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CA 2646741 A1 2007/10/04

(21) **2 646 741**

(12) **DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION**

(13) **A1**

(86) Date de dépôt PCT/PCT Filing Date: 2007/03/09
(87) Date publication PCT/PCT Publication Date: 2007/10/04
(85) Entrée phase nationale/National Entry: 2008/09/19
(86) N° demande PCT/PCT Application No.: US 2007/063708
(87) N° publication PCT/PCT Publication No.: 2007/112192
(30) Priorité/Priority: 2006/03/24 (US11/388,920)

(51) Cl.Int./Int.Cl. *C12N 5/00* (2006.01),
C12M 3/02 (2006.01), *C12N 5/08* (2006.01)
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(54) Titre : PROCEDE DE CREATION DE SYSTEMES DE MICROVAISSEaux POUVANT ETRE PERFUSES
(54) Title: METHOD FOR CREATING PERFUSABLE MICROVESSEL SYSTEMS

(57) **Abrégé/Abstract:**

Microvessel networks are produced in vitro from tissue-engineered parent vessels (200, 202) sprouting into a supporting matrix, as for example gels, of extracellular matrix proteins. The microvessel systems are integrated into devices that allow for controlled perfusion with fluids. The vessels may include cells from one cell type, for example, endothelial cells, or from combinations of two or more cell types.



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
4 October 2007 (04.10.2007)

PCT

(10) International Publication Number
WO 2007/112192 A3

(51) International Patent Classification:

C12N 5/00 (2006.01) *C12N 5/08* (2006.01)
C12M 3/02 (2006.01)

(21) International Application Number:

PCT/US2007/063708

(22) International Filing Date: 9 March 2007 (09.03.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

11/388,920 24 March 2006 (24.03.2006) US

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(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM,

AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- *with international search report*
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:
4 December 2008

(54) Title: METHOD FOR CREATING PERFUSABLE MICROVESSEL SYSTEMS

(57) Abstract: Microvessel networks are produced in vitro from tissue-engineered parent vessels (200, 202) sprouting into a supporting matrix, as for example gels, of extracellular matrix proteins. The microvessel systems are integrated into devices that allow for controlled perfusion with fluids. The vessels may include cells from one cell type, for example, endothelial cells, or from combinations of two or more cell types.



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METHOD FOR CREATING PERFUSABLE MICROVESSEL SYSTEMS

Statement Regarding Federally Sponsored Research

This invention was made with government support under Grant No. 1 R21 HL081152-01 awarded by NIH National Heart, Lung, and Blood Institute. The US
5 government has certain rights in the invention.

Field of the Invention

The present invention relates to methods for the study of physiological and pathological vascular growth, and vascular growth in response to angiogenic or angiostatic factors.

10 Background of the Invention

During normal processes of vascular growth (e.g., the menstrual cycle, placentation, changes in adiposity, wound repair, inflammation), the creation of new blood vessels is regulated and eventually ceases. Significantly, the deregulation of vascular growth is a critical element of pathology. For example, tumor growth,
15 diabetic retinopathies, arthritis, and psoriasis involve excessive proliferation of blood vessels that contributes directly to the pathological state. In contrast, impairment of vascular growth, characteristic of aged individuals, compromises the healing of wounds and the revascularization of tissues rendered ischemic by trauma or disease. Therefore, an understanding of the mechanisms that direct the assembly new blood
20 vessels, and the processes that start and stop vascular growth, are central to the development of strategies to control vascularization in disease.

During the growth of new blood vessels (*angiogenesis*), sprouts arise from endothelial cells that line the lumens of capillaries and postcapillary venules – the smallest branches of the vascular system. Angiogenesis is a complex, multi-step
25 process. Although published studies of angiogenesis number in the many thousands, the cellular mechanisms that mediate and regulate angiogenic growth and morphogenesis are poorly understood.

The details of angiogenic sprouting are difficult to observe in "real-time" *in vivo* because of the opacity of most tissues. Tissue sections are difficult to reconstruct in
30 3D and do not communicate the dynamic nature of vascular growth. Moreover, the region near the tips of angiogenic sprouts – a critical area of control of vascular invasion and morphogenesis – is rarely found in tissue sections. In order to

overcome the limitations of conventional histology, a variety of "models" of angiogenesis *in vivo* and *in vitro* have been developed.

Models of angiogenesis *in vivo*: To circumvent the opacity of living tissues, investigators have observed angiogenesis through "windows" in living animals that include the naturally transparent tails of amphibian larvae (Clark and Clark 1939), or specialized viewing chambers either implanted into rabbit ears (Clark and Clark 1939), mouse skin (Algire, Chalkley et al. 1945) and hamster cheek pouches (Greenblatt and Shubi 1968) or developed from rabbit corneal pockets (Gimbrone, Cotran et al. 1974) or chick chorioallantoic membranes (Ausprunk, Knighton et al. 1974). From these early, largely descriptive studies came validation of the central paradigm of tumor-induced vascular chemotaxis and the corresponding discovery of diffusible, tumor-derived molecules that promote vascular growth. Newer assays of angiogenesis *in vivo* measure vascular ingrowth into polymeric sponges or plugs of gelled basement membrane proteins implanted subcutaneously into rodents (Passaniti, Taylor et al. 1992; Andrade, Machado et al. 1997; Akhtar, Dickerson et al. 2002; Koike, Vernon et al. 2003). For all of their elegance, approaches *in vivo* are made difficult by: (1) intra-species variation in angiogenic response from animal to animal; (2) the lack of translation of results from one species to another; (3) high costs of animal purchase and maintenance; (4) public disapproval of the use of animals for research purposes; and (5) complexities encountered in animal surgeries and in the visualization and evaluation of results.

Two-dimensional (2D) models of angiogenesis *in vitro*: In an effort to understand the molecular mechanics of angiogenesis, endothelial cells isolated from large vessels were cultured in flat dishes until they formed confluent, pavement-like monolayers that simulated the endothelial linings of blood vessels (Jaffe, Nachman et al. 1973; Gimbrone 1976). Although useful as models of proliferative responses to endothelial injury in large blood vessels (Gimbrone, Cotran et al. 1974; Fishman, Ryan et al. 1975; Madri and Stenn 1982; Madri and Pratt 1986; Jozaki, Marucha et al. 1990; Rosen, Meromsky et al. 1990), monolayer cultures of endothelial cells on rigid substrata do not typically organize into capillary-like tubes in simulation of angiogenesis. In 1980, however, following successful long-term culture of capillary endothelial cells (Folkman, Haudenschild et al. 1979), it was reported that 20–40 day cultures of bovine or human capillary endothelial cells developed a 2D cellular

network on top of the confluent cellular monolayer, a process termed "angiogenesis *in vitro*" (Folkman and Haudenschild 1980). The endothelial cells of the network appeared as "tubes" with "lumens" filled with a fibrillar/amorphous material that was interpreted to be an endogenously-synthesized network of "mandrels" on which the cells organized. Later studies reported similar 2D network formation by endothelial cells from large vessels (Maciag, Kadish et al. 1982; Madri 1982; Feder, Marasa et al. 1983) and by endothelial cells seeded on top of malleable, hydrated gels of basement membrane proteins (e.g. Matrigel® gel)(Kubota, Kleinman et al. 1988).

Although 2D models of vascular development remain in use today (the Matrigel®-based assay (Kubota, Kleinman et al. 1988) is available commercially), such models lack the following 5 defining characteristics of true angiogenesis:

1. **Invasion** – Endothelial cells in 2D models form networks on top of extracellular matrix and show little propensity to burrow into the extracellular matrix (Vernon, Angello et al. 1992; Vernon, Lara et al. 1995).
2. **Directionality** – In 2D models, the networks of endothelial cells form *in vitro* more or less simultaneously throughout a field of pre-positioned cells, whereas angiogenesis *in vivo* involves the vectorial invasion of extracellular matrix by filamentous sprouts that arborize by multiple levels of branching.
3. **Correct polarity** – Although the 2D models make unicellular tubes that markedly resemble capillaries (Maciag, Kadish et al. 1982; Feder, Marasa et al. 1983; Sage and Vernon 1994), their polarity is "inside-out", that is, they deposit basement membrane material on their luminal surfaces and have their thrombogenic surfaces facing outward to the surrounding culture media (Maciag, Kadish et al. 1982; Feder, Marasa et al. 1983) – opposite to the situation *in vivo*.
4. **Lumen formation** – Evidence that 2D models generate endothelial cell (EC) tubes with patent lumens is weak. Typically, the endothelial cell tubes have "luminal" spaces that are filled with extracellular matrix (either exogenous or synthesized by the cells)(Maciag, Kadish et al. 1982; Madri 1982; Feder, Marasa et al. 1983; Sage and Vernon 1994; Vernon, Lara et al. 1995). Where present, patent lumens usually appear as slit-like or narrow cylindrical spaces bounded by thick walls of endothelial cell cytoplasm – quite different from the inflated, thin-walled endothelial cell tubes that typify capillaries *in vivo*.

5. **Cell specificity** – The cellular networks in 2D models are generated by mechanical processes that may be accomplished by non-EC cell types (Vernon, Angello et al. 1992; Vernon, Lara et al. 1995). Indeed, mathematical modeling has shown that any adherent cell type capable of applying tensile forces to malleable, 2D extracellular matrix (either synthesized endogenously or supplied (e.g., Matrigel® gel)) can generate networks under optimal conditions (Manoussaki, Lubkin et al. 1996).

Three-dimensional (3D) models of angiogenesis *in vitro*: The recognition that angiogenesis *in vivo* occurs within a 3D extracellular matrix has led to a variety of models in which sprouting is induced within 3D gels of extracellular matrix *in vitro*. In an early 3D model, endothelial cells dispersed within collagen gels (Montesano, Orci et al. 1983) formed networks of cords and tubes (Elsdale and Bard 1972). Although the endothelial cell tubes exhibited correct polarity, the characteristics of invasion and directionality were lacking (the endothelial cells were pre-embedded and evenly dispersed in the extracellular matrix). Nonetheless, this approach has proven useful in studies of lumen formation (Davis and Camarillo 1996) and of responses of endothelial cells to growth factors (Madri, Pratt et al. 1988; Merwin, Anderson et al. 1990; Kuzuya and Kinsella 1994; Marx, Perlmutter et al. 1994; Davis and Camarillo 1996).

In an alternative approach, 1 mm sections (rings) of rat aorta embedded in a 3D plasma clot generated branching, anastomosing tubes (Nicosia, Tchao et al. 1982). Sprouts from the aortic rings exhibited angiogenesis-like invasion and directionality in addition to polarity. Explant models utilizing aortic rings from rats or microvascular segments from mice have been used to study the influence of tumors, growth factors, various extracellular matrix supports, and conditions of aging on angiogenesis (Nicosia, Tchao et al. 1983; Mori, Sadahira et al. 1988; Nicosia and Ottinetti 1990; Nicosia, Bonanno et al. 1992; Villaschi and Nicosia 1993; Nicosia, Bonanno et al. 1994; Nicosia, Nicosia et al. 1994; Nicosia and Tuszynski 1994; Hoying, Boswell et al. 1996; Arthur, Vernon et al. 1998).

A variety of models exist that induce purified endothelial cells (as monolayers or aggregates) to sprout invasively into underlying or surrounding 3D extracellular matrix gels (Montesano and Orci 1985; Pepper, Montesano et al. 1991; Montesano, Pepper et al. 1993; Nehls and Drenckhahn 1995; Nehls and Herrmann 1996; Vernon

and Sage 1999; Vernon and Gooden 2002). Each of these models has specific limitations that include difficulty in visualizing sprout formation, limited sprouting, a requirement for sectioning, or lack of effectiveness with certain types of endothelial cells.

5 Wolverine and Gulec have disclosed a 3D angiogenesis system (US 2002/0150879 A1) that involves embedding a fragment of tumor tissue into a matrix. The outgrowth of microvessels can be characterized to assay the angiogenic potential of the tissue. However, this approach does not provide luminal perfusion of the microvessels.

10 Neumann (the inventor here) et al. 2003, has disclosed the possibility of creating perfused microvessels *in vitro* that can be included in an artificial tissue. Neumann et al. 2003 teaches using 127 micrometer nylon fishing line as mandrels held by shrink tubing for making microvessels. The vessels were made from rat aortic smooth muscle cells embedded in agar. These microvessels were of an exploratory
15 nature and not suitable for creating a human vessel graft.

Angiogenesis *in vitro*: a new approach: Two-dimensional models of vascular growth *in vitro* do not establish the defining characteristics of angiogenesis listed previously, whereas existing 3D models reproduce some or most of the characteristics. Importantly, none of the 3D models currently available reconstruct a
20 parent blood vessel that contains a pressurized, flowing, circulatory fluid. Consequently, none of the existing *in vitro* 3D models permit study of the contribution of luminal pressure and flow to vascular growth and morphogenesis.

The present invention overcomes the limitations of existing models of angiogenesis by combining proven methods for generating invasive, tubular,
25 microvascular sprouts in 3D extracellular matrix (ECM) with novel methodologies for the fabrication of an tissue-engineered parent vessel that will be the source of luminal flow. Via the perfusate, angiogenesis-modulatory compounds can be administered to the luminal surface of endothelial cells where specific target receptors are known to reside. The presence of a luminal flow of nutrient medium will substantially increase
30 the survival time of capillary tubes *in vitro*. The disclosed angiogenesis system can be used evaluate a variety of experimental parameters that include hypoxia/hyperoxia, test of specific soluble bioactive compounds, use of genetically modified cells, and gene delivery via viral transfection. The system allows the study of

angiogenesis relative to wound repair, aging, cancer, and atherosclerosis. Importantly, a model following the teachings of the present invention may be adapted to provide fully functional vascular systems capable of being incorporated into bioengineered artificial tissues.

5 The present invention also provides new and novel approaches, including a manifold design for making microvessels, making microvessels from endothelial cells and making larger vessels (e.g. having the size of coronary arteries). These and other important new teachings, including, for example, a method for creation of microvascular networks are evident from the specification and claims hereinbelow.

10 Summary of the Invention

A method for creating networks of perfusable microvessels *in vitro*, said method comprising the steps of:

creating at least one parent vessel by culturing cells onto and around a set of mandrels;

15 embedding the at least one parent vessel into a matrix;

inducing the at least one parent vessel the at least one parent vessel to create sprouts in the matrix;

extracting the set of mandrels; and

subjecting the at least one parent vessel and sprouts to luminal perfusion to

20 imitate natural blood flow from the arterial end to the venous end of the capillary bed so as to create a microvessel network.

Brief Description of the Drawings

FIG. 1A, FIG. 1B and FIG. 1C schematically show an example of parent-vessel creation employed in accordance with the method of the invention.

25 FIG. 2A, FIG. 2B, FIG. 2C and FIG. 2D schematically show an example of a known heat-shrink process employed in accordance with the method of the invention.

FIG. 3A schematically shows a known design for mounting culture/perfusion devices employed in accordance with the method of the invention.

30 FIG. 3B schematically shows a design used in a manufacturing method for mounting culture/perfusion devices employed in accordance with the method of the invention.

FIG. 4A and FIG. 4B schematically show creation of manifolds for culture/perfusion devices employed in accordance with the method of the invention.

FIG. 5A, FIG. 5B and FIG. 5C schematically show an alternative design for microfabricated culture/perfusion devices constructed in accordance with the method of the invention.

FIG. 6 schematically shows a cell-seeding procedure employed in accordance
5 with the method of the invention.

FIG. 7 shows a schematic of a capillary network between two bioartificial parent vessels in accordance with the method of the invention.

FIG. 8A shows an *in vitro* image of an example of a plurality of mandrels after seeding with smooth muscle cells in accordance with the method of the invention.

10 FIG. 8B shows an example of a perfused muscle plate made in accordance with the method of the invention.

Detailed Description of the Preferred Embodiments

The examples presented herein are for the purpose of furthering an understanding of the invention. The examples are illustrative and the invention is not
15 limited to the example embodiments. The method of the present invention is useful for the study of physiological and pathological vascular growth, and vascular growth in response to angiogenic or angiostatic factors. Other useful applications are to methods that evaluate the angiogenic potential of cancer tissues and the response to antiangiogenic drugs. Additionally, the method of the invention may be used to
20 construct various wound-healing devices and for vascularization of tissue-engineered constructs.

In one example a method and device for the creation of perfusable three-dimensional microvessel networks is disclosed. As used herein "EC" refers to endothelial cells, "SMC" refers to smooth muscle cells and "CAS" refers to coronary-
25 artery substitutes.

Generally, the devices for the culture and perfusion of microvessel networks consist of a chamber holding one or more mandrels in the center (as best shown in FIG.1). The chambers can be fabricated from any biocompatible material and by a number of techniques, for example, by sandwiching laser-cut frames. The mandrels
30 are assembled within the chamber in such way that they are retractable. This can be achieved by fitting the ends of the mandrels into tubing, as for example, by heat shrinking, (as demonstrated in FIG.2). The diameter of the mandrels depends on the desired vessel caliber. The setup can be modified to accommodate single vessels,

two vessels, or up entire arrays of vessels in 2D or 3D. Mandrels can be of various materials, such as polymer fibers, glass fibers, wires or the like.

Microvessels are created by seeding cells onto the mandrels, stimulating the cells to multiply around the mandrels, and extracting the mandrels when cells have formed vessel walls. The vessels are then embedded in a matrix. Depending on the culture conditions, the composition of the matrix, and the presence of angiogenic stimuli (e.g. growth factors), the parent vessels will sprout into the surrounding matrix. The sprouts will anastomose with each other and, thus leading to the formation of capillary networks. After removal of the mandrels, the devices are connected to a perfusion system, and vessels are subjected to luminal fluid flow.

Referring now to FIG. 1A, FIG. 1B and FIG. 1C, there shown is an example schematic of parent-vessel creation employed by the method of the invention FIG. 1A shows endothelial cells 1 in a culture growth medium 100, seeded onto mandrel 2 held by shrink tubing 4 in a device body 3. FIG. 1B shows that the cells 1 have multiplied and formed a circular layer in the form of cell-sleeve 102. FIG. 1C shows the cell-sleeve after extraction of the mandrel 2 in an extracellular matrix (ECM) gel 110 being perfused with culture growth medium 100. The invention comprises the engineering of perfusable bioartificial vessel structures for tissue-engineering applications and research models. The general principle of the invention involves the culture of cells in layers around removable mandrels that are tightly fit into thin-wall tubing or other fittings. Once the cell layers have reached a desired wall thickness, the mandrels are removed, and the hereby-created bioartificial vessels (BAVs) may be perfused with culture medium, blood, blood substitutes, or other fluids by aid of a perfusion system. The method of the present invention allows for the production of mass manufactured or custom-created blood vessels, perfused *in vitro* angiogenesis models, wound healing devices, tissue components, whole tissues and organs, as well as research models.

Manufacture of culture/perfusion devices

Referring now to FIG. 2A, FIG. 2B, FIG. 2C and FIG. 2D there shown is an example schematic of a known heat-shrink process used in one embodiment of the method of the invention. As shown specifically in FIG. 2A each culture/perfusion device (CPD) may comprise one or more mandrels 2 held by a supporting frame 12. The mandrels 2 of the diameter of the desired vessel caliber are fit with their ends

tightly into medical-grade shrink tubing segments 4. The mandrels 2 may comprise biocompatible fibers (e.g. polymer, glass), wires or equivalents having diameters from several micrometers up to several millimeters depending on the vessel size being emulated.

5 As shown in the more detailed drawing of FIG. 2B, a central portion 14 of each shrink tubing segment 4 is heat-shrunk around one of the mandrels 2. Subsequently, as specifically shown in FIG. 2C, the mandrel 2 is retracted, and the tubing cut. FIG. 2D shows the situation after re-positioning the mandrel such that both ends of the mandrel are enclosed by the now cut-and-separated shrink tubing segment 4. The
10 frames 12 may be fabricated using various materials and techniques. The setup may be modified to accommodate either single bioartificial vessels or arrays of bioartificial vessels. Similarly, by layering several planes of mandrel arrays, a thick, perfusable tissue may be generated with vascular networks.

Machining of perfusion chambers

15 Referring now to FIG. 3A a known setup for the perfusion of several mandrel/shrink-tubing assemblies 11 is shown. A frame 20 may advantageously be milled from polycarbonate or equivalent materials. Distribution chambers 30 may be included into the design, which allows for simultaneous perfusion of many bioartificial vessels. Ends of a set of threads comprising the mandrels 2 are gathered in a silicon
20 tube 23.

Laser cutting of Mylar frames

Referring now to FIG. 3B a novel design used in a manufacturing method for mounting culture/perfusion devices employed in accordance with the method of the invention is schematically shown. A single vessel design, CPD 70, may
25 advantageously be created by sandwiching a mandrel 2 held by heat-shrink tubing 4 between two laser-cut Mylar® frames 22. A cylindrical epoxy manifold 21, constructed as detailed below, may advantageously be used for holding the mandrel/shrink-tubing assembly 11.

Mandrel/shrink-tubing assemblies may be sandwiched between two frames of
30 a polyester film or the like, such as Mylar®, with adhesive sides pressed together such that each mandrel is suspended in the frame window 76 by two shrink-tubing segments 4' at each end. The two shrink-tubing segments 4' are stabilized and strengthened by inclusion of at least one thin stabilizing wire 26 in the frame 22 and

by encapsulation in cylindrical epoxy manifolds that are cast around the shrink-tubing and the at least one thin stabilizing wire 26 by use of a mold of silicone tubing. The two shrink-tubing segments 4' will eventually become the inflow and outflow ports for the CPD 70.

5 Referring now to FIG. 4A and FIG. 4B, there schematically shown is a method for creation of manifolds for the devices in accordance with the method of the invention. FIG. 4A particularly shows a plurality of shrink-tubing/mandrel assemblies 11 pulled through a sleeve of, for example, silicone tubing 50. An epoxy glue 40 is injected to fill the silicone tubing 50 and allowed to harden.

10 FIG. 4B particularly shows the condition after the epoxy glue 40 has hardened and the silicone tubing 50 is slit open and removed. Remaining is a hardened epoxy rod 44. The epoxy rod 44 is cut after the mandrels have been retracted behind the cutting spot leaving channels 42 created by the shrink tubing. The ends 46 of many shrink tubes may be integrated to form a manifold 21. Stacking of individual CPDs or
15 CPD frame assemblies can be used to create 3D vessel arrays.

Alternative methods

Referring now to FIG. 5A, FIG. 5B and FIG. 5C there schematically shown is an alternative design for microfabricated culture/perfusion devices in accordance with the method of the invention. FIG. 5A particularly shows a set of mandrels 2 introduced
20 through small perforations 54 in a frame where the perforations have sleeves 56, which substitute for the shrink tubing. FIG. 5B particularly shows a CPD before cell seeding including a set of mandrels 2 mounted in a frame wall 52.

FIG. 5C particularly shows an alternate example of a culture/perfusion device with vessels 62 where microfabricated manifolds 64 may be attached to the sleeves
25 56 on the outside of the frame 52. The vessels 62 are grown on mandrels as shown herein and remain after the mandrels are removed. Microfabrication methods, such as micro molding, may be used for the mass production of such CPD frame assemblies.

Vessel creation and perfusion

Referring now to FIG. 6 there schematically shown is a cell-seeding procedure
30 in accordance with the method of the invention. In order to prepare the CPDs 70 for cell seeding, they are first cleaned and then UV-sterilized. Under sterile conditions, the CPDs are fixed to a surface, e.g. the bottom of the Petri dish 72. The inner window 76 (as shown in FIG. 3B) of the CPD frame assembly 70 is then filled with a

solution that contains an attachment-protein, such as laminin-1. One or more spacers 77 may be used as necessary. After an incubation period, the attachment-protein containing solution is removed, and a suspension of the desired cell type (e.g. smooth muscle cells or endothelial cells) in culture medium is then transferred into the window 76 of the CPD 70.

Cell seeding may be done by filling a volume of cell suspension into the window 76, and flipping the CPD frame assembly 70 upside down, thus creating a hanging droplet 80. During an incubation period of about 45 min., a large number of cells will attach to the mandrel/shrink tubing assemblies within the CPD frame assembly. Excessive cells will sink into the tip of the hanging drop and may be easily collected and discarded. The Petri dish, containing one or more CPD frame assemblies, is then returned into an upright position, filled with culture medium until the CPD frame assemblies are flooded, and incubated. The incubation conditions in one example were in an environment of 5% CO₂ at 37°C. The cells attached to the mandrel/shrink tubing assemblies will spread out and multiply, forming concentric monolayers (e.g. endothelial cells) or multilayers of 150 µm and more in thickness (e.g. smooth muscle cells).

At the desired wall configuration or thickness the mandrels are extracted, thereby creating hollow cellular tubes. Thinner walls may be protected from rupture by casting a gel such as, for example, agarose, collagen, a gel of basement membrane proteins or the like, around the cell sleeves prior to mandrel extraction. The manifolds of the CPD frame assemblies are then connected to a perfusion system and perfused with the fluid of choice, such as growth medium.

In another embodiment of the invention, a method for the creation of endothelial "parent" vessels from human vascular endothelial cells (HUVEC) comprises the steps wherein:

The culture device is first cleaned and then sterilized by UV exposure for 30 min. from each side. Under sterile conditions, the device is fixed to the bottom of a Petri dish with sterile strips.

The inner window of the device is then filled with an attachment-protein solution of laminin-1 (other attachment proteins, such as fibronectin, fibrin, or gelatin can be used instead).

After overnight incubation, the attachment-protein containing solution is removed, and a suspension of human vascular endothelial cells in culture medium is then transferred into the window of the device.

5

The Petri dish is then flipped upside down, thus creating a hanging drop of cell-medium suspension in the window of the device. After a 45 min. incubation period in a cell culture incubator (5% CO₂, 37°C) a large number of cells will be attached to the mandrel/shrink tubing assemblies within the devices.

10

The Petri dish is then brought back into the upright position, and filled with growth medium for human vascular endothelial cells until the device is submerged.

15

Cells not bound to the mandrels will float off and can be aspirated and discarded.

The Petri dish is then placed in an incubator (5% CO₂, 37°C). The cells attached to the mandrels will spread out and multiply, forming concentric monolayers of human vascular endothelial cells.

20

The culture medium is then removed from the Petri dish. A collagen solution is filled into the window of the culture device, and allowed to solidify, thus embedding the mandrel with the cell layer.

25

The human vascular endothelial cells will form sprouts into the collagen gel. The mandrel is then slowly extracted, leaving behind a perfusable "parent" microvessel of human vascular endothelial cells.

30

The manifolds of the device are then connected to a perfusion system and perfused with human vascular endothelial cells growth medium.

Perfusion system

The CPDs may be attached to perfusion systems either in linear or in circulatory mode. A linear setup may be created with a gravity flow system, or a commercially available or custom-built syringe pump. Syringes are filled with perfusion medium, mounted into the syringe pump and connected to the upstream
5 ends of the CPDs via gas-tight tubing. The CPDs may be stored in an incubator under sterile conditions or a sterile cell culture environment may be established within the CPD. The downstream manifold of the CPDs are connected to end reservoirs that collect the perfusate. A circulatory system may be built by using a peristaltic pump. Both, the linear and the circulatory system may be fitted with devices for gas
10 exchange. Gas concentration, perfusion pressure, flow, temperature, and the concentration of nutrients and metabolic byproducts are measured with sensors. The collected data may be fed into a feedback loop, allowing for tight control of the desired parameters.

Specific Applications

15 Models for angiogenesis related research

Referring now to Fig. 7, FIG. 7 shows a schematic of a capillary network between two bioartificial parent vessels 200, 202 in accordance with the method of the invention. The fluid perfusate 204 is re-routed through the capillaries 206 by decreasing the flow (f) into the "venous" parent vessel 202, and increasing the
20 resistance (R) in the "arterial" parent vessel 200. Consequently, the perfusate 204 is driven from the vessel with higher pressure to the vessel with lower pressure, simulating natural blood flow from the arterial end to the venous end of the capillary bed.

The mandrel method may be also used for the development of models for
25 angiogenesis research, as needed for pharmaceutical testing and research in wound repair, aging, and diseases like cancer, diabetes, arthritis, and psoriasis. Using endothelial cells only, or combinations of endothelial cells, smooth muscle cells, and pericytes, parent bioartificial microvessels (BMVs) can be cultured around micron-diameter mandrels, and embedded into a supportive gel of extracellular matrix. The
30 mandrels will then be extracted, leaving behind patent endothelial cell tubes within the extracellular matrix gel 210 (collagen gel, basement-membrane matrices (BMMs), or others). The extraction may be done by hand, or by aid of an automated device, and with speeds varying from extremely slow to extremely fast. Other variations may

include the extraction of the mandrel from bioartificial microvessels in a frozen state, coating of the mandrels with a thermo-responsive polymer, or pulling on either end of the mandrel, and thereby thinning it until rupture.

5 The sprouting of the parent vessels into the surrounding gel 210 will be induced by compounds, such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and phorbol 12-myristate-13-acetate (PMA), which are added to the gel and/or perfusate (e.g. growth medium).

10 Complex capillary networks 222 may be created by establishing a pressure difference between two adjacent parent bioartificial microvessels, thereby imitating arterial and venous blood flow. The fluid flow will then be re-directed from the "arterial" bioartificial microvessel through the interconnected sprouts into the "venous" bioartificial microvessel.

The perfusate may advantageously comprise oxygenated cell growth medium, free of serum and angiogenic or angiostatic substances. In another example the
15 perfusate may be an oxygenated cell growth medium, supplemented with serum, and/or angiogenesis influencing compounds. In yet another example embodiment the perfusate may be an oxygenated physiological salt solution. In another example the perfusate may include oxygenated blood, blood components, or blood substitutes. In yet another example embodiment the perfusate may not be an oxygenated, and
20 oxygenation of the system is achieved by diffusion through the matrix. In yet another example embodiment angiogenic or angiostatic compounds may be added to a perfusate.

In one example embodiment, angiogenic and angiostatic compounds or the like are added to the matrix. In yet another example embodiment cells comprise
25 genetically modified cells that release products into a perfusate or into the matrix. In yet another example embodiment the matrix may advantageously comprise fibrin, collagen, and gelatin. One type of useful matrix is Matrigel® gel. In another example embodiment the matrix may comprise agar, agarose, alginate, or silica gel.

In one example embodiment, the cells may be selected from the group
30 consisting of endothelial cells, smooth muscle cells, pericytes, human cells, animal cells, plant cells, stem cells and genetically modified cells. The matrix may be populated with cells selected from the group consisting of human cells, animal cells, and plant cells, either dispersed throughout the matrix, or locally concentrated. In

some cases a fragment of healthy or diseased tissue, such as cancer tissue is embedded into the matrix.

Sprouting from parent vessels may be microscopically studied in vitro, in sectioned material or in whole-mount preparations. Perfusion of the bioartificial microvessels with fluorescent solutions (e.g. fluorescent dextrans) aids analysis of the sprout diameter, the patency of sprout lumens, and the degree of anastomization. 3D reconstruction of sprout morphologies may be performed by z-axis stacking of epifluorescence images taken by a confocal microscope. The synthesis of a pericellular basement-membrane matrix by sprouts may be monitored in whole mounts and in histological (paraffin) sections by immunolabeling with anti-laminin and type IV collagen primary antibodies and fluorescent or peroxidase-tagged second antibodies.

In composite EC/SMC sprouts, the spatial relationships between the two cell types may be examined by labeling endothelial cells with a FITC-monoclonal antibody (MAb) to human CD31 (clone P2B1 – Chemicon) or FITC-UEA 1 agglutinin – a specific marker for human endothelial cells. smooth muscle cells may be labeled with a MAb to human alpha-SM actin followed by FITC-anti-mouse second antibodies. Details of luminal structure and interaction between endothelial cells and smooth muscle cells may be obtained from paraffin sections labeled with the aforementioned reagents.

The described fabrication methods are the foundation for commercial mass-production of angiogenesis devices with a high repeatability. With suitable preservation (e.g. cryostorage), pre-grown parent vessels or whole capillary networks could be made available to researchers in off-the-shelf fashion.

Coronary-artery substitutes

For the creation of coronary-artery substitutes, mandrels with an outer diameter selected to yield a coronary artery substitute having a vessel lumen with an inner diameter of approximately 4 mm to 5.5 mm. Alternatively, the mandrel may be a hollow tube that is perfused and permeable enough to allow for exchange of nutrients and gases during the growth period of the coronary-artery substitute. The coronary-artery substitutes may be grown either solely from smooth muscle cells, thus presenting a structure analog to the media layer in blood vessels, or made as composite structures from two or three cell types.

Smooth muscle cells are seeded onto the mandrels and grown to circular layers of 300-400 μm . In order to speed up the creation of coronary-artery substitutes, the SMC-phenotype may be manipulated in such way that the cells are brought into a highly proliferative phenotype during the initial growth phase, and then
5 switched to a differentiated state after the vessel wall has reached the desired thickness. The phenotype switch will cause the smooth muscle cell's to dramatically slow down their growth rate, and induce the production of extracellular matrix proteins, such as collagen and elastin, which are essential for the right mechanical properties of the vessels. The phenotype switch may be achieved by controlling the
10 expression of certain genes. For example, with aid of a tetracycline-responsive promoter, gene expression (e.g. for elastin) may be suppressed until the vessel wall has reached the desired thickness. Omitting tetracycline from the growth medium will then activate the inserted gene. Over-expression of elastin, for instance, will inhibit further cell proliferation and exert structural and signaling functions within the vessel
15 wall. Mechanical conditioning, e.g. pulsatile flow may be used to strengthen the coronary-artery substitutes, and induce physiological alignment of the cells. Other external or internal "phenotype switches" may be potentially used, as well.

Endothelial cells may be seeded into the SMC sleeves either directly after removal of the mandrel, or after the conditioning and restructuring of the smooth
20 muscle cells. Endothelial cell seeding may be done by infusion of an endothelial cell suspension into the SMC sleeve. The flow is then stopped for a period of time to allow proper attachment of the endothelial cells. If necessary, the vessels may be rotated, or repeatedly flipped upside down in order to facilitate an even distribution of the endothelial cells.

25 Alternatively, endothelial cells may be seeded onto the mandrel first. In that case smooth muscle cells are seeded onto a confluent endothelial cell layer. For this method, it will be necessary to prevent the endothelial cells from migration towards the periphery of the coronary-artery substitute, which is richer in oxygen and nutrients.

If desired, seeding fibroblast cells onto the outside of the SMC sleeves can
30 create an adventitial layer.

The cells for creating coronary-artery substitutes may be derived from autologous, heterologous, or xenogeneic material. The cells may be stem cells,

precursor cells, or differentiated cells. The cells may be genetically modified to achieve a specific phenotype or to lower the immune response of the host organism.

The method of the present invention provides the option for mass-producing off-the-shelf vessel substitutes, or vessel substitutes that are custom designed for the recipient. The method of the present invention is also suitable for the development of models for tissue engineering of coronary-artery substitutes, for research in atherogenesis, arteriogenesis, for research in the interaction of different vascular cell types with each other and with extracellular matrix components, for studies on the effects of nitric oxide, and for the study of various pharmaceuticals.

Blood and lymphatic vessels of different size or type

The method of the present invention may be used to create blood vessels in diameter and type other than coronary arteries. Changing the diameter of the mandrel will vary the vessel diameter. The type of the vessel (arterial, venous, lymphatic) may be varied with the phenotype of the cells, and/or the time point when the proliferation of the cells is inhibited. Veins, for example, contain only a small smooth muscle cell layer.

Other tubular-like tissues

The method of the present invention may be used for the engineering of other tubular tissues, such as bile duct, lacrimal duct, pharyngotympany tube, oviduct, vas deferens, ureter, urethra etc. The method of the present invention may prove useful for the generation of nerve conduits from different cell types, including glial cells, for guidance of neural growth and repair.

BAV systems for engineered tissues

The method of the present invention may be used for the engineering of tissues and organs by using arrays of removable mandrels as scaffold. The cells of the desired tissue/organ (muscle, liver, kidney, lung, skin, etc.) are seeded onto the attachment-protein coated mandrels. These mandrels may be made from solid fibers or wires, or, alternatively from perfusable permeable tubes, such as cellulose. The mandrels are separated from each other in a precise spacing that allows the single cell sleeves to merge. With this method, sheets or blocks of tissue may be formed. The mandrels are then extracted (or differently removed), and the bioartificial tissue is internally perfused by aid of a perfusion system.

Wound healing device

Pre-manufactured bioartificial vessel systems may be used to assist in wound healing, such as for chronic ulcers in diabetic patients. Bioartificial capillary networks could be embedded into patches of supportive materials (e.g. from extracellular matrix gels, enriched with angiogenic growth factors), and placed onto the wound. Autonomously perfused with oxygenized nutrient solutions, the bioartificial vessel would facilitate the sprouting of the donor vasculature and skin. Alternatively, such a bioartificial vessel patch could be sandwiched between the wound and a skin graft, and facilitate the in-growth of the graft.

Gene-therapy device

Bioartificial vessels could be used for implantable drug delivery devices. Cells, taken from a patient, could be genetically modified in vitro to produce a certain protein (hormone, enzyme etc.). These cells may be then grown into bioartificial vessels or vascular networks, using the aforementioned method. Re-implanted into the host, the cells continue to produce the target substance and release it locally or systemically.

Artificial Tissues and Organs

Tissue engineered vascular networks, as described above, may be used for the creation of tissues, or even whole organs. One approach is the creation of one or more *in vitro* perfused parent vessels. Stroma cells from the desired tissue or organ are seeded around the parent vessels, as for example, in a gel. The stroma cells are supplied with nutrients and oxygen via the parent vessels. When the stroma cells multiply, the demand for nutrients and oxygen increases. The cells release angiogenic factors, and stimulate the vessels to sprout. The vessel system sprouts in the same rate, as the tissue grows -very similar to the natural growth. Therefore, this system would be also a good model for studies in developmental biology.

Another approach utilizes parallel arrays of mandrels as a scaffold for stroma cells. As the stroma cells multiply, cell layers are formed around the mandrels. Eventually the space between all the mandrels is filled with stroma cells, resulting in a sheet of tissue. Upon removal of the mandrels, the tissue may be perfused through the channels, left behind by the mandrels. Those channels can become endothelialized through luminal seeding. The approach is not limited to 2D. Either several sheets may be stacked, or 3D scaffolds may be used. The inventor herein has used 2D arrays as well as 3D arrays for the engineering of muscle tissue.

In yet another approach, layers of tissue and layers of vascular networks could be created independently, and then intermittently stacked. All these approaches can produce either simple models with one or two cell types, or rather complex constructs composed of several cell types.

5 Upon implantation, the tissues or organs, engineered with these methods could be either connected directly to the blood stream, or kept perfused by a perfusion system until the host vasculature has grown into the graft.

Example of Perfused Tissue Engineered Muscle Construct

Referring now to FIG. 8A, an *in vitro* image of an example of a plurality of
10 mandrels after seeding with smooth muscle cells in accordance with the method of the invention is shown. A plurality of mandrel-and-shrink tubing units M were sandwiched on a Mylar® frame. The distance between the mandrels M was adjusted to approximately 100 µm. The ends of all shrink tubing segments were combined in one upstream and one downstream manifold (not shown). The Mylar frame was
15 sterilized, laminin coated and seeded with a suspension of 5×10^6 rat aortic smooth muscle cells SM (RASMCs)/ml. The cells SM attached to each individual mandrel M and multiplied, thus forming circular layers. After 10 days, the individual layers had merged and resulted in one thick sheet or plate of smooth muscle cells. After additional 7 days in growth medium, the medium was supplemented with 50 U/ml
20 heparin for another 7 days. Then, all mandrels were extracted, and the tissue perfused with heparin-medium at a rate of 10ml/day. The perfusion chamber was kept fixed to the bottom of a 100-mm Petri dish filled with heparin-medium. The SMC plate was perfused for 11 days. Over that time, the channels CH remained functional and remained clearly visible *in vitro* (as best shown in Fig. 8B).

25 Referring now to FIG. 8B, an example of a perfused muscle plate MP made in accordance with the method of the invention is shown. Fluid is shown perfused through the tubing ends (T) into channels (CH) left behind by the extracted mandrels.

The invention has been described herein in considerable detail in order to comply with the Patent Statutes and to provide those skilled in the art with the
30 information needed to apply the novel principles of the present invention, and to construct and use such exemplary and specialized components as are required. However, it is to be understood that the invention may be carried out by specifically different equipment, and devices and reconstruction algorithms, and that various

modifications, both as to the equipment details and operating procedures, may be accomplished without departing from the true spirit and scope of the present invention.

The complete disclosures of all references cited in this specification are hereby incorporated by reference. In the event of an otherwise irreconcilable conflict, however, the present specification shall control.

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Claims

What is claimed is:

1. A method for creating networks of perfusable microvessels *in vitro*, said method comprising the steps of:

5 creating at least one parent vessel (200) *in vitro* by culturing cells (1) onto and around a set of mandrels (2);

 embedding the at least one parent vessel (200) into a matrix;

 inducing the at least one parent vessel (200) to create sprouts (220) in the matrix;

 extracting the set of mandrels (2); and

10 subjecting the at least one parent vessel (200) and sprouts to luminal perfusion to imitate natural blood flow from the arterial end to the venous end of the capillary bed so as to create a microvessel network.

2. The method of claim 1, wherein the step of creating at least one parent vessel (200) creates two or more parent vessels and sprouts anastomize and form a
15 capillary network.

3. The method of claim 1, wherein the step of further creating at least one parent vessel (200) comprises creating multidimensional arrays of perfusable vessels.

4. The method of claim 1, wherein the perfusate (204) comprises oxygenated cell growth medium, free of serum and angiogenic or angiostatic substances.

20 5. The method of claim 1, wherein the step of subjecting the at least one parent vessel (200) and sprouts to luminal perfusion includes using a perfusate (204) including oxygenated cell growth medium, supplemented with serum, and/or angiogenesis influencing compounds.

6. The method of claim 1, wherein the step of subjecting the at least one parent
25 vessel (200) and sprouts to luminal perfusion includes perfusing with an oxygenated physiological salt solution.

7. The method of claim 1, wherein the step of subjecting the at least one parent vessel (200) and sprouts to luminal perfusion includes perfusing with oxygenated blood, blood components, or blood substitutes.

30 8. The method of claim 1, wherein the step of subjecting the at least one parent vessel (200) and sprouts to luminal perfusion includes perfusing with an un-oxygenated perfusate (204), and oxygenation of the system is achieved by diffusion through the matrix.

9. The method of claim 1, wherein the step of subjecting the at least one parent vessel (200) and sprouts to luminal perfusion includes adding at least one of angiogenic and angiostatic compounds to a perfusate (204).

10. The method of claim 1, wherein the step of subjecting the at least one parent vessel (200) and sprouts to luminal perfusion includes adding at least one of angiogenic and angiostatic compounds to the matrix.

11. The method of claim 1, wherein the cells comprise genetically modified cells that release products into a perfusate (204) or into the matrix.

12. The method of claim 1, wherein the matrix comprises material selected from the group consisting of fibrin, collagen, gelatin, gelled basement membrane, agar, agarose, alginate, basement membrane proteins, silica gel and combinations thereof.

13. The method of claim 1, wherein the cells are selected from the group consisting of endothelial cells, smooth muscle cells (SMC), pericytes, human cells, animal cells, plant cells, stem cells and genetically modified cells.

14. The method of claim 1, wherein the matrix is populated with cells selected from the group consisting of human cells, animal cells, and plant cells, either dispersed throughout the matrix, or locally concentrated.

15. The method of claim 1, wherein a fragment of healthy or diseased tissue is embedded into the matrix.

16. The method of claim 1, wherein a fragment of cancer tissue is embedded into the matrix.

17. The method of claim 1 wherein a fluid perfusate (204) is re-routed through the capillaries (206) by decreasing the flow into a venous parent vessel (202), and increasing the resistance in an arterial parent vessel (200) so that the perfusate (204) is driven from the vessel with higher pressure to the vessel with lower pressure.

18. The method of claim 1 wherein the microvessel network comprises a selected one of a bioartificial microvessel, patent endothelial cell tubes, and smooth muscle cell tubes.

19. The method of claim 1 wherein microvessel networks are comprised of normal or genetically modified cells that release factors into the perfusate (204).

20. The method of claim 1, wherein the step of subjecting the at least one parent vessel (200) and sprouts to luminal perfusion includes using normal or genetically modified cells to release factors into a perfusate (204).

21. A method for the creation of endothelial parent vessels from human vascular endothelial cells *in vitro* comprising the steps of:

cleaning and sterilizing a culture device having an inner window;

fixing the culture device under sterile conditions to the bottom of a container with
sterile strips;

filling the inner window with an attachment-protein solution;

removing the attachment-protein containing solution after incubation;

transferring a suspension of human vascular endothelial cells (EC) in culture
medium into the window;

creating a hanging drop of cell-medium suspension in the window of the culture
device;

bringing the container back into the upright position, and filling it with growth
medium for human vascular endothelial cells (EC) until the culture device is
submerged;

placing the container in an incubator until cells attached to the mandrels (2)
spread out and multiply, forming concentric monolayers of human vascular
endothelial cells;

removing the culture medium;

filling the window with a collagen solution, and allowing it to solidify, thus
embedding the mandrel with the cell layer;

slowly extracting the mandrel, leaving behind a perfusable parent microvessel of
human vascular endothelial cells which sprouts into the collagen gel; and
perfusing with human vascular endothelial cells growth medium.

22. The method of claim 21 wherein the attachment-protein solution is selected from
the group consisting of laminin-1, fibronectin, fibrin, and gelatin.

23. A method for creating a tissue comprising the steps of:

juxtaposing a plurality of mandrels (2) on a frame where the mandrels (2) are
substantially parallel to each other spaced apart at a predetermined distance, and
where each of the plurality of mandrels (2) is held at both ends by a tube;

combining the ends of all tubes in one upstream and one downstream
manifold;

sterilizing the frame;

coating the frame;

seeding the frame with a suspension of cells;
allowing the cells attached to each mandrel to multiply and form circular layers
until individual layers merge into a tissue;
subjecting the tissue to a growth medium;
5 extracting the mandrels (2); and
perfusing the tissue with perfusate (204).

24. The method of claim 23 wherein the perfusate comprises heparin.

25. The method of claim 23 wherein the cells are selected from the group consisting
of human smooth muscle cells (SMC), endothelial cells (EC), stroma cells and human
10 vascular endothelial cells.

26. A method for creating human coronary-artery substitutes *in vitro*, said method
comprising the steps of:

creating a cell tube by culturing human smooth muscle cells (SMC) *in vitro* onto
and around a mandrel sized to form a human coronary artery;
15 embedding the cell tube into a matrix;
extracting the mandrel; and
subjecting the cell tube to luminal perfusion.

27. The method of 26, wherein the cell tube includes a luminal surface, further
comprising the step of coating the luminal surface with endothelial cell cells by
20 seeding in a culture medium.

28. The method of 26, wherein the cell tube includes an outer surface, further
comprising the step of coating the outer surface with fibroblast cells by seeding in a
culture medium.

29. The method of claim 26 wherein the mandrel is sized to form a cell tube having a
25 luminal diameter in the range of 4 mm to 5.5 mm.

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FIG. 1A

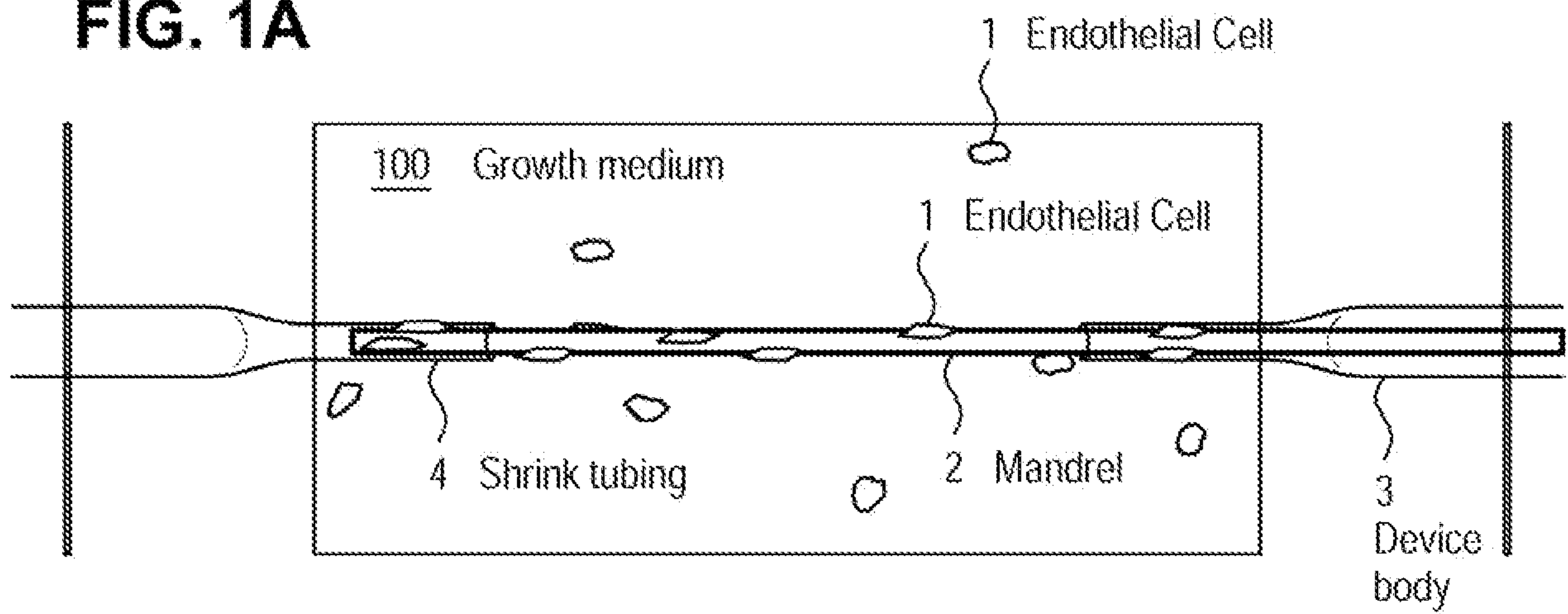


FIG. 1B

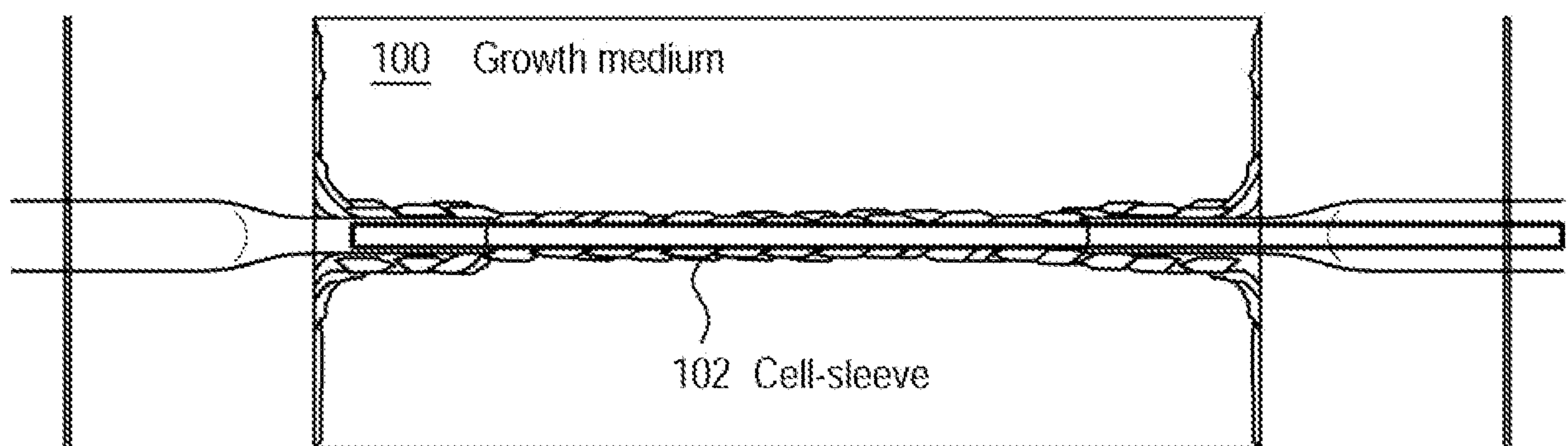
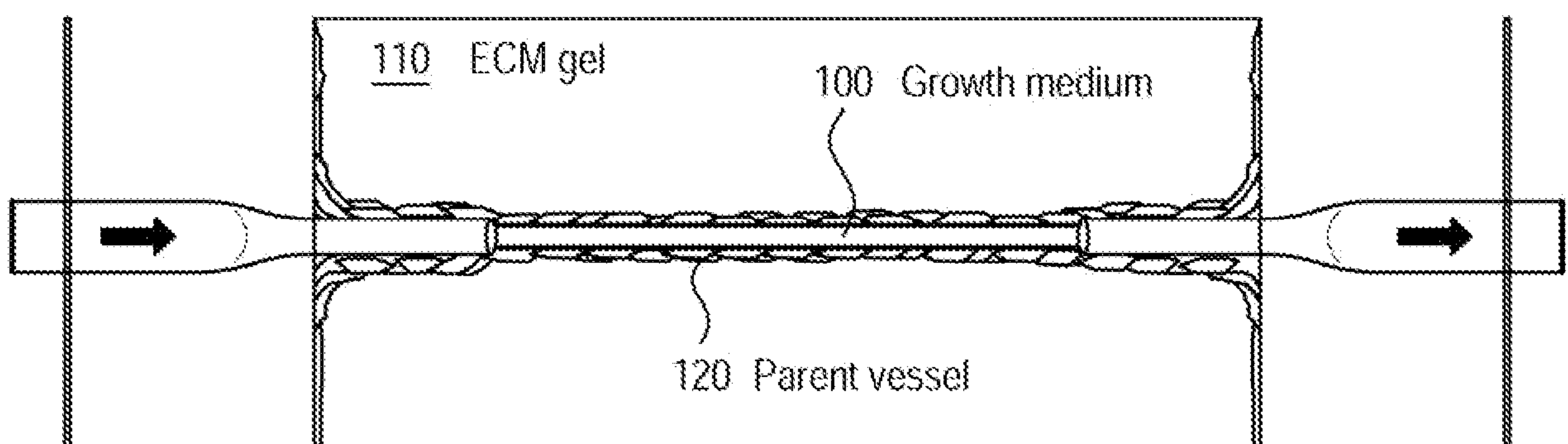
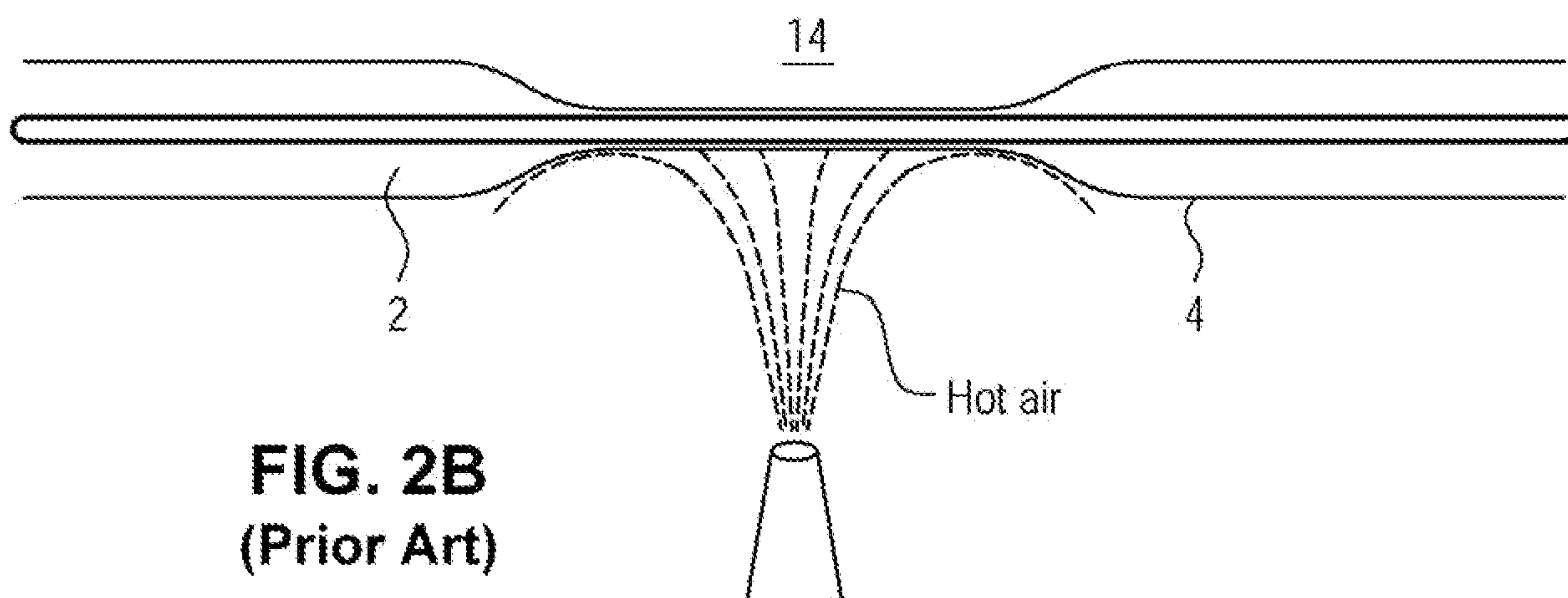
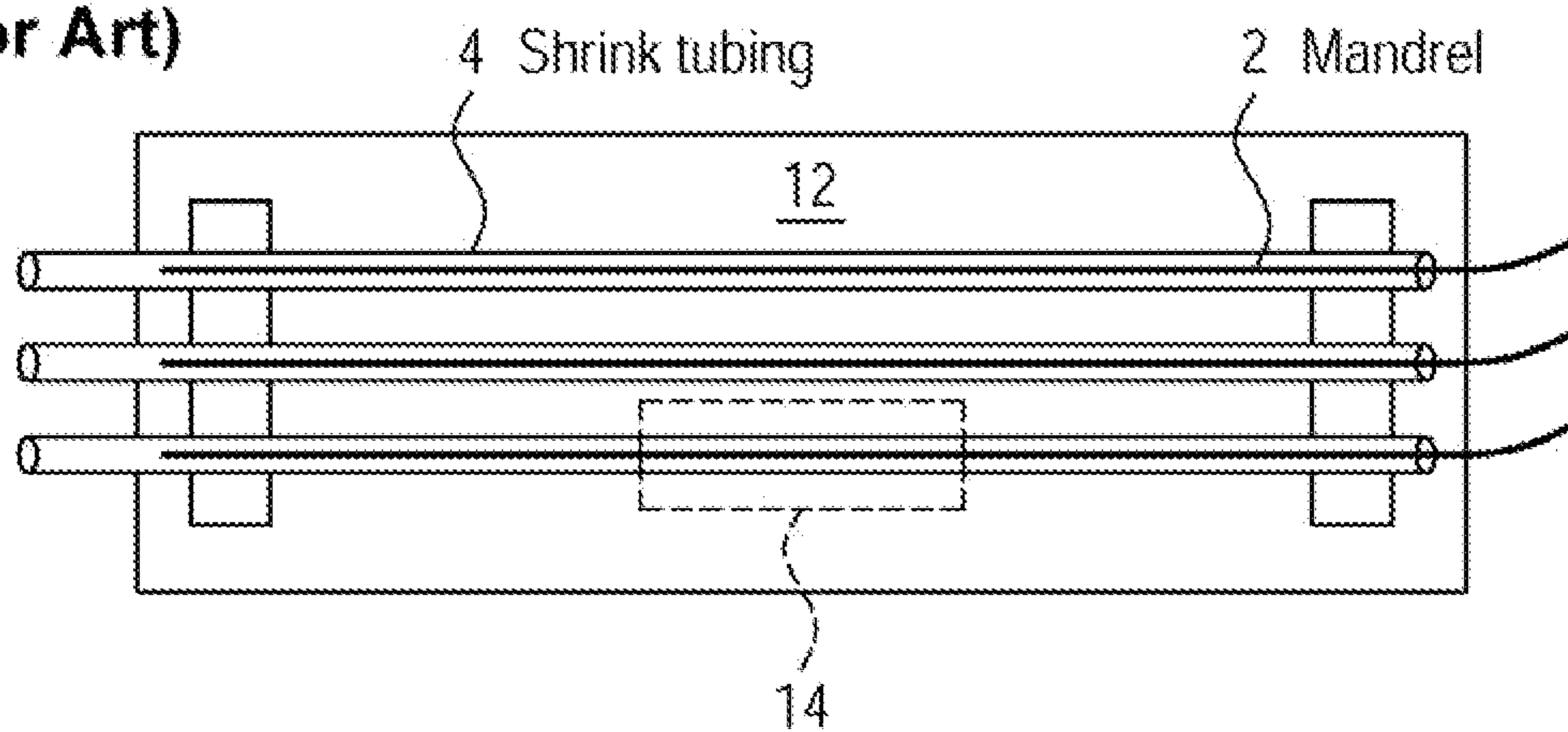
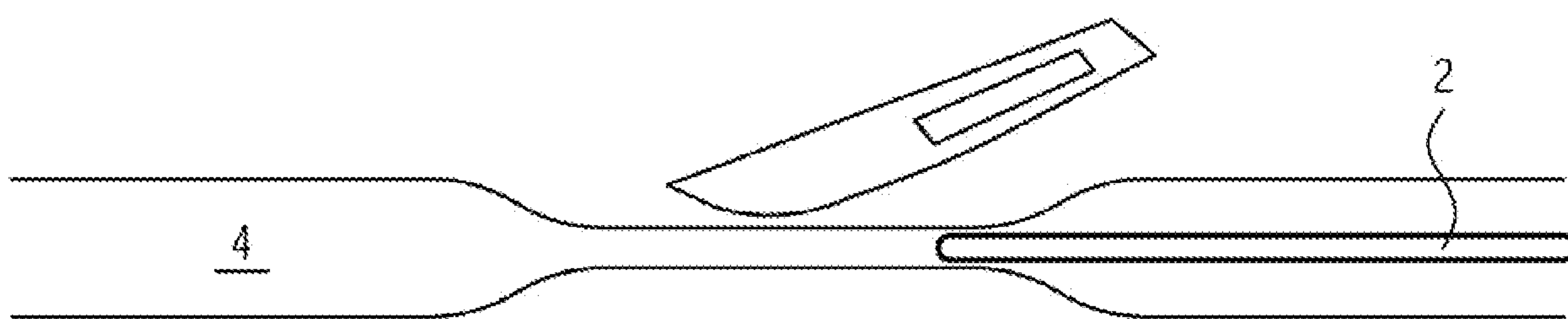
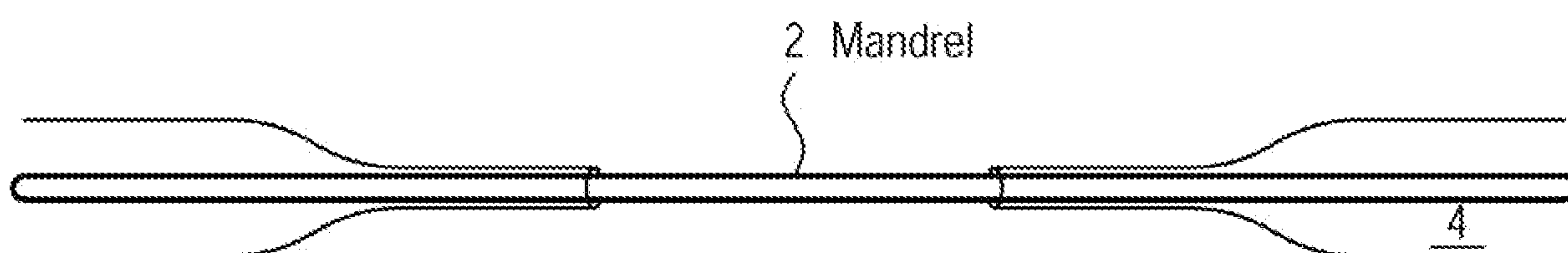
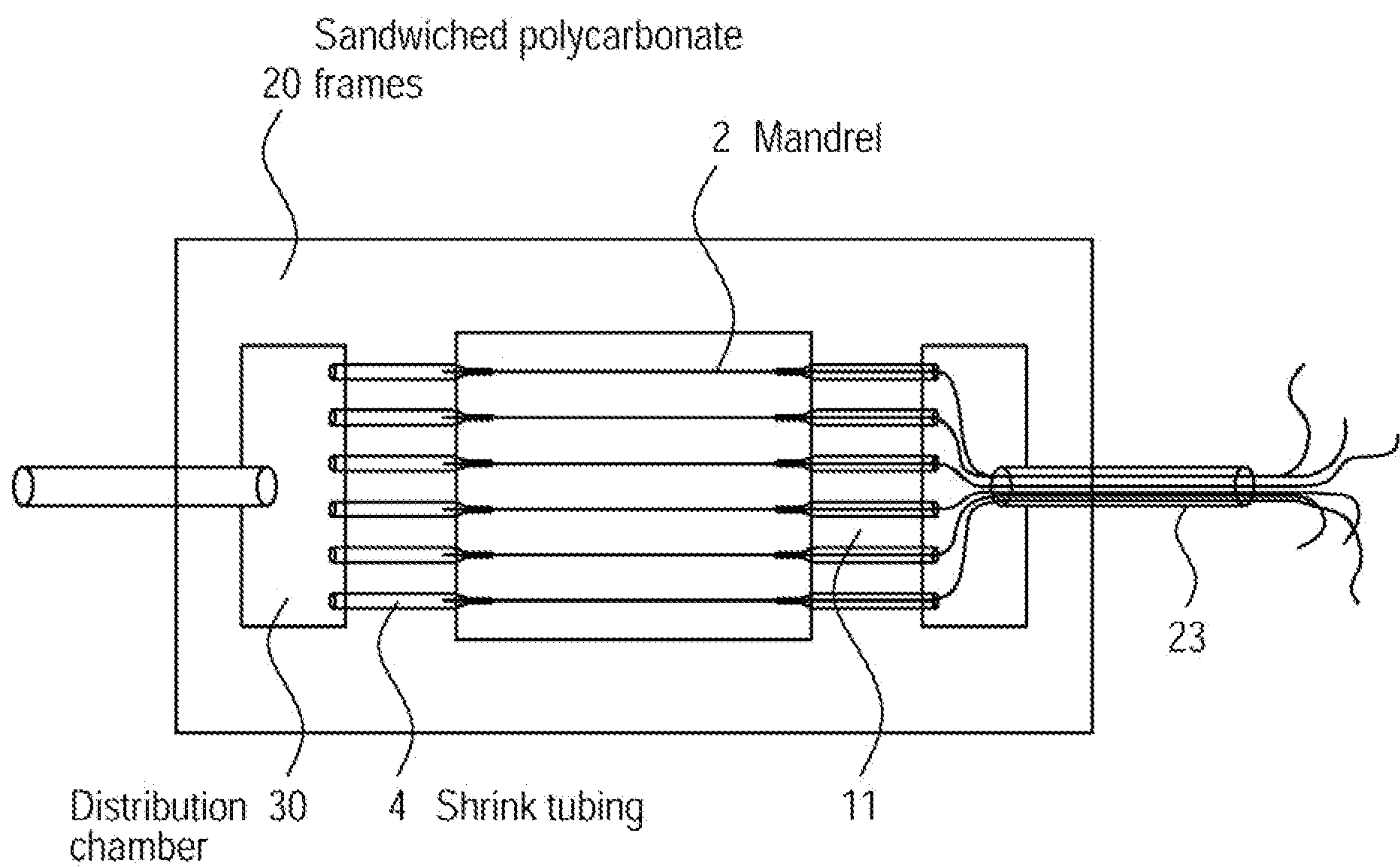
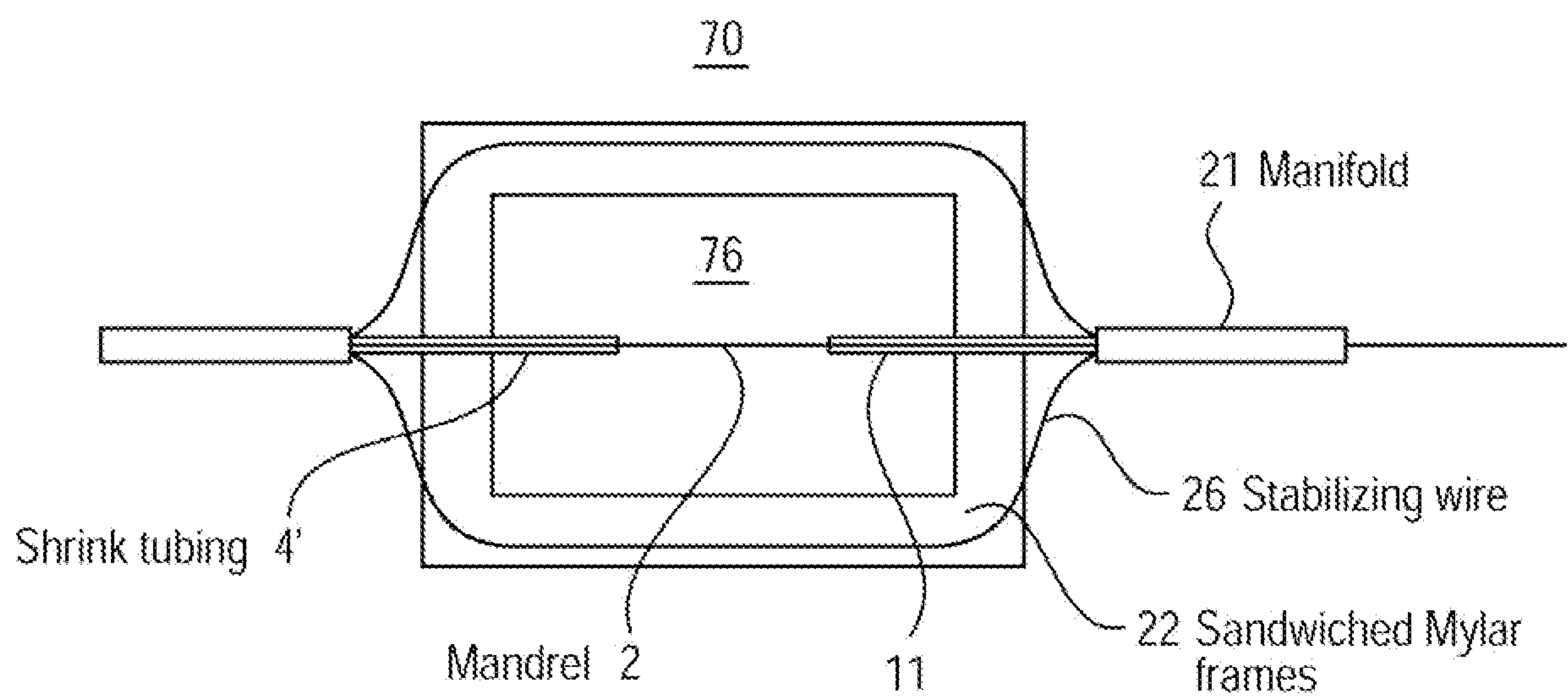


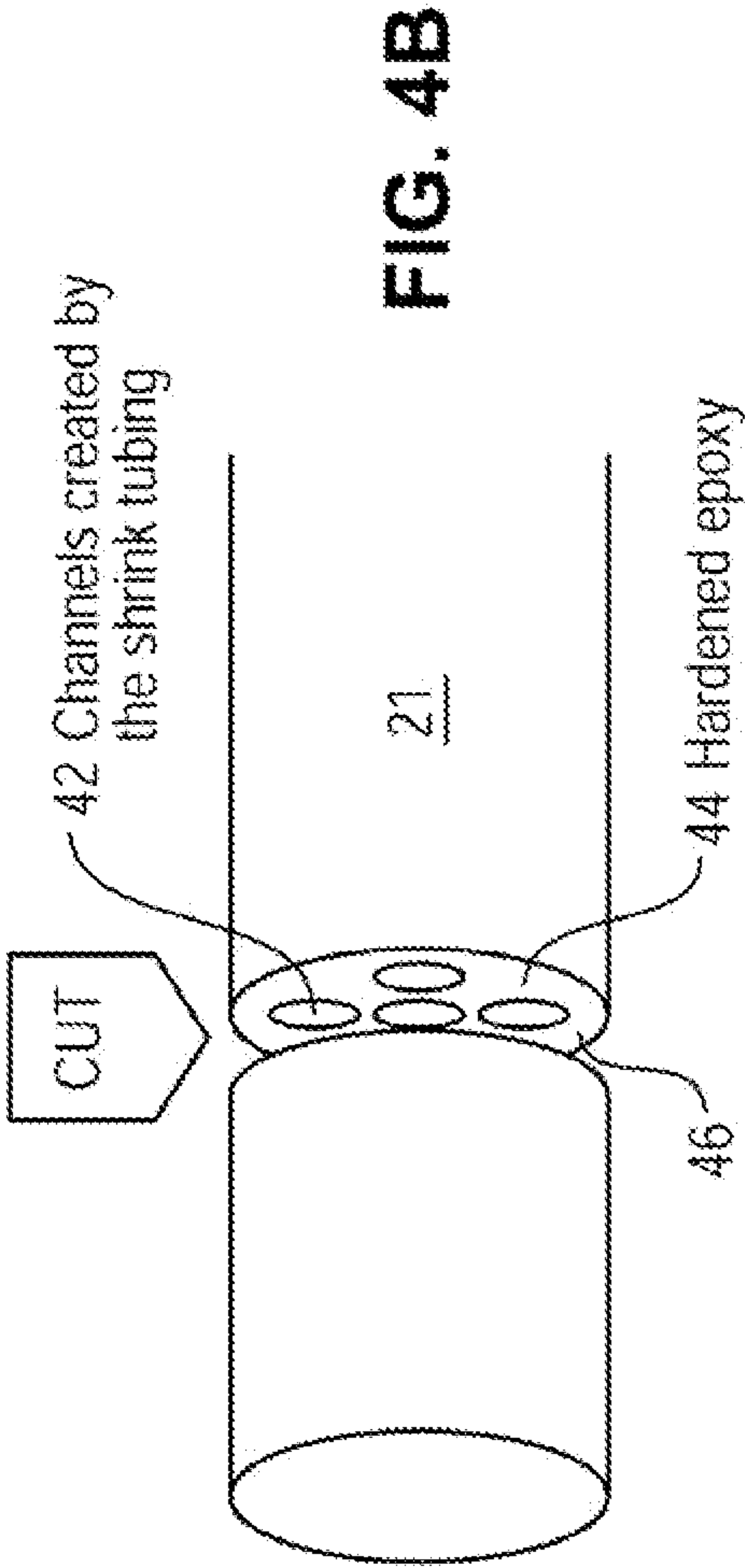
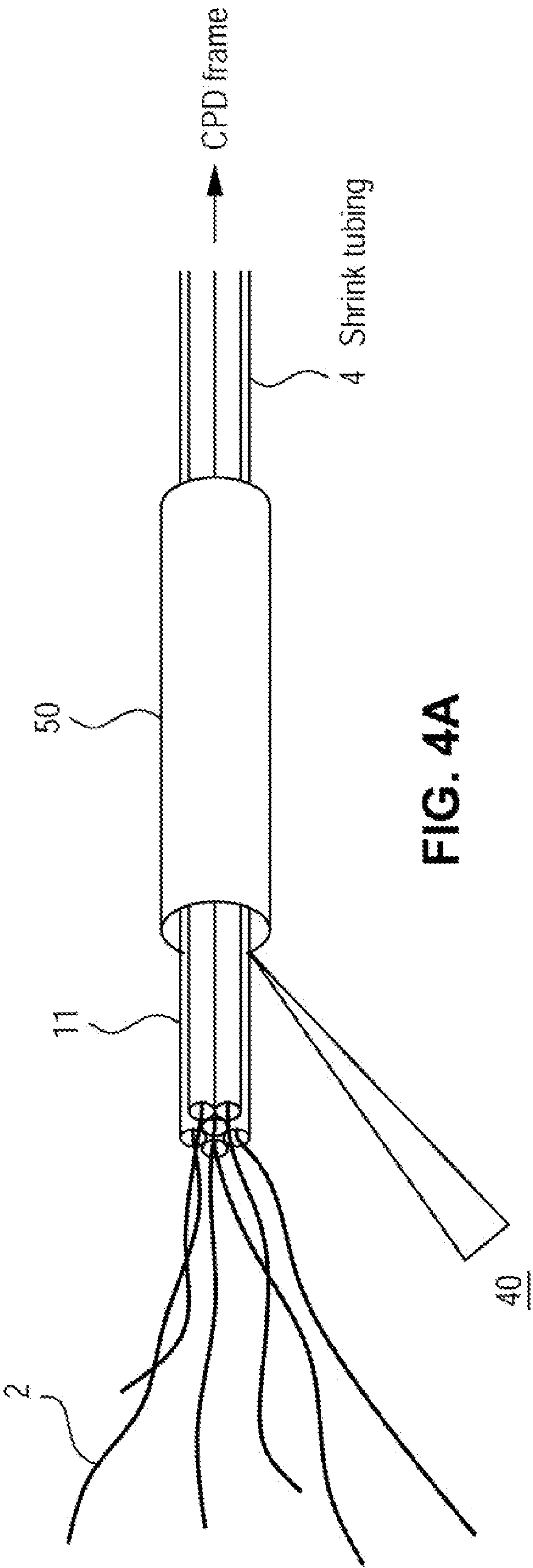
FIG. 1C



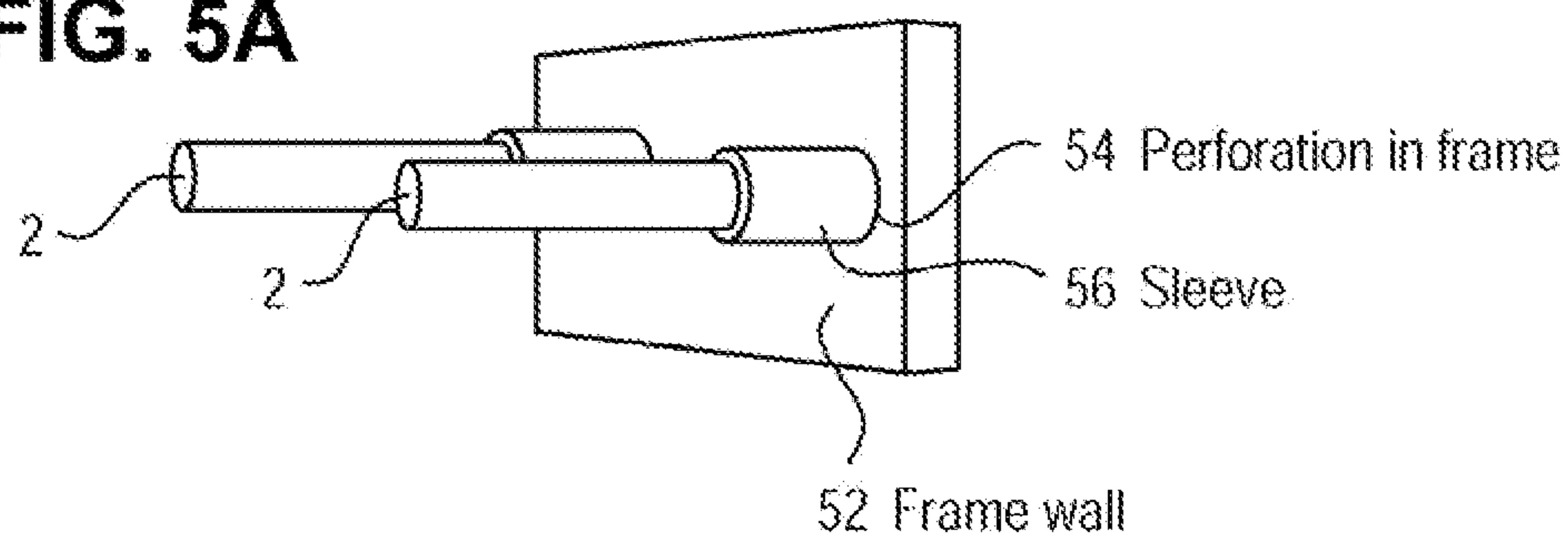
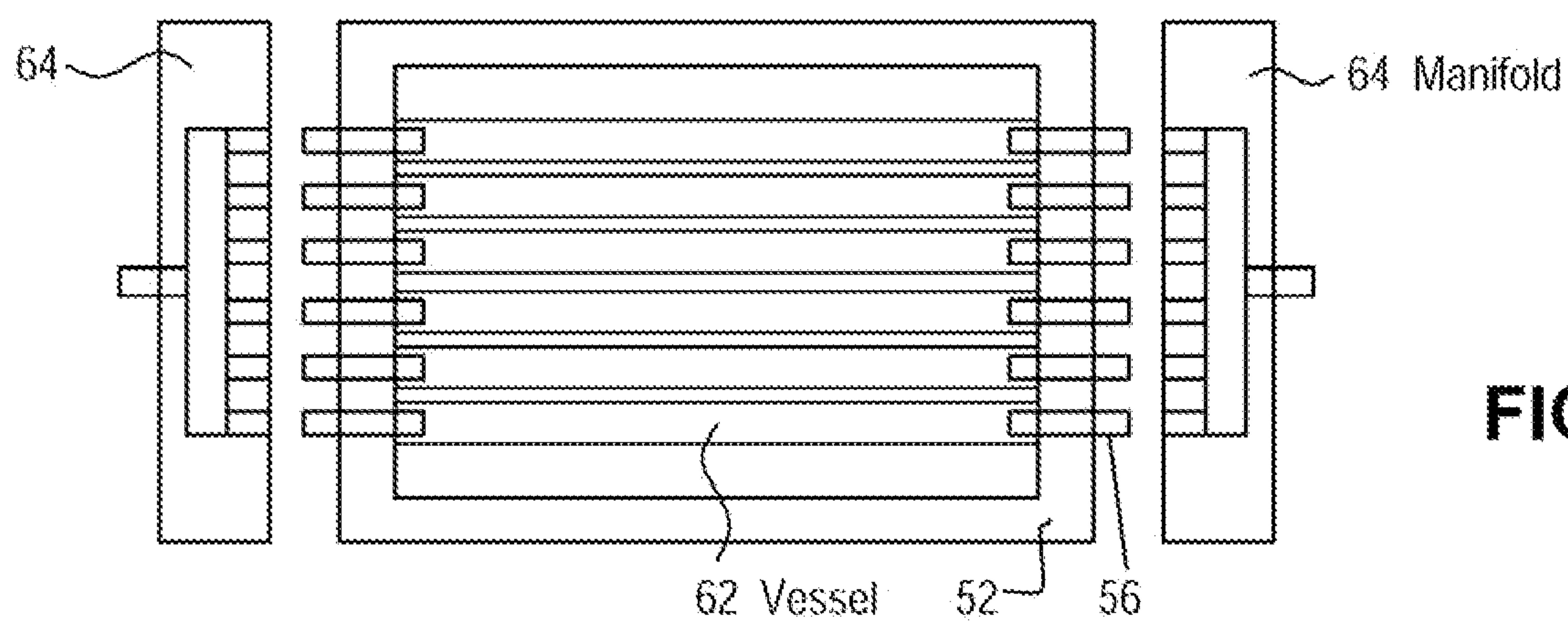
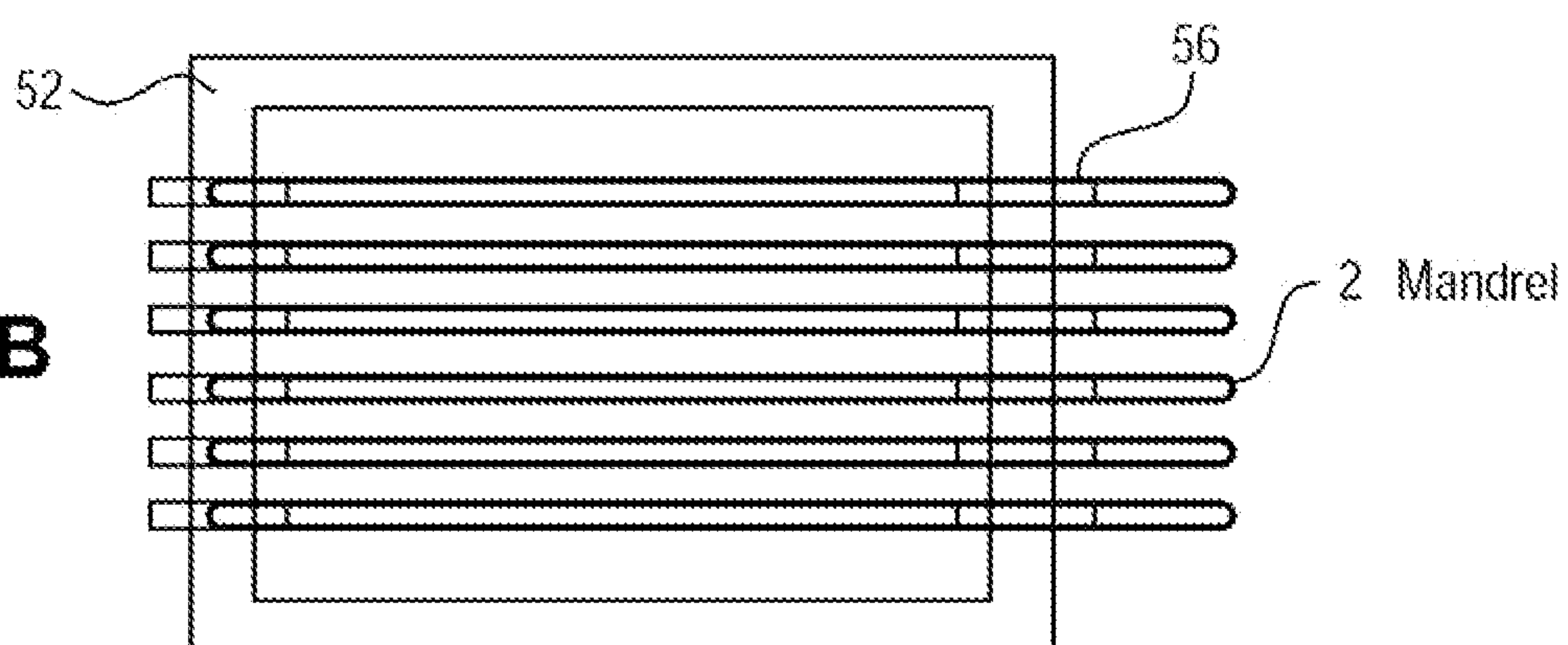
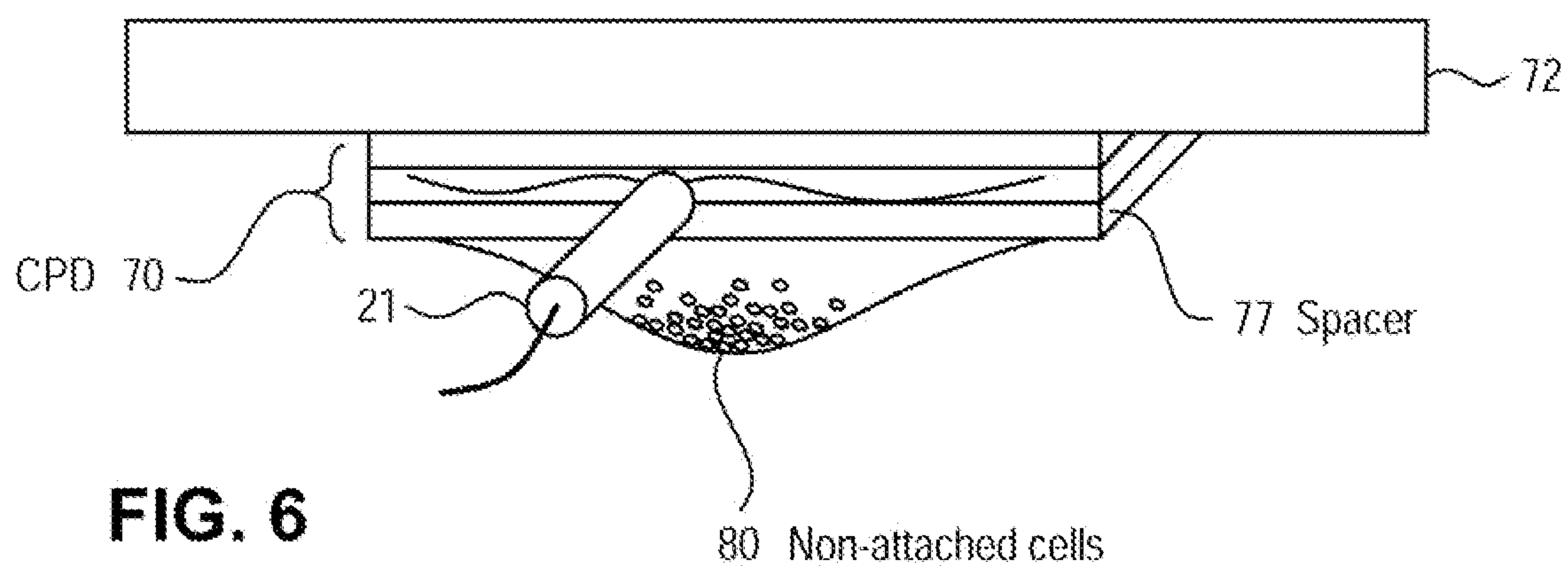
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FIG. 2A
(Prior Art)**FIG. 2B**
(Prior Art)**FIG. 2C**
(Prior Art)**FIG. 2D**
(Prior Art)

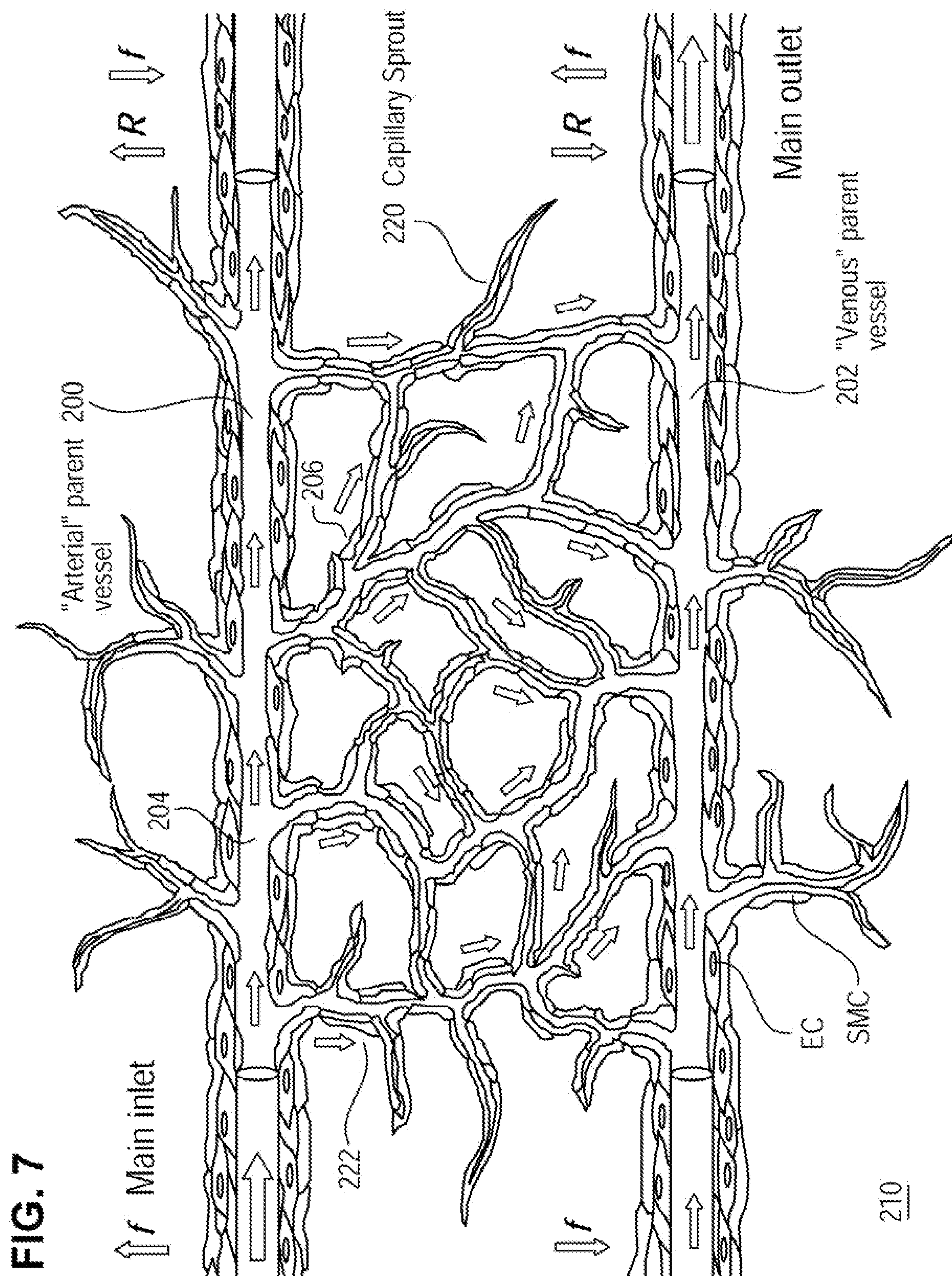
3/7**FIG. 3A**
(Prior Art)**FIG. 3B**



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FIG. 5A**FIG. 5B****FIG. 5C****FIG. 6**

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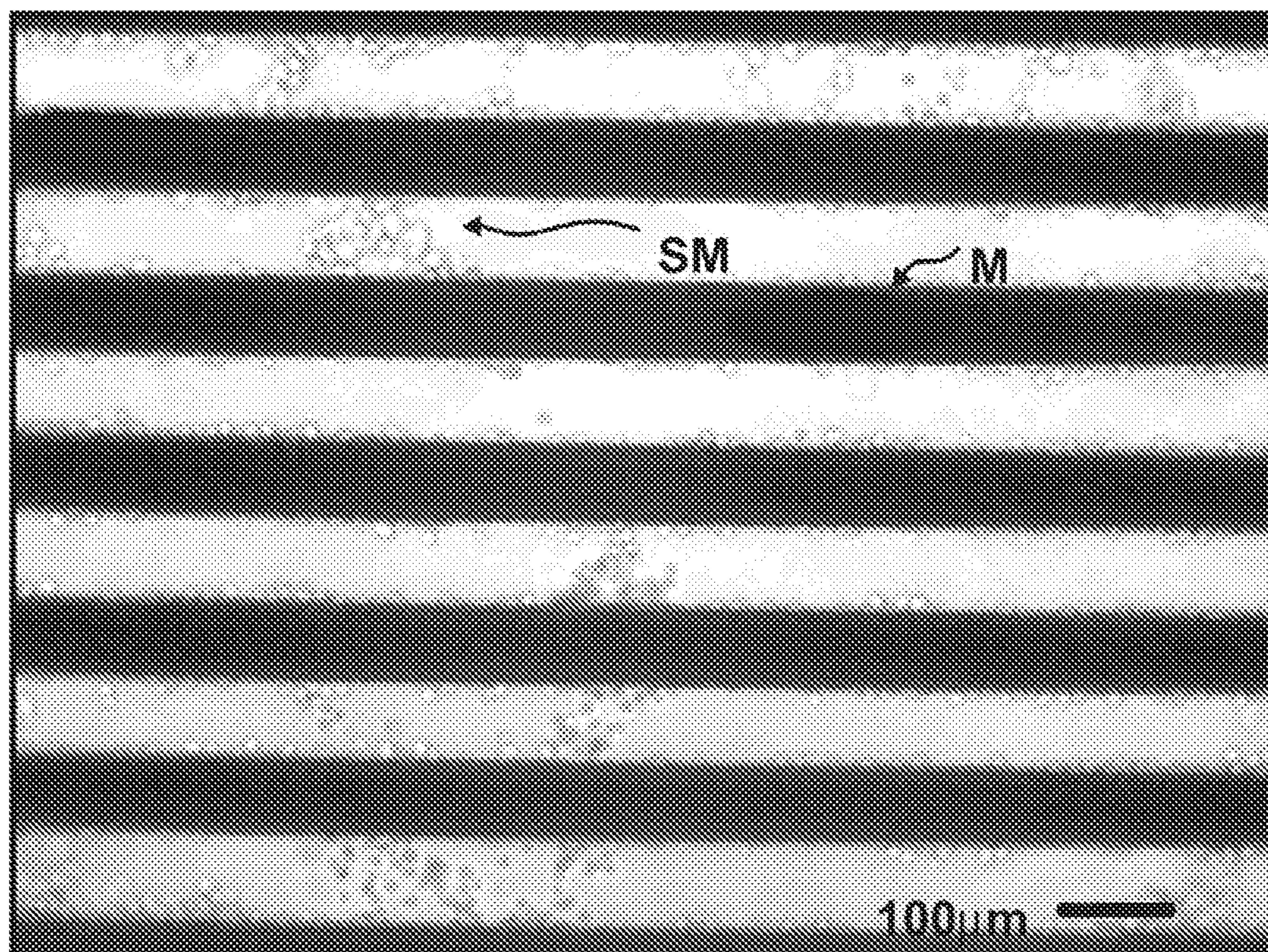


FIG. 8A

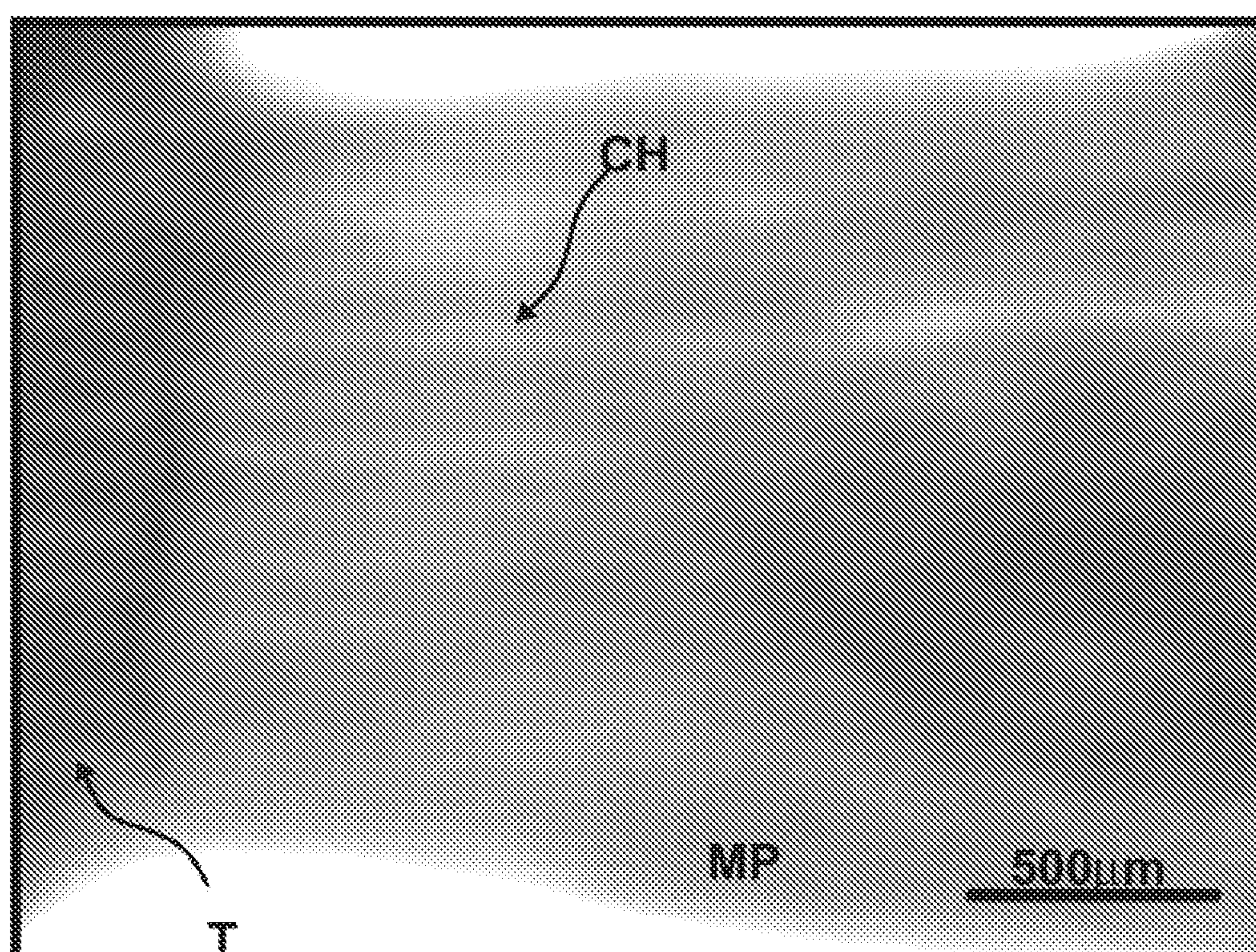


FIG. 8B