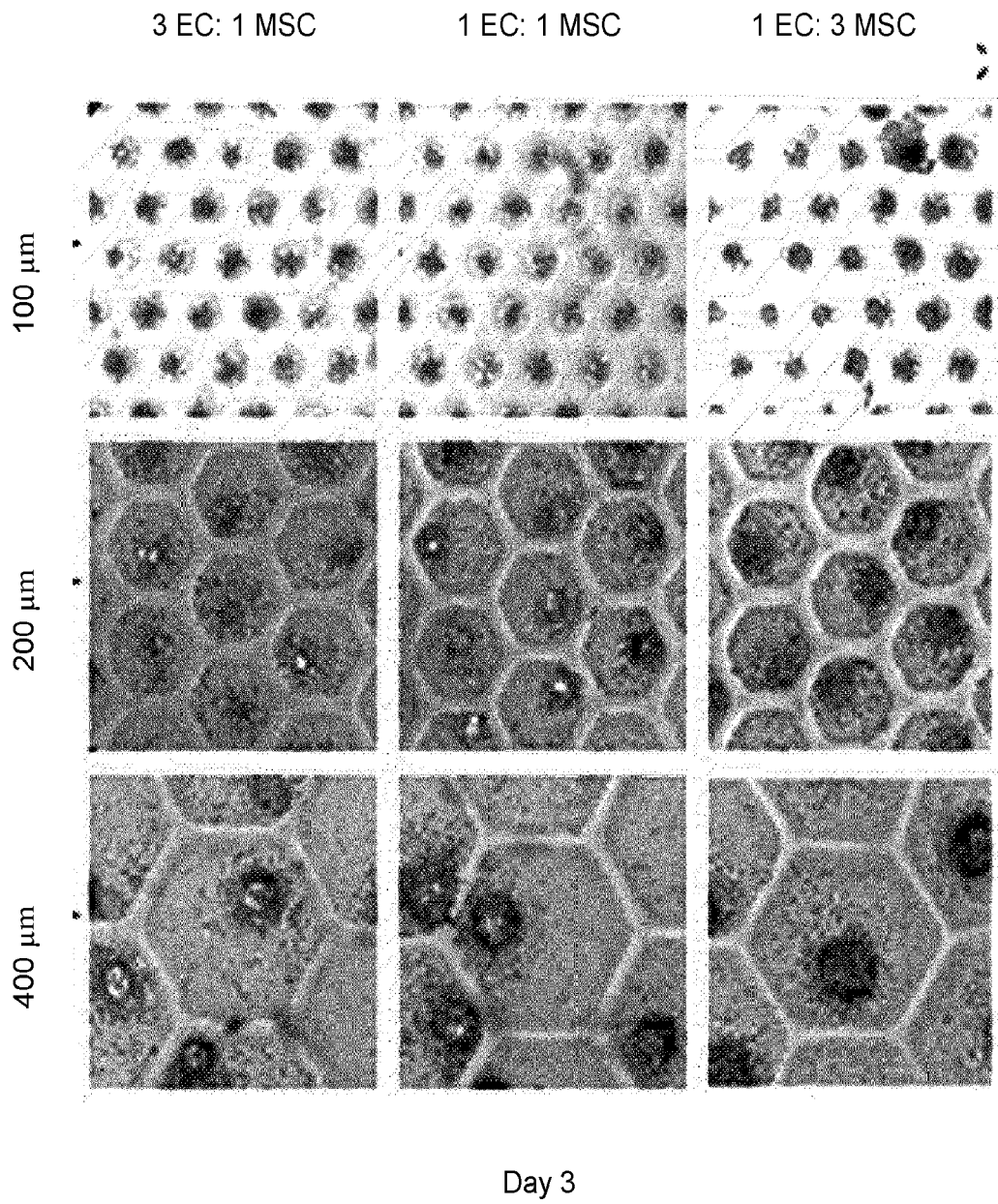
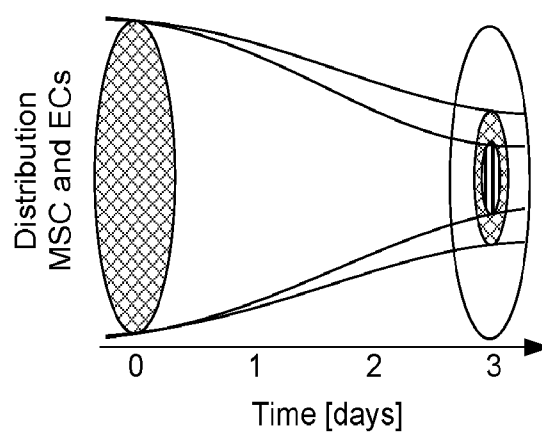
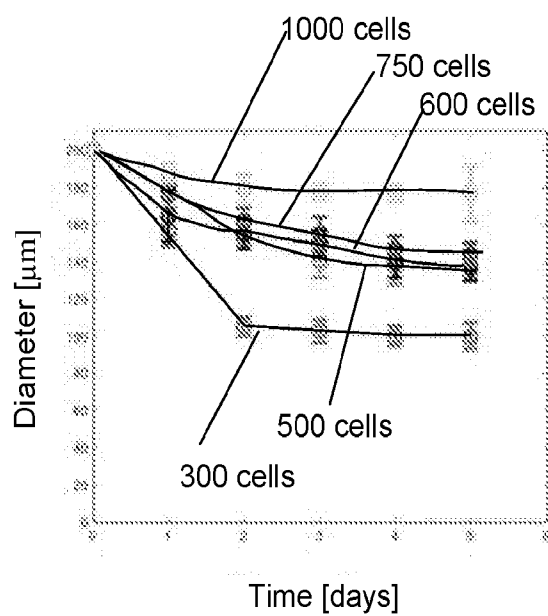
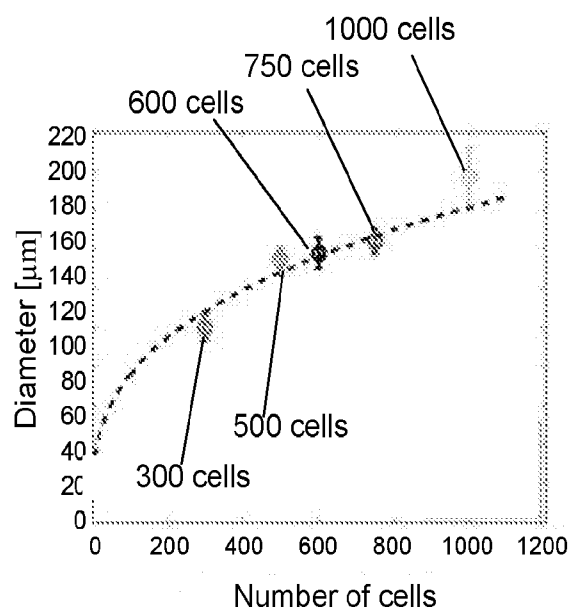


FIG. 7

**FIG. 8**

**FIG. 9**

**FIG. 10****FIG. 11****FIG. 12**

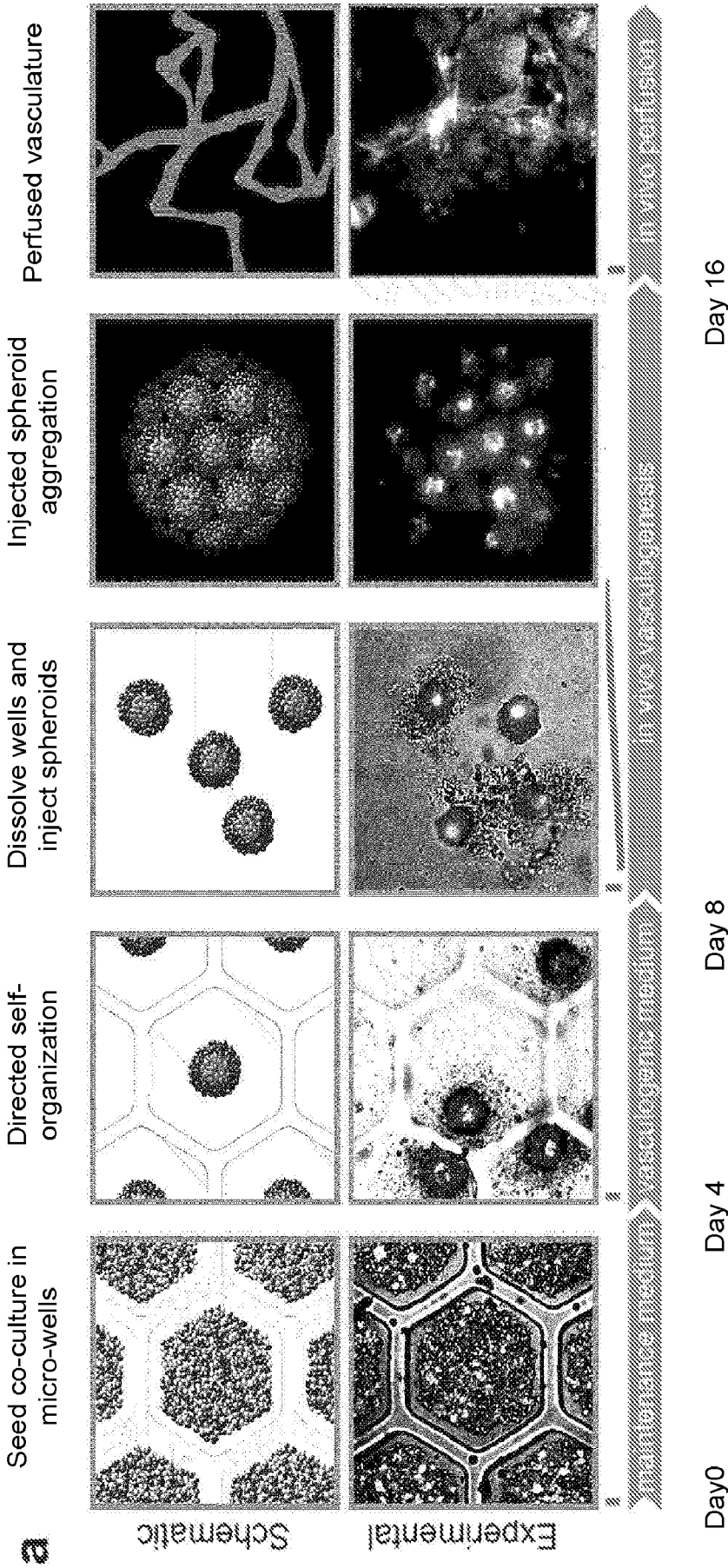


FIG. 13

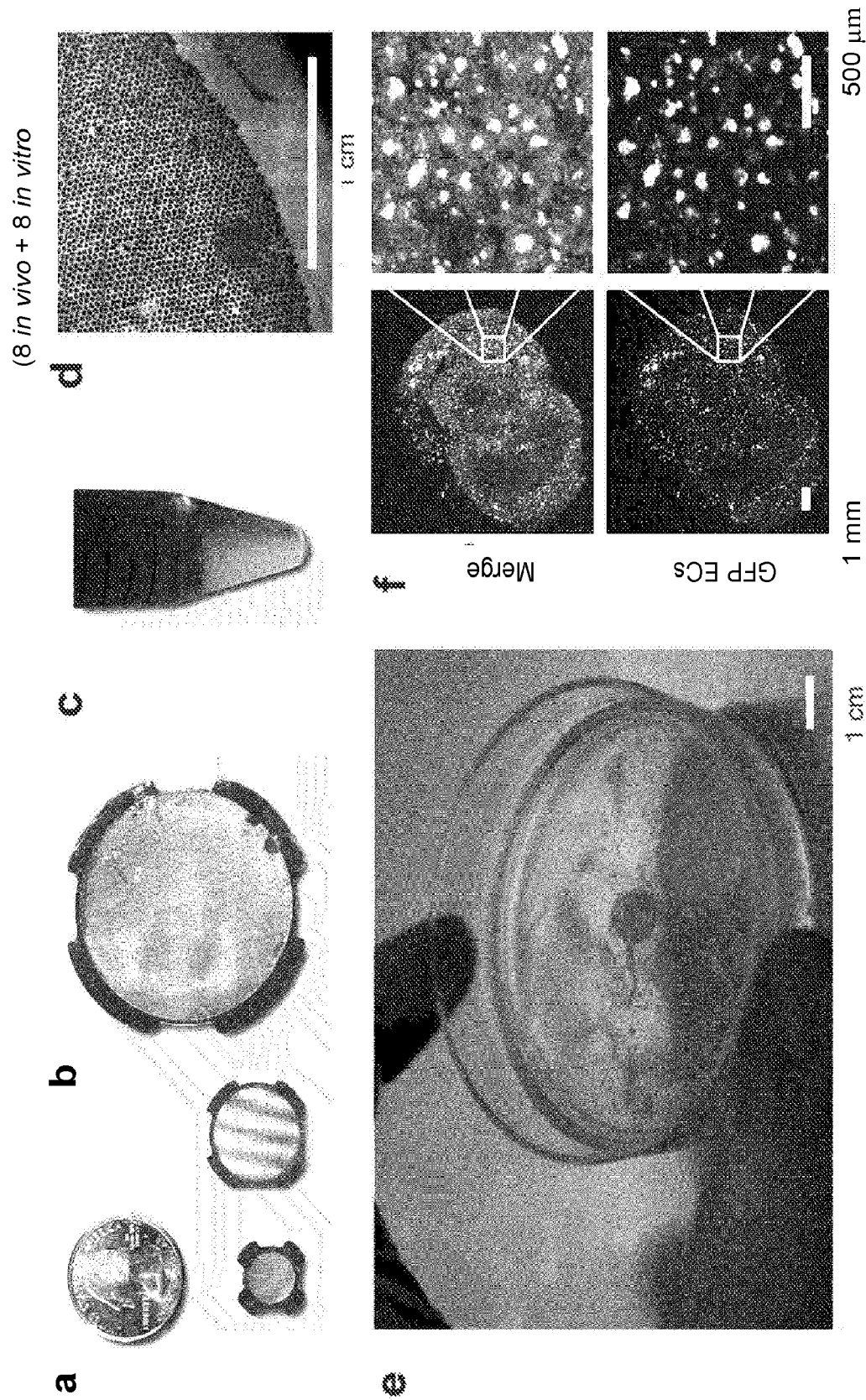
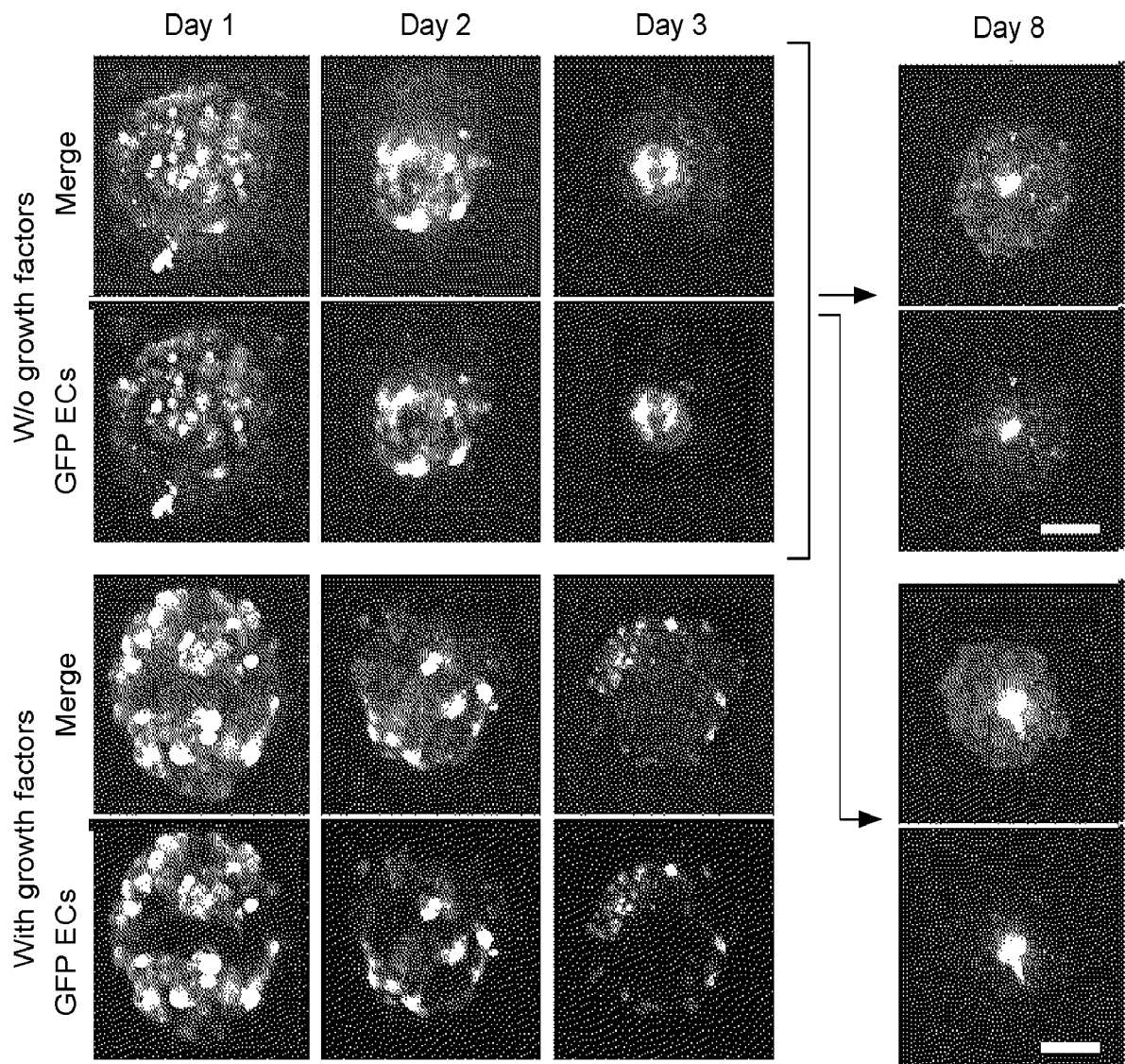
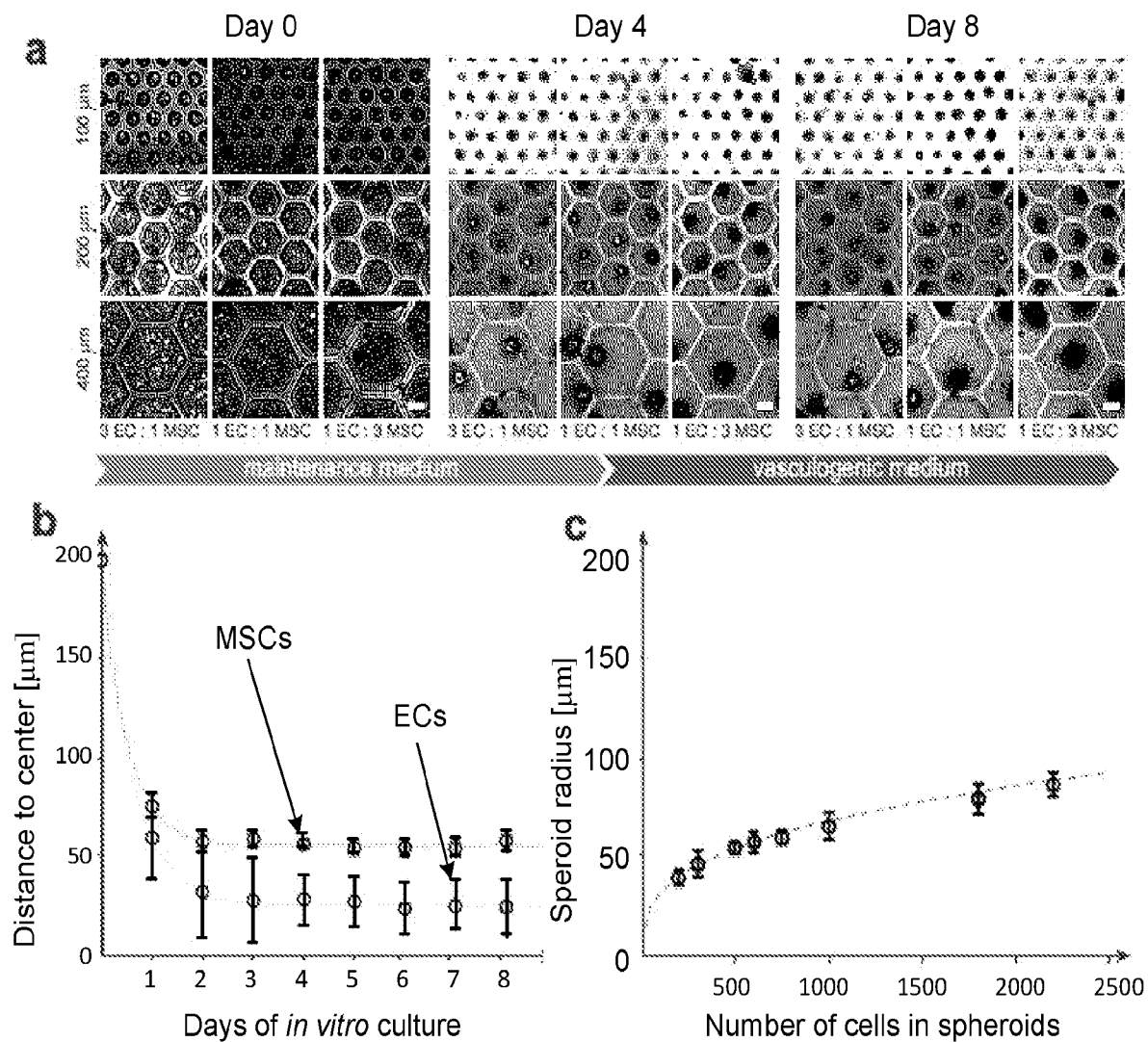


FIG. 14

**FIG. 15**

**FIG. 16**

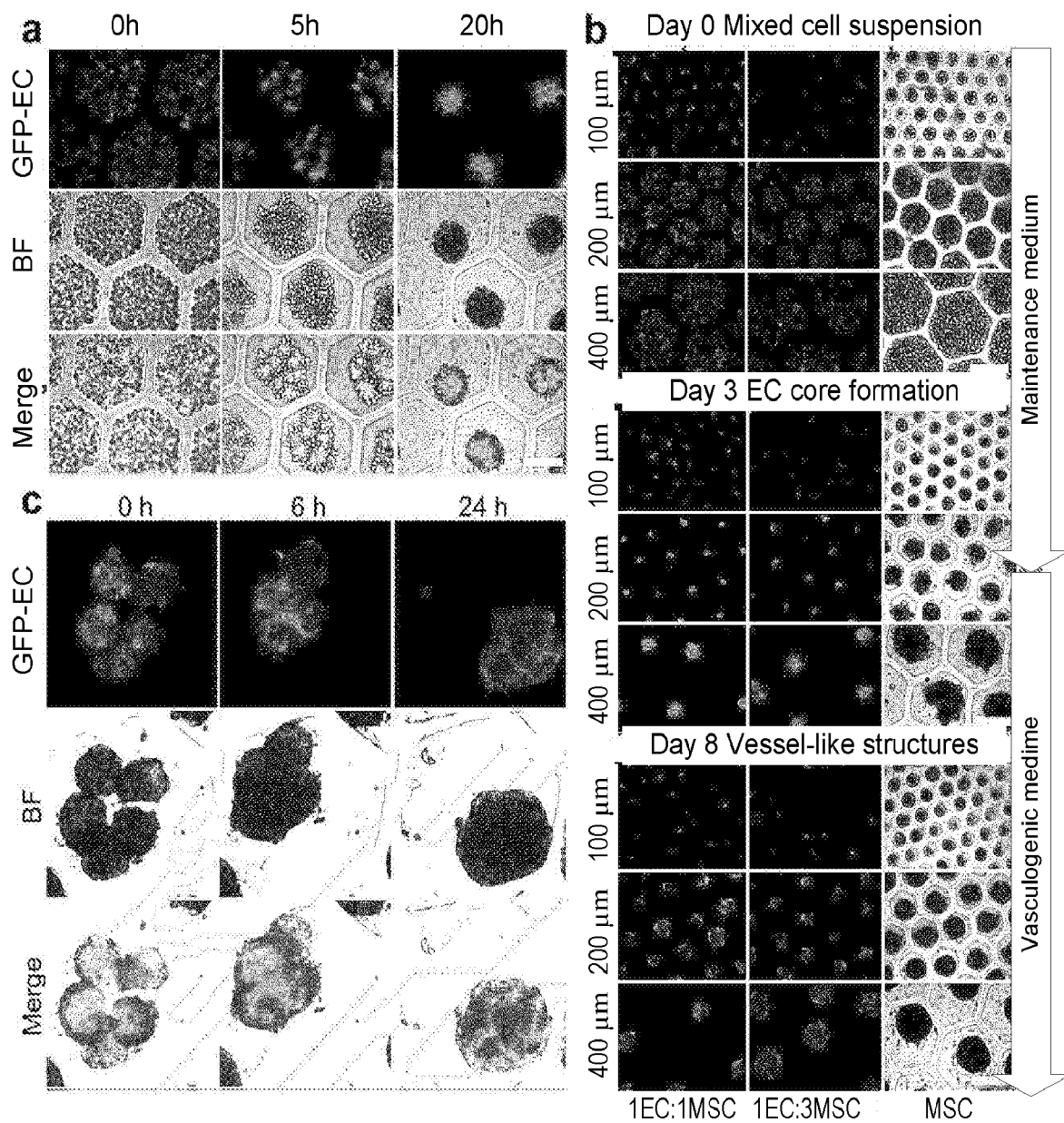
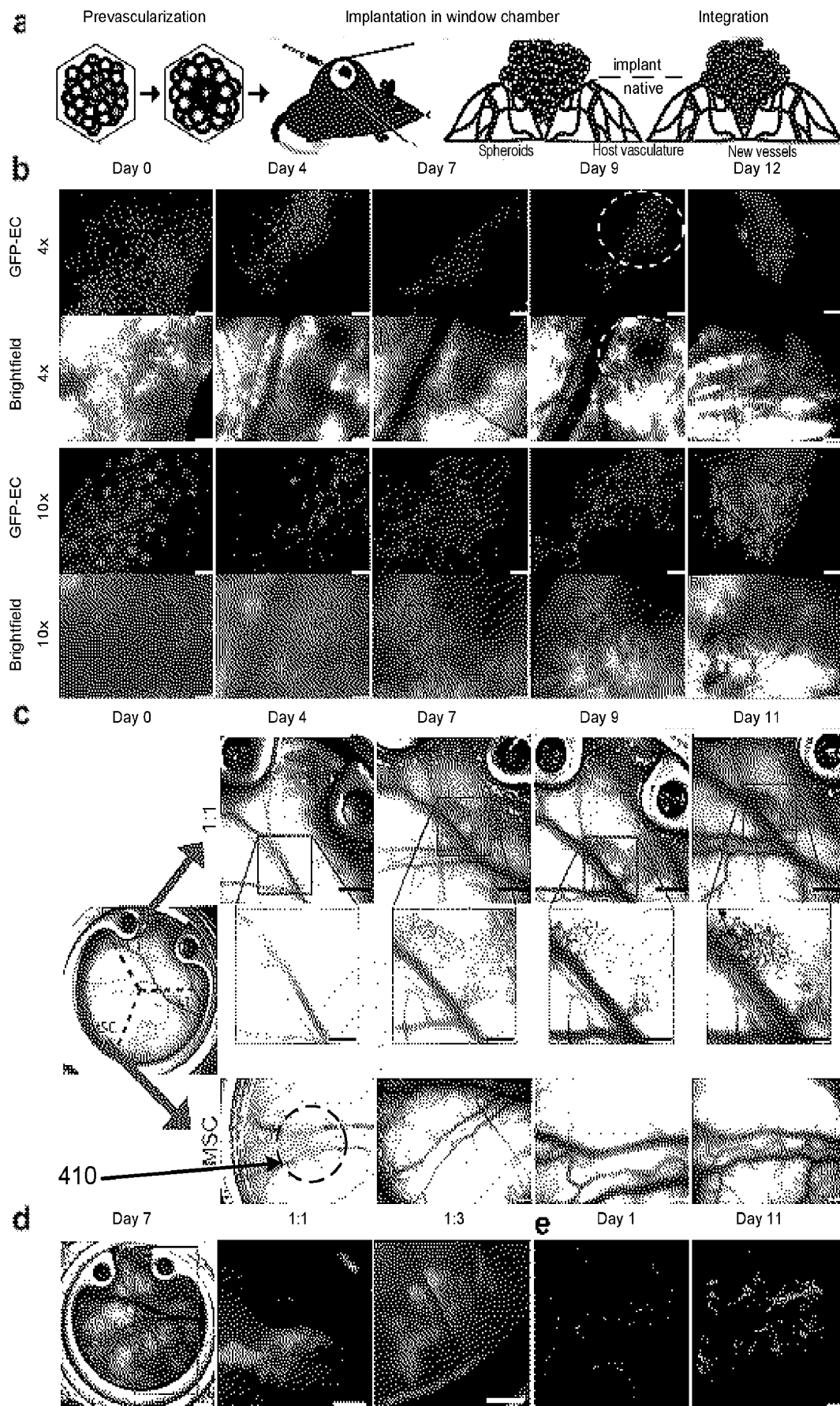
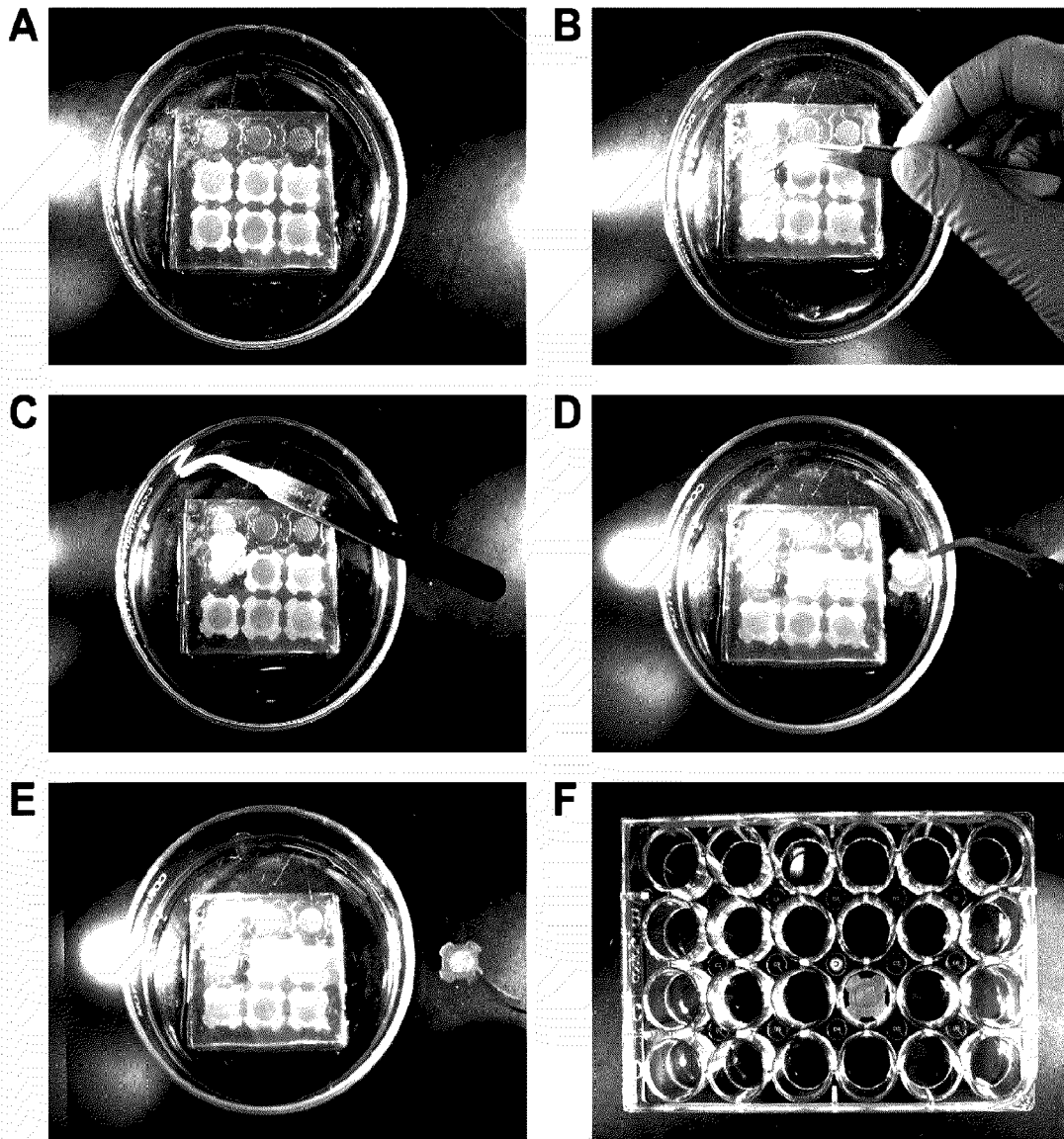


FIG. 17

**FIG. 18**

**FIG. 19**

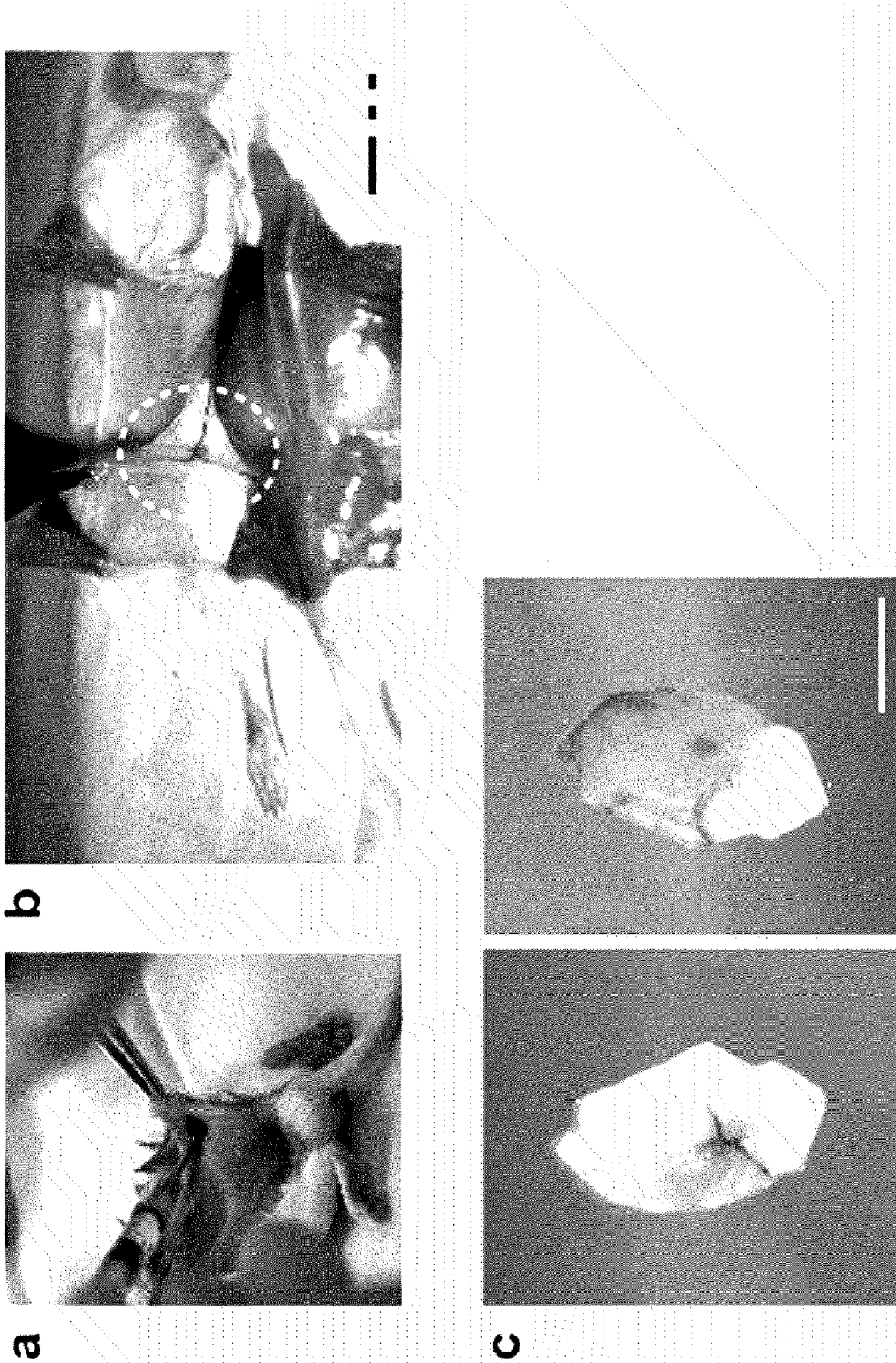


FIG. 20 (a-c)

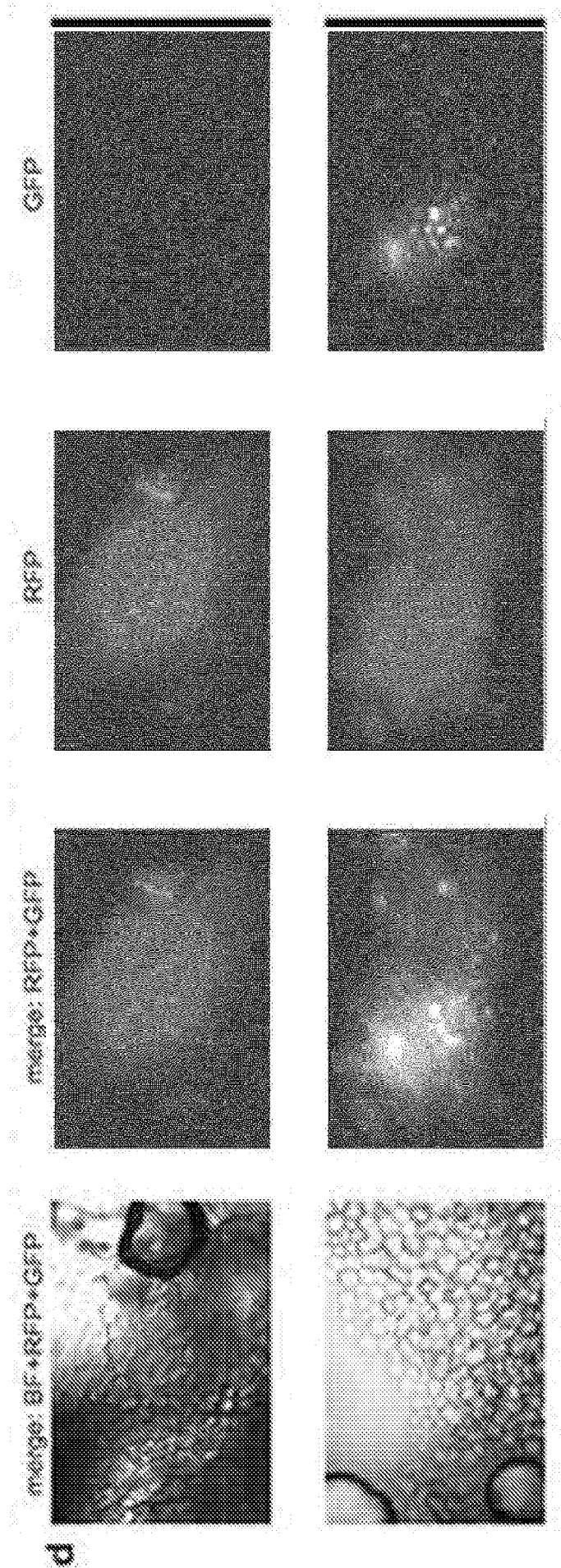
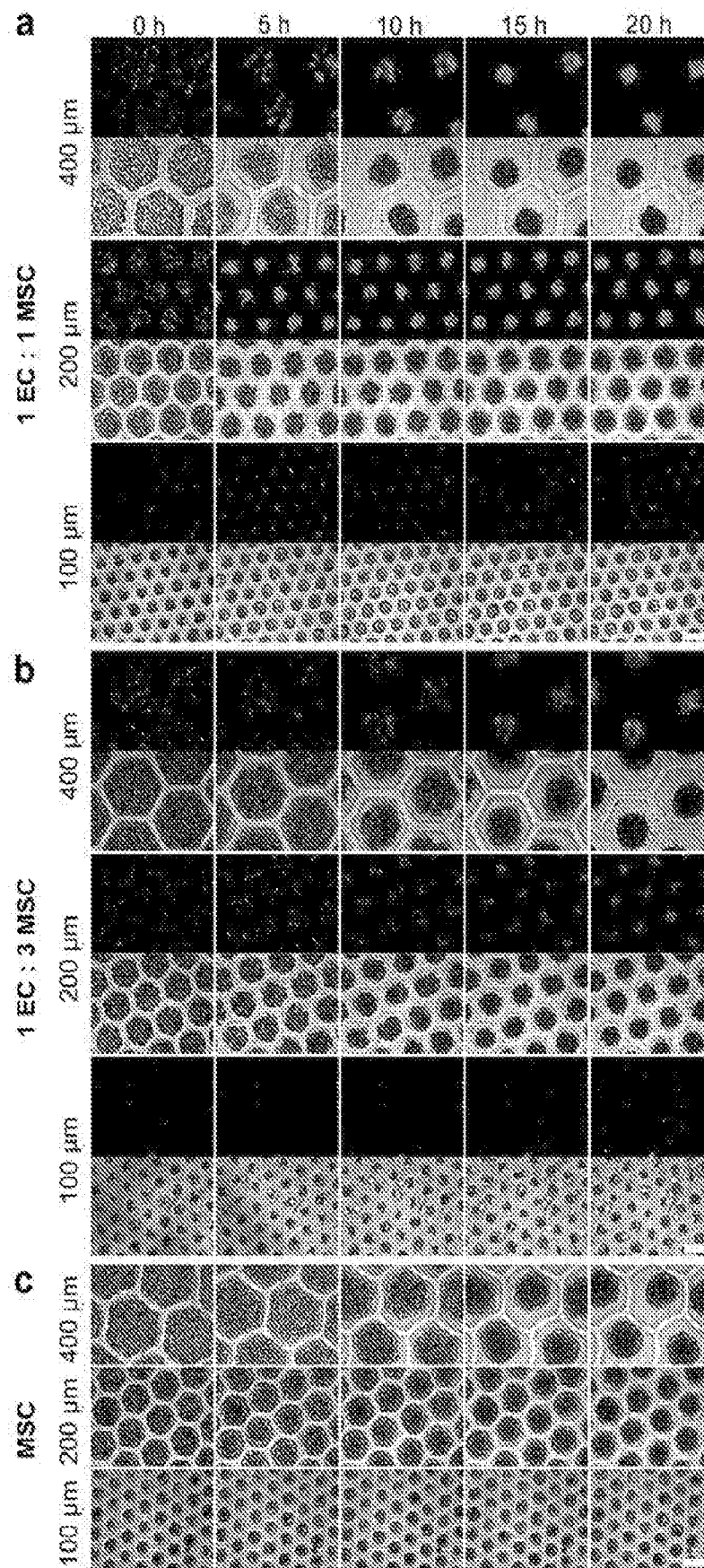


FIG. 20 (d)

**FIG. 21**

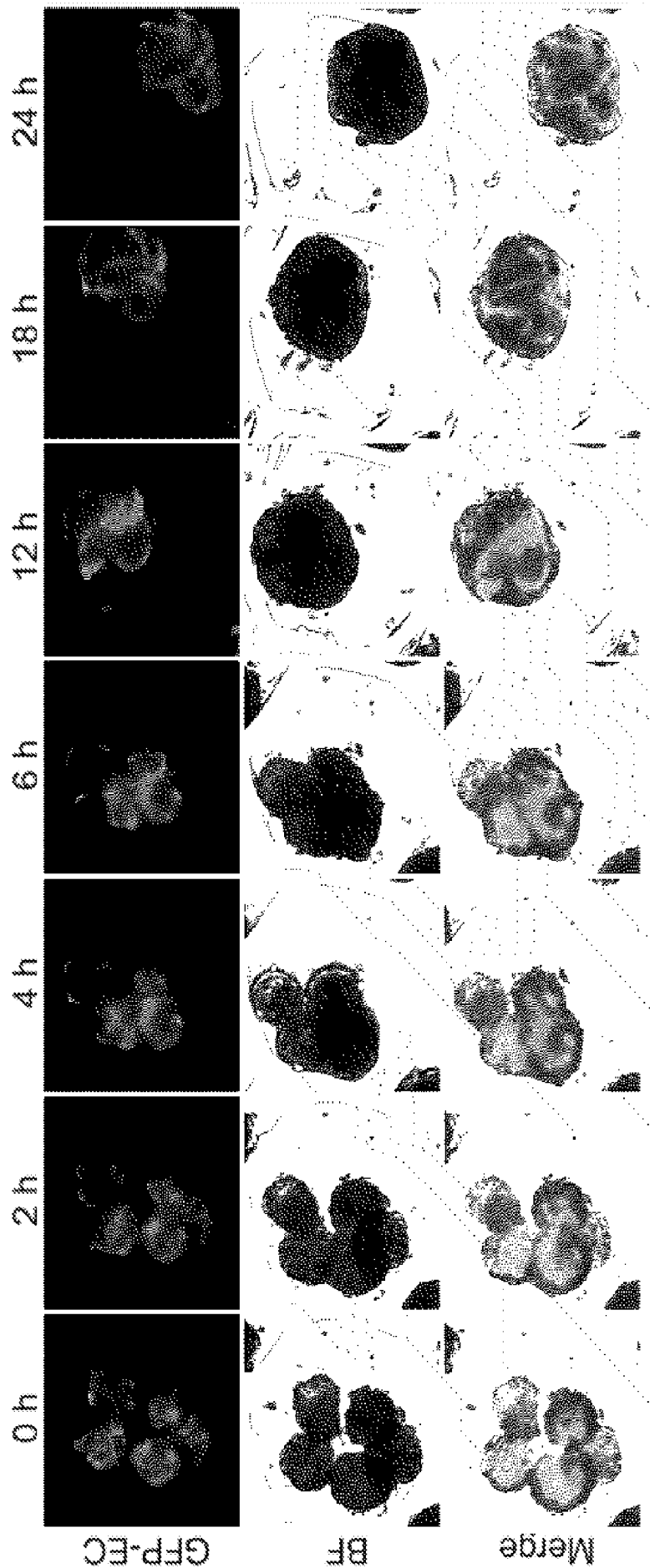
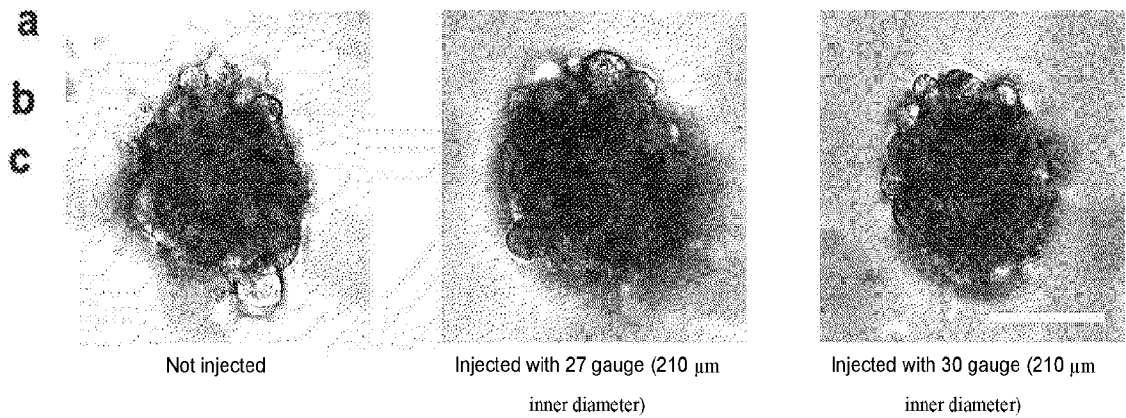
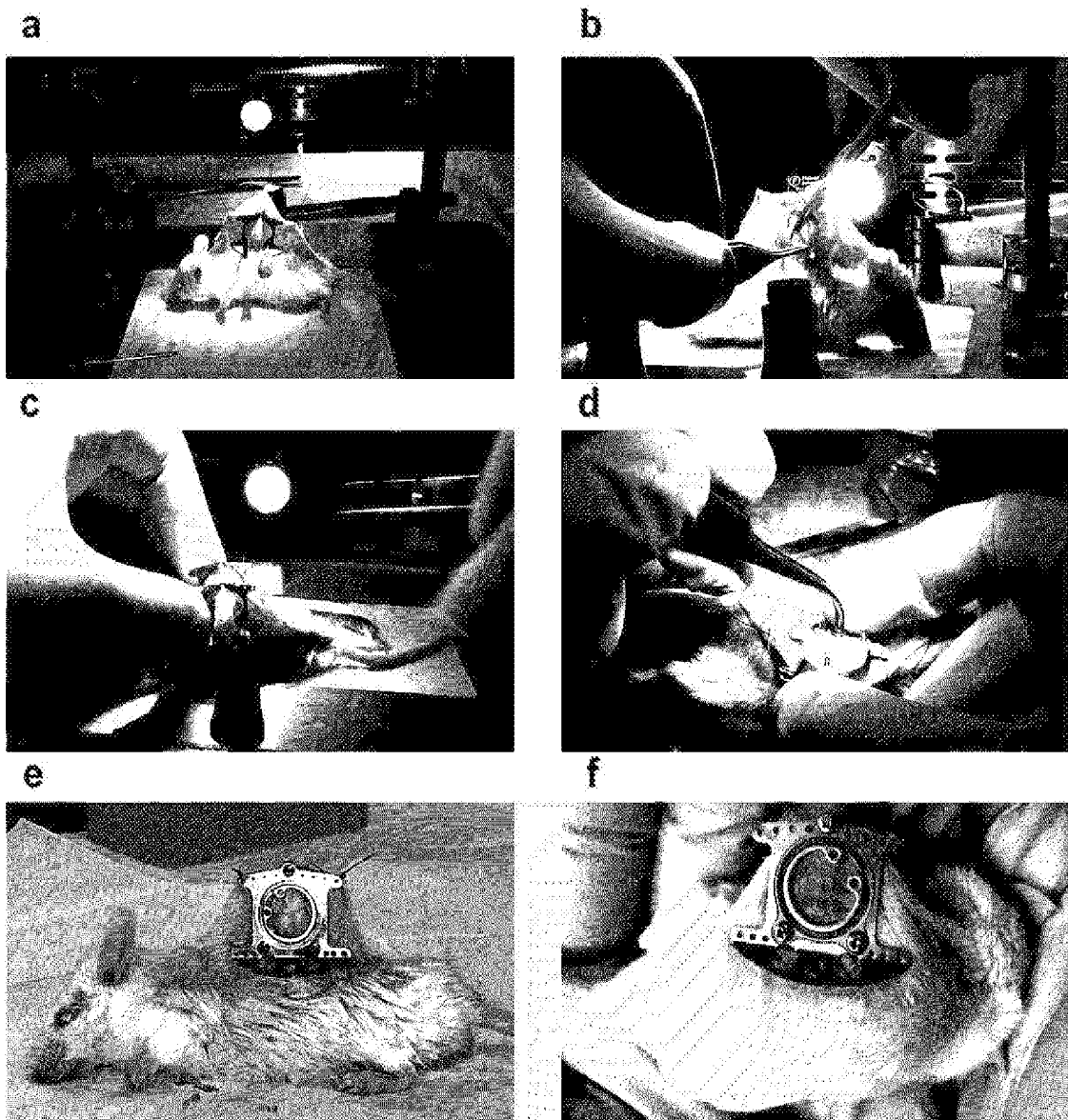


FIG. 22

**FIG. 23****FIG. 24**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US15/63266

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61P 3/04; C12N 5/074, 5/0775 (2016.01)

CPC - A61K 35/44, 35/35; C12N 5/0653

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): A61P 3/04; C12N 5/02, 5/074, 5/0775 (2016.01)

CPC: A61K 35/12, 35/44, 35/35; C12N 5/0653, 2502/28, 2506/1384, 2513/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); EBSCO Discovery; Google/Google Scholar; Pubmed

KEYWORDS: inject, microtissue, spheroid, endothelial, cell, vascular, tissue, 'second cell', 'blood vessels', form

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	WO 2014/026201 A1 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) February 13, 2014; abstract; paragraphs [0007]- [0011], [0017], [0018], [0021], [0022], [0026], [0039], [0061], [0078], [0105], [0111], [0114], [0115], [0120]; figures 1, 3	1, 2, 4, 6, 7, 9-31, 74, 75, 77, 79, 80, 82-88, 90, 92, 93, 95, 105-108, 110, 112, 113, 115, 140/1-2, 140/4, 140/6-7, 140/9-31, 140/74-75, 140/77, 140/79-80, 140/82-88, 140/90-95, 140/105-108, 140/110, 140/112-113, 140/115 --- 3, 5, 8, 76, 78, 81, 89, 91, 94, 109, 111, 114, 140/3, 140/5, 140/8, 140/76, 140/78, 140/81, 140/89, 140/91, 140/94, 140/109, 140/111, 140/114
Y	US 2014/0369970 A1 (CYTORI THERAPEUTICS, INC.) December 18, 2014; abstract; paragraph [0242]	5, 78, 91, 111, 140/5, 140/78, 140/91, 140/111
Y	US 2008/0063716 A1 (MORO, D et al.) March 13, 2008; abstract; paragraphs [0109], [0114], [0198]	3, 8, 76, 81, 89, 94, 109, 114, 140/3, 140/8, 140/76, 140/81, 140/89, 140/94, 140/109, 140/114

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

14 March 2016 (14.03.2016)

Date of mailing of the international search report

29 MAR 2016

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US15/63266

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2007/0116674 A1 (CASTEILLA, L et al.) May 24, 2007; entire document	1-31, 74-95, 105-115, 140/1-31, 140/74-95, 140/105-115
A	US 8,173,119 B2 (TAMARAT, R et al.) May 8, 2012; entire document	1-31, 74-95, 105-115, 140/1-31, 140/74-95, 140/105-115

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US15/63266

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 141-151, 181, 182
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

-Please See Supplemental Page-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-31, 74-95, 105-115, 140(in-part)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US15/63266

---Continued from Box No. III: Observations where unity of invention is lacking---

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-31, 74-95, 105-115 and 140 (in-part) are directed toward an injectable microtissue; a method of forming said microtissue, and a method of treating a patient comprising injecting said microtissue.

Group II: Claims 32-73 122-139, 140 (in-part) and 152-180 are directed toward a biological tissue product prepared by a process comprising: co-culturing therapeutic and endothelial cells in a cluster in maintenance medium depleted in vasculogenic growth factors, and a method for producing a biological tissue therewith.

Group III: Claims 96-104 and 140 (in-part) are directed toward an array of microtissue spheroids, comprising: a plurality of spheroids, each comprising a plurality of cells of a first cell type and a plurality of cells of a second cell type, the first cell type being endothelial cells that are concentrated in an interior region of the each spheroid; each of the plurality of spheroids being isolated from others in a respective well of an array of microwells in a substrate.

Group IV: Claims 116-121 and 140 (in-part) are directed toward a method of growing a tissue structure in a living host, comprising: co-culturing cells of multiple types in respective microwells, including controlling a composition of a nutrient medium such that the cells in each microwell organizes into a respective cluster responsively to growth factors; combining in a solution; injecting the solution, containing the clusters, into a living host.

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Group I include a blood normal medicament, which is not present in any other Group, the special technical features of Group II including a vasculogenic medium, which is not present in any other Group, the special technical features of Group III including an array, which is not present in any other Group, the special technical features of Group IV including co-culturing cells of multiple types in respective microwells, which is not present in any other Group.

Groups I-IV share the technical features including: spheroids comprising cells of one type concentrated in the center or interior region; and microwells. Groups I, III and IV share the technical features including: a plurality of spheroids comprising cells of a first type and a second type. Groups I, II and IV share the technical features including: a method of forming or creating an injectable or implantable microtissue; co-culturing cells of at least two types in microwells; combining the cells in a solution or medicament; and implanting or injecting microtissue into a host or patient. Groups I and II share the technical features including endothelial cells; spheroids of less than 200 microns; depositing cells of a first and second type into a microwell of a construct, the construct or surfaces of the microwell including a material to which the deposited cells are non-adherent; during a first time period, culturing the deposited cells in a culture media without one or more growth factors; and during a second time period after the first time period, further culturing the spheroid with one or more growth factors to form the microtissue; and forming a vascular network or interconnections in the microtissue.

However, these shared technical features are previously disclosed by WO 2014/026201 A1 to The Trustees of Columbia University in The City of New York (hereinafter 'Columbia').

Columbia discloses spheroids (spherical 3D cell aggregates (spheroids); paragraph [0008]; figure 1, which discloses spheroid aggregates) comprising cells of one type concentrated in the center or interior region (comprising endothelial cells, which have migrated into the center of the aggregates (comprising cells of one type concentrated in the center or interior region); paragraph [0018]); and microwells (paragraph [0011]); a plurality of spheroids comprising cells of a first type and a second type (a plurality of spheroid cell aggregates comprising EC's and differentiated brown adipose tissue cells (a plurality of spheroids comprising cells of a first type and a second type); paragraph [0008]; figure 1, discloses spheroid aggregates); a method of forming or creating an injectable or implantable microtissue (a method of forming an injectable cellular aggregate microtissue; paragraphs [0007], [0009]); co-culturing cells of at least two types in microwells (co-culturing EC's and stem cells or BAT cells in microwells; paragraphs [0009], [0011]); combining the cells in a solution or medicament (recovering the cultured cells in a solution; paragraphs [0009], [0011], [0112]); implanting or injecting microtissue into a host or patient (paragraph [0007]); the use of endothelial cells (ECs (endothelial cells); paragraph [0008]); the use of spheroids (figure 1) of less than 200 microns (of 50 microns; paragraph [0008]); depositing cells of a first and second type into a microwell of a construct (depositing and expanding stem cells and endothelial cells mixed in suspension into a microwell of an array; paragraphs [0009], [0011]), the construct or surfaces of the microwell including a material to which the deposited cells are non-adherent (the construct comprising a non-adhesive microwell; paragraph [0009]); during a first time period, culturing the deposited cells in a culture media without one or more growth factors (culturing white adipose tissue in media lacking angiogenic factors, including VEGF and bFGF; paragraph [0017]); and during a second time period after the first time period, further culturing the spheroid with one or more growth factors (resuspending co-cultured cells, including differentiated cells, and further culturing in microwells in the presence of factors, for promoting vascularization (during a second time period, after the first time period, further culturing the spheroid with one or more growth factors); paragraphs [0009], [0010], [0011]) to form the microtissue (paragraph [0011]); and forming a vascular network or interconnections in the microtissue (paragraph [0011]).

Since none of the special technical features of the Groups I-IV inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the Columbia reference, unity of invention is lacking.