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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 August 2017 (24.08.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/142410 A1

(51) International Patent Classification:
C12M 1/32 (2006.01) *C12M 1/00* (2006.01)
B01L 3/00 (2006.01) *C12M 1/12* (2006.01)

(21) International Application Number:
PCT/NL2017/050098

(22) International Filing Date:
17 February 2017 (17.02.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2016281 18 February 2016 (18.02.2016) NL

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, 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, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

(54) Title: MEANS AND METHODS FOR SPHEROID CELL CULTURING AND ANALYSIS

(57) Abstract: The invention relates to means and methods for cell culturing and cell analysis, in particular to devices designed for growth, maintenance and/or microscopic analysis of 3D cell culture models, like cell aggregates or spheroids. Provided is an insert plate for detachably attaching to a standardized microplate and having a plurality of wells, the insert plate having a main body comprising a plurality of tubes for receiving a sample, each tube having a liquid impermeable optically clear bottom portion to allow for microscopic imaging and wherein at least part of said bottom portion has a concave arcuate surface, the plurality of tubes being positioned to fit into and align with a corresponding well of the microplate when the microplate and the insert plate are attached. Also provided is an assembly of the insert plate and a microplate.

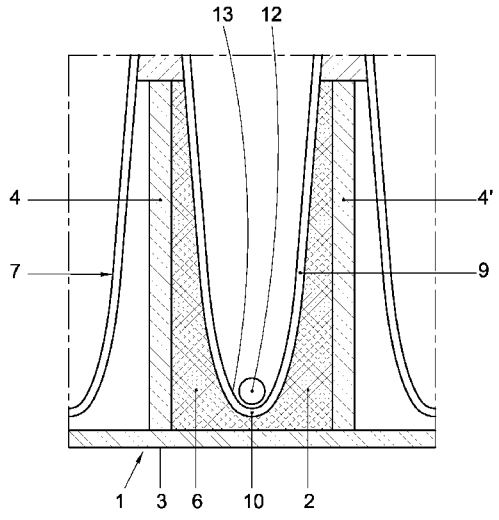

Fig. 3

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Title: Means and methods for spheroid cell culturing and analysis.

The invention relates to means and methods for cell culturing and cell analysis. More in particular, it relates to devices designed for growth, maintenance and/or microscopic analysis of 3D cell culture models, like cell aggregates or spheroids.

In vitro cellular and tissue models for various drug testing and screening experiments are often central to the development of novel therapeutics in the pharmaceutical industry. Currently, however, most in vitro studies are still performed under conventional two-dimensional (2D) cell culture systems, which are often not physiological models and/or hard to relate to functional tissues and tumors. Drug studies involving such models may therefore not produce accurate or realistic readouts. To obtain more meaningful results, *in vivo* studies involving animals are often utilized. However, next to the ethical aspects of animal models, a major drawback of *in vivo* studies is the time-consuming and expensive nature of these experiments. To bridge this gap between the non-physiological conventional 2D models and in vivo experiments, 3D *in vitro* models that provide more therapeutically predictive and physiologically relevant results for drug testing and screening in the pharmaceutical industry are needed. One way to create 3D cell culture models is through the formation of so-called spheroids, or 3D clusters or aggregates of cells or more complex and functional organoids.

A primary purpose of growing 3D cell spheroids *in vitro* is to test pharmacokinetic and pharmacodynamic effects of drugs in preclinical trials. Toxicology studies known in the art have shown 3D cell cultures to be nearly on par with *in vivo* studies for the purposes of testing toxicity of drug

compounds. When comparing LD50 values for 6 common drugs, the 3D spheroid values correlated directly with those from *in vivo* studies. Although 2D cell cultures have previously been used to test for toxicity along with *in vivo* studies, the 3D spheroids are better at testing chronic exposure toxicity because of their longer life spans. The three-dimensional arrangement allows the cultures to provide a model that more accurately resembles human tissue *in vivo* without utilizing animal test subjects

Scaling up of spheroid culture in a manner suitable for certain applications such as high-throughput screening and testing has several drawbacks. One way of traditional spheroid formation involves cultivation of suspended cells in hanging drops on the underside of a Petri dish lid. This process requires inverting of the lid following placement of the drops. As a result, the drops are susceptible to perturbation, resulting in falling, spreading, and merging with neighboring drops. Although inexpensive, this method is labor-intensive, does not permit efficient scalable production, and is not compatible with automated instruments for high-throughput screening. Because it is difficult to perform media exchange without damaging the spheroids, this method usually requires another labor-intensive step of transferring the spheroids manually, one by one, to a multi-well culture plate for longer-term culture, treatment, analysis, and harvest. An alternative is to induce the formation of spheroids under continuous agitation of cell suspension in bioreactors, such as spinner flasks and rotary culture vessels. This method requires the consumption of large quantities of culture media. It also requires specialized equipment and the size and uniformity of the spheroids are hard to control. The high variability in spheroids prohibits their use in many applications.

Methods are also available to produce spheroids using 3D microwell structures and planar micropatterns. Microplates allow for a uniform, single spheroid formation across all wells and the culturing and assaying of

spheroids in the same microplate, without the need for transfer to a new plate. A special (e.g. Ultra-Low Attachment; ULA) coating can be applied on the well surface to avoid cellular adhesion and promote cellular aggregation by gravitational force. The microplate format also enables media exchange or spheroid treatment with test compounds, drugs or assay reagent. For example, US2014/0322806 discloses a spheroid cell culture article including a frame having a chamber including an opaque side wall surface, a top aperture, a gas-permeable, transparent bottom, and optionally a chamber annex surface and second bottom, and wherein at least a portion of the transparent bottom includes at least one concave arcuate surface.

Spheroid analysis by automated visualization plays an important role in drug testing and screening applications. For instance, majority of high-throughput spheroid assays involve microscopic detection of viable or dead cells, (morphological) changes of cells or cell organelles. Most automated imagers for analyzing standardized microplates are designed for "reading" the well through an optically clear ultra-flat bottom plate. Typically, this reading uses high numerical aperture (NA) water-immersion objectives and requires all well access.

The microplate standards govern various characteristics of a microplate including well dimensions (e.g. diameter, spacing and depth) as well as plate properties (e.g. dimensions and rigidity), which allows interoperability between microplates, instrumentation and equipment from different suppliers, and is particularly important in laboratory automation. In 2010, the Society for Biomolecular Sciences (SBS) merged with the Association for Laboratory Automation (ALA) to form a new organisation, the Society for Laboratory Automation and Screening (SLAS). Henceforth, the microplate standards are also known as ANSI/SLAS standards.

Currently known (standardized) microplates designed for spheroid handling suffer from the drawback that their application for automated visualization is not optimal. More specifically, the present inventors observed that the arcuate surface of round bottom well plates designed to promote spheroid formation (e.g. each well bottom of a plate according to US2014/0322806) significantly limits their compatibility with commonly used automated plate imagers.

Therefore, the inventors set out to develop a new spheroid plate concept that allows not only for convenient spheroid culturing and handling, but also for automated microscopic spheroid analysis by commonly used automated plate imagers, in particular those that rely on imaging with water immersion lenses like the Operetta imaging system and similar high content imaging systems.

This goal was met by the provision of a round-bottom, optically clear insert plate to seed and grow spheroids, which insert plate can be combined to fit into, a flat microplate designed for analysis using conventional high content imaging systems. The optical imaging is performed by focusing through the flat, clear-bottom of the microplate on the spheroids contained in the wells of the insert plate. Herewith, a convenient and cost-effective spheroid culturing and imaging system is provided.

Accordingly, in one aspect the invention provides an insert plate for detachably attaching to a microplate having dimensions established by the Society of Biomolecular Sciences (SBS Standards) or the SLAS/NIST Microplate Standards, and having a plurality of wells, the insert plate having a main body comprising a plurality of tubes for receiving a sample, each tube having a liquid impermeable optically clear bottom portion to allow for microscopic observation (visualization/imaging of the sample) and wherein at least part of said bottom portion has a concave arcuate surface, the

plurality of tubes being positioned to fit into and align with a corresponding well of the microplate when the microplate and the insert plate are attached.

The indefinite article "a" or "an" and its corresponding definite article "the" as used herein means at least one, or one or more, unless specified otherwise. Abbreviations, which are well known to one of ordinary skill in the art, may be used (e.g., "h" or "hrs" for hour or hours, "g" or "gm" for gram(s), "mL" for milliliters, and "rt" for room temperature, "nm" for nanometers, and like abbreviations).

For the purpose of clarity and a concise description features are described herein as part of the same or separate embodiments, however, it will be appreciated that the scope of the invention may include embodiments having combinations of all or some of the features described. Specific and preferred values disclosed for components, ingredients, additives, dimensions, conditions, and like aspects, and ranges thereof, are for illustration only; they do not exclude other defined values or other values within defined ranges. The products and methods of the disclosure can include any value or any combination of the values, specific values, more specific values, and preferred values described herein, including explicit or implicit intermediate values and ranges.

An insert plate of the invention is among others characterized by a plurality of tubes for receiving a sample, each tube having a liquid impermeable optically clear bottom portion to allow for microscopic observation (visualization/imaging of the sample contain therein, i.e. spheroids, and wherein at least part of said bottom portion has a concave arcuate surface The tube and tube bottom portion ultimately terminates, ends, or bottoms-out in a spheroid "friendly" rounded or curved surface, such as a dimple or a

pit. In one aspect, the concave arcuate surface of the bottom portion comprises a hemi-spherical surface or a conical surface.

It is well known that the formation of uniformly-sized spheroids of, for example, tumor cells, can be facilitated if the cells are cultured in a round-bottom vessel that has low-adhesion properties. For example, ultra-low-adhesion (ULA)-coated 96-well clear, round-bottom, polystyrene microplates (Corning, Inc.) were used to successfully produce tumor spheroids that enabled target validation and drug evaluation (see Vinci *et al.*, BMC Biology, 2012, 10:29). The spheroids enhanced the biological relevance of the tumor cell cultures and facilitated a range of functional assays. The round-bottom well shape along with gravity and the ultra-low attachment coat or coating, encouraged the cells to come together and self-assemble into the spheroid shape rather than to form a monolayer of cells. Therefore, in a preferred embodiment, the tubes of an insert plate comprise a low-adhesion or no-adhesion coating on the concave arcuate bottom surface to facilitate spheroid formation and/or spheroid maintenance.

The tubes can have any desired design as long as they fit into and align with a corresponding well of the microplate. For example, the tubes have a circular cross section or square base tapered to round bottom.

The plurality of tubes of the insert plate provided herein are positioned to fit into and align with a corresponding well of the microplate when the microplate and the insert plate are attached. As will be understood, for optical imaging of the contents of the tubes it is preferred that the bottom surfaces of the tubes are centered with respect to the wells and not too far away from the bottom of the microplate through which the imaging view is cast. Therefore, the tubes are designed to fit into the (center of the) corresponding wells with a significant depth, e.g. into at least the bottom one-third or bottom one-fourth of the wells. In a specific aspect, the outside surface of the bottom portion of the tubes is essentially in physical

contact with the bottom portion of the well. Hence, each of the tubes has a depth to position the concave arcuate surface at a desired distance from the base surface of the microplate when the microplate and the insert plate are attached. Preferably, to ensure uniform imaging quality between the wells/tubes, said desired distance is essentially the same for each of the tubes. In one embodiment, the desired distance is 0 to 2 mm, preferably 0 to 1 mm.

The skilled person will appreciate that the absolute number, pattern and dimensions of the plurality of tubes will depend on the specifics of the wells of the microplate to be used in combination with the insert plate.

In one embodiment, the insert plate comprises 96 or 384 tubes to fit into an SBS-standardized or SLAS/NIST-standardized microplate comprising, respectively, 96 or 384 wells. In one preferred aspect, the insert plate comprises 96 tubes arranged in 8 rows and 12 columns. In another preferred aspect, the insert plate comprises 384 tubes arranged in 16 rows and 24 columns.

In one embodiment, e.g. for a 384-well format, the tube depth is between 10 to 14 mm, preferably between 12 and 13.5 mm. In one embodiment, e.g. for a 384-well format, the tube diameter at the base of the tube (i.e. opposite of the bottom portion of the tube) is between 3 to 3.7 mm.mm.

The insert plate may comprise one or more additional structural features, in particular those that contribute to the handling or (automated) processing of the plate. For example, the insert plate comprises a plate orientation notch in the lower left corner. As another example, the insert plate has an outer rim provided with a pressure sensitive adhesive to allow for securely attaching the insert to the microplate.

In a specific aspect, the insert plate includes a skirt portion about a periphery of the main body of the plate, preferably wherein said skirt portion is generally perpendicular to main body. A skirt portion provides additional stability to the insert plate such that during handling (e.g. when culturing spheroids) the plate can be used as "stand alone" spheroid culturing article. The skirt portion of the insert plate is suitably removed, e.g. by cutting, prior to imaging of the spheroids to allow for assembly with the microplate. In another embodiment, the insert plate is a skirtless object such that it can be assembled with the microplate without any modification.

The insert plate can be made from any moldable, optical transparent material, such as a plastic polymer. In a preferred embodiment, the material is a thermoformable polymer. Suitable preferred materials include plastic polymers such as polyethylene, polypropylene, polystyrol, thermoplastic elastomer and the like. Preferably, the plate is made from a material selected from polypropylene, polystyrene, polyethylene terephthalate, glycolyzed poly(ethylene terephthalate) (GPET) and polyolefins.

The insert plate is preferably constructed from a single piece of material. There are several methods that can be used to make an insert plate having a main body comprising a plurality of tubes for receiving a sample, each tube having a liquid impermeable transparent (optically clear) bottom portion to allow for microscopic imaging and wherein at least part of said bottom portion has a concave arcuate surface. The insert element may be molded, for example by injection molding, or shaped by thermoforming. Thermoforming is a production method where you force a plastic part with pressure and heat (and/or vacuum) into a certain shape. This enables a fast and cost-efficient manufacturing of the multiwell plate device. Any optically clear foil or sheet suitable for thermoforming can be used.

The invention accordingly also relates to a method for manufacturing an insert plate as described herein above. In one embodiment, the invention provides a method for providing an insert plate as a unitary piece, comprising subjecting an optically transparent polymer sheet to (vacuum) thermoforming technology to introduce a plurality of tubes for in the main body. Optically transparent polymers are known in the art. In a specific aspect, the method comprises thermoforming a glycolyzed poly(ethylene terephthalate) (GPET) sheet or a polyolefin sheet. Preferably, said polymer sheet has a thickness of between 0.3 and 1 mm, preferably between 0.5 and 0.8 mm.mm.

In a still further embodiment, the invention provides an assembly of (i) an insert plate according to the invention, the plate being attached to (by insertion into) (ii) a standardized microplate having a plurality of wells, each well having a flat, transparent bottom portion and opaque side wall surfaces. The multiwell microplate contains at least a plurality of wells in the suitable pattern to accommodate for the tubes of the insert plate. Typically, the number of tubes of the insert plate corresponds to the number of wells of the microplate.

In a preferred embodiment, the dimension of the multiwell plate device, the well dimension, and/or the well spacing conform to the SBS (Society for Biomolecular Screening)standard (also known in the art as ANSI/SLAS Microplate Standards. The dimensions for typical multiwell plates conforming to the SBS standard are approximately 85 mm (width) x 125 mm (length) with the wells arranged in a standardized format depending on the total number of wells. This ensures that the multiwell plate device is compatible for use in workstations or automated sample preparation systems specifically adapted to handle SBS conforming multiwell plates.

The design of the wells can vary as long as they can accommodate the tubes of the insert plate according to the invention. In one embodiment, the microplate is a 96- well or 384-well SBS-standardized microplate.
For example, the wells can have a circular or square cross section. In a specific aspect, the microplate is 384-well SBS-standardized microplate containing 384 wells having a square cross section. In another embodiment, the microplate is 96-well SBS-standardized microplate containing 96 wells having a circular cross section.

The bottom portion of each well is flat and transparent to allow for accurate microscopic imaging. Preferably, the clear bottom portion of the microplate is ultra-flat. High optical-quality film bottom microplates are ideal for performing high content cell-based assays using imaging systems. The film bottom provides a flat and optically clear surface that reduces autofocus time. In a preferred embodiment, the bottom portion of the microplate comprises cyclic olefin polymer. The downward facing bottom surface of the microplates is designed to allow for all well access when using water immersion and high NA objectives and without objectives colliding with the plate skirt.

The side wall surfaces of the microplate wells are opaque so that light cannot be transmitted between adjacent wells through the side walls. In certain types of luminescence and fluorescence analysis it is preferred that the side walls of wells be non-reflective, in which case the side walls of the wells of the microplate are preferably formed from a black or dark-colored polymer. The dark polymer may be formed by the addition of carbon black in mounts ranging from about 0.5 weight % to about 15 weight %.
In a preferred aspect, the microplate is a Low bottom height (200±10 µm), black cyclic olefin microplate with optically clear, cyclic olefin foil bottom (188 µm). Suitable plates for use in an assembly according to the invention

are known in the art and commercially available. For example, it can be the CellCarrier-384 ultra plate (PN 6057300, PerkinElmer)

In one embodiment, at least one tube of the insert plate, optionally in assembly with the microplate, comprises a plurality of living cells. For example, the cells are cardiomyocytes, tumor cells or cancer cell lines, hematopoietic cells, endothelial cells, insulin producing beta cells, neuronal cells, glial cells, kidney cells, hepatocytes, vascular progenitor cells or derivatives (such as hematopoietic stem cells or endothelial cells). The cells may be in suspension or they form a 3D-structure, like aggregate or cluster or spheroid. Preferably, at least one of the tubes contains spheroids.

The assembly may further comprise a seal, lid or cover plate that is positioned over said plurality of tubes, for instance to protect the cells and/or spheroids during culturing, incubating, handling and/or imaging.

In a still further aspect, at least one of the microplate wells is provided with an optically clear matrix, like a liquid, a gel or solidifying material, to fill at least part of the void volume formed by the interior space of the well not occupied by the tube of the insert plate. The optically clear matrix can reduce optical distortion of the incoming beam of light, thereby improving (auto)focussing and accurate microscopic imaging. It also avoids condensation of humid air in the void that could disturb the imaging quality.

The invention also provides a method of spheroid handling, comprising:
a) inserting a plurality of cells into at least one tube of an insert plate of the invention, the insert plate preferably being comprised in an assembly with a microplate, and allowing the formation of spheroid formation and

b) performing on one or more of the spheroids a handling method selected from the group consisting of spheroid culturing, spheroid maintaining, spheroid analysis, spheroid testing, and combinations thereof.

The formation of spheroids can be achieved by methods known in the art, typically involving gravity and/or cellular interactions. Depending on the type of cells, one or more agents can be added to the growth medium to promote spheroid formation or maintenance. For example, it has been shown in the art that growth factors support the clustering and/or proliferation of cells.

A method of spheroid handling as provided herein may comprise contacting the spheroid(s) with a potential therapeutic agent. In one embodiment, the potential therapeutic agent is at least one of:

a. a molecule selected from the group consisting of an endogenous ligand or ligands, a biological sample suspected of containing a native or endogenous ligand or ligands, a combinatorial library of small molecules, a hormone, an antibody, a polysaccharide, an anti-cancer agent, a natural product, a terrestrial product and a marine natural product;

b. a molecule that binds with high affinity to a biopolymer selected from the group consisting of a protein, a nucleic acid, and a polysaccharide; and,

c. a purified biological molecule selected from the group consisting of a protein, a nucleic acid, a silencing RNA (siRNA), a micro RNA (miRNA), and a short hairpin RNA (shRNA).

Preferably, the spheroid handling method further comprises assaying for a marker indicative of modulation of a cellular target of said potential therapeutic agent or screening for activity in modulating the phenotype of a spheroid. In one embodiment, the cells forming the spheroid(s) of cells, are transformed with at least one heterologous nucleic acid molecule that encodes one or more biomarkers associated with a

phenotype of interest. In a preferred embodiment, the recombinant nucleic acid molecule(s) are chromosomally integrated into the genome of the cell.

Preferably the biomarkers are linked to an indicator that can be detected in situ following expression of the biomarker. The term "indicator" is meant to refer to a chemical species or compound that is readily detectable using a standard detection technique, such as dark versus light detection, fluorescence or (chemi)luminescence spectrophotometry, scintillation spectroscopy, chromatography, liquid chromatography/mass spectroscopy (LC/MS), colorimetry, and the like. Representative indicator compounds thus include, but are not limited to, fluorogenic or fluorescent compounds, chemiluminescent compounds, calorimetric compounds, UV/VIS absorbing compounds, radionucleotides and combinations thereof. Exemplary indicators for use in the screening methods of the present invention are proteins including Red Fluorescence Protein (RFP), which fluoresces when exposed to light of wavelength 558 nm, Green Fluorescent Protein (GFP) which fluoresces when exposed to light of wavelength 395 nm, and luciferase, which produces light in the conversion of luciferin and oxygen to oxyluciferin. These indicators, when coupled with an automated plate reading mechanism, form an embodiment of the present invention that is readily amenable to both robotic and very high throughput systems.

Whereas any cell type of interest can be used, they are preferably capable of sustained growth under tissue culture conditions. For example, the cells are neoplastic cells. Cells may be tumor cells that have been isolated from a human. In a specific embodiment, the cells are human malignant tumor cells selected from the group consisting of breast cancer, lung cancer, prostate cancer, colon cancer, melanoma cancer, and cancer of the bone and connective tissues. In another specific aspect, the cells are stem cells selected from the group consisting of embryonic stem cells or adult stem

cells, progenitor cells, bone marrow stromal cells macrophages, fibroblast cells, endothelial cells, epithelial cells, and mesenchymal cells.

The invention also provides the use of an insert plate, assembly or method as described herein in a high throughput high throughput drug discovery program. For example, it provides an industrially applicable high through put screening method for assaying the non-, pro-, or anti-apoptotic or proliferative or necrotic activity of test compounds in spheroid cells. Compounds discovered using the screening methodologies of this invention can be developed into therapeutics that systemically target the inhibition of metastasis. Additionally, these techniques can be adapted to conduct individualized treatment on patients in the clinical setting. For example, primary breast carcinoma cells isolated from patients may be cultured in the 3D screening systems of the present invention and screened against known lead compounds from previous chemical library screenings as well as clinical agents in use today in order to identify patients that are likely or unlikely to respond to one or more agents available to the treating physician ("personalized medicine/therapy").

DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B schematically show a top view and a longitudinal cross-section of an embodiment of a microplate forming part of an embodiment of an assembly according to the invention, respectively,

Figs. 2A and 2B schematically show a top view and a longitudinal cross-section of an embodiment of an insert plate according to the invention, which insert plate forms another part of an embodiment of an assembly according to the invention, respectively, and

Fig. 3 schematically shows a cross-section of a part of an assembly according to the invention in which the insert plate of Figs. 2A and 2B has been detachably inserted into the microplate of Figs. 1A and 1B.

Please note that the Figures are merely schematically and are not necessarily to scale.

In Figs. 1A and 1B a standardized microplate 1 is shown in top view and cross-section, respectively. The microplate 1 has a plurality of wells 2, which plurality preferably is 96 or 384, although in other embodiments another number of wells can be used. In case the number of wells is 96 they preferably are arranged in 8 rows and 12 columns. When the microplate 1 comprises 384 wells, they are preferably arranged in 16 rows and 24 columns.

In the shown embodiment each well 2 has a square cross-section, although in other embodiments the wells can be circular. Each well 2 has a flat, optically clear bottom portion 3 and opaque side wall surfaces 4, 4'. The clear bottom portion 3 of the shown microplate 1 is formed by a clear bottom plate 3A which is attached to the bottom of the microplate 1 and extends below all the wells 2. Each clear bottom portion 3 of the microplate 1 is ultra-flat. In case a clear bottom plate 3A is used, this clear bottom plate 3A is ultra-flat.

The shown microplate 1 has a footprint of approximately 127.76 x 85.48 mm, and as shown in Figs. 1A and 1B is a Low bottom height (200±10 μm), black cyclic olefin microplate with optically clear, cyclic olefin foil bottom, such as a 384-well SBS-standardized microplate. The micro plate 1 comprises a microplate orientation notch 5 in the lower left corner when the plate is oriented with well 1A in the upper left hand corner.

Prior to use, the wells 2 can be provided with an optically clear matrix 6 (liquid, gel, solidifying material) such that during use (see Fig. 3) at least part of a void volume formed of the interior space of the well 2 not occupied by the tube is filled to avoid optical distortion of an incoming beam

of light, thereby improving (auto)focussing and accurate microscopic imaging.

In Figs. 2A and 2B an embodiment of an insert plate 7 according to the invention is shown in top view and cross-section, respectively. The insert plate 7 is arranged to be detachably attached or inserted into the microplate 1 described above to form an assembly of microplate 1 and insert plate 7, as schematically shown in part in Fig. 3.

The insert plate 7 has a main body 8 comprising a plurality of tubes 9 for receiving a sample. Each tube 9 has a liquid impermeable optically clear bottom portion 10 (Fig. 3) to allow for microscopic imaging. As can be clearly seen in Fig.3 at least part of said bottom portion 10 has a concave arcuate surface, which comprises a hemi-spherical surface or a conical surface.

The number of tubes 9 of the insert plate 7 corresponds to the number of wells 2 of the microplate 1, and amounts to e.g. 94 or 384, and the plurality of tubes 9 is arranged to be detachable attached or inserted, such as to fit into and align with a corresponding well 2 of the microplate 1 when the microplate 1 and the insert plate 7 are attached as shown in Fig. 3. To effect an easy and defined insertion into the microplate 1 the insert plate 7 comprises a insert plate orientation notch 11 in the lower right corner.

In the shown embodiment the tubes 9 have a circular cross section tapered to the concave arcuate surface or round bottom. Moreover in the embodiment shown, the tube depth is between 10 and 13.5mm, preferably about 12 mm, and the tube diameter at the base (i.e. open end) of the tube 9 opposite of the bottom portion 10 is between 3 and 3.7 mm. The tubes 9 preferably taper such that the bottom of the tubes fit into the bottom one-third of the corresponding wells 2 of the microplate 1, in other words the bottom of the tubes 9 occupy one third of the bottom of the wells 2. It will be appreciated that in other embodiments tubes can have of square base.

The depth of each of the tubes 9 is chosen such as to position the concave arcuate surface 10 at a desired distance between 0 to 2 mm, preferably 0 to 1 mm, from the base surface or clear bottom plate 3, 3A of the microplate 1 when the microplate 1 and the insert plate 7 are attached.

Although the desired distance can vary between various tubes, in the shown embodiment the desired distance is essentially the same for each of the tubes 9.

The insert plate 7 is fabricated as a unitary piece from an optically transparent polymer, preferably selected from glycolyzed poly(ethylene terephthalate) (GPET) and polyolefins. For manufacturing the microplate 1 preferably thermoforming plastic foil technology is used.

To facilitate spheroid formation and/or spheroid maintenance during use of the insert plate 7 or during use of the assembly of micro plate 1 and insert plate 7, during which use at least one tube 9 comprises a plurality of living cells (schematically depicted as single item) 12, the at least one tube 9 comprises a low-adhesion or no-adhesion coating 13 on the inner side of the concave arcuate bottom surface 10. In this manner, the cells 12 can form a 3D-structure, like an aggregate or a cluster or a spheroid.

The insert plate or assembly may further comprise a seal, lid or cover plate (not shown) that is positioned over said plurality of tubes, for instance to protect the cells and/or spheroids during culturing/incubating, handling and/or imaging.

Thus the insert plate according to the invention as well as the assembly according to the invention provide a rounded-bottom, thermoformed, in particular injection-molded, optically clear insert plate to seed and grow spheroids, which insert plate can be combined to fit into a flat microplate designed for analysis using conventional high content imaging systems. The optical imaging is performed by focusing through the flat, clear-bottom of the microplate on the spheroids contained in the wells of the

insert plate. Herewith, a convenient and cost-effective spheroid culturing and imaging system can be provided. Fig. 4 shows representative fluorescent images of Spheroids of HepG2 cells grown in the 384-well coated insert plate, stained with Hoechst-33342 nuclear counterstain and imaged at the Operetta. HepG2 spheroids are visible in the middle of the insert that is positioned in the well. An overview of 5 by 5 wells is shown (panel A), where clearly the round HepG2 spheroids can be seen as bright white dots. Furthermore a zoomed image is shown (panel B). After two days ,the HepG2 spheroids have a diameter of 200 µm.

EXPERIMENTAL SECTION

Example 1: Manufacture of an insert plate

The insert plate is suitably manufactured by using thermoforming technology. Thermoforming line includes heating, forming, cutting and stacking of selected plastic material. The plastic material is based on specific customer requirements. Basic raw materials in thermoforming process are polypropylene, polystyrene, polyethylene terephthalate. The raw material is handled in material rolls. Conventional material roll stand can accommodate rolls with a diameter of max. 1200 mm.

The heating method for thermoforming line that manufactures insert plate is radiation heating. In radiation heating the transmission is effected by electromagnetic waves in the infrared range. Infrared rays are absorbed by plastics. The absorption rate is a function of material thickness and wave length of the rays. The thicker the material, the higher the absorption rate. Each material version has its own absorption curve.

Forming can be divided to four steps; pre-forming of heated material by pre-stretching, part forming, cooling of formed part, demolding of formed part.

Three are different ways of pre-forming:

- pre-blowing = formation of a bubble by pressure air
- pre-suction = formation of a bubble by vacuum
- pre-stretching by using a pre stretching plug, also called plug assist or pre-stretcher.
- pre-stretching with mold itself
- combination of all possibilities

Forming possibilities include vacuum forming, forming by pressure air, forming by pressure air and vacuum and forming by embossing.
Colling possibilities of formed parts can be achieved by contact with forming tool, with forming tool + air, by contact with forming tool + cooled air or free cooling by air.
Cutting with a steel rule die is a so called knife cut. Conventional terms for this type of cutting tools: knife cutting tool, steel rule cutting tool.

After forming and punching, thermoformed parts are broken out of the web. This can either be done manually or by forming product stacks in a stacking device. The layout of the stacking station considers the admissible depths of draw. Stacking of positive or negative parts is possible.

Example 2: Ultra Low Attachment (ULA) Coating of 384-well insert plates

Thermoformed 384-tube insert plates were put into 384-well CellCarrier ultra microplates (6057300, PerkinElmer, USA). Next, 50 µl of an ultra low attachment ('ULA') coating solution, such as 0.5% poly 2-hydroxyethyl methacrylate (poly-HEMA) (see Ivascu A., and Kubbies M., Journal of Biomolecular Screening 2006:922-932) or Lipidure-CM5206 (NOF Europe GmbH, Frankfurt, Germany) in 100% ethanol (Sigma-Aldrich, USA), was added to all tubes by dispensing with a Thermo Scientific MultiDrop-384 dispensing station (Thermo Fisher Scientific Oy, Vantaa,

Finland). Plates containing ULA coating solution were incubated for at least one minute at room temperature. Next the coating solution was discarded and the insert plates were dried for two days to obtain ULA coated 384-tube insert plates.

Example 3: Spheroid Formation

HepG2 cells (ATCC HB-8065, LGC Standards GmbH, Wesel, Germany) were cultured in DMEM/F12 + GlutaMax culture medium (31331-028, Life Technologies, Bleiswijk, The Netherlands) supplemented with 10% Fetal Bovine Serum (9215-50, ID-bio, Limoges, France) and 1% Penicillin-Streptomycin (15070-063, Life Technologies) in T25 culture vessels (734-2064, Nunc, Roskilde, Denmark). When HepG2 cells reached a confluency of approximately 80%, cells were washed with PBS and dissociated by incubating for 5 minutes with 0.5% trypsin-EDTA solution in PBS (15400-054, Life Technologies) resulting in single cells. Cells were counted with the Coulter Particle Counter (Beckman Coulter, Woerden, The Netherlands) and subsequently diluted to 4000 cells / ml in culture medium. 30µl to 50µl of cell suspension was added to the tubes of the ULA coated 384-tube insert plate to obtain 120 to 200 cells per tube. The cells were spun down to the bottom of the tubes by a centrifugation step at 800rpm for 2 minutes, and cells were placed in the CO_2 – incubator at 37°C. Spheroid formation was assessed by daily monitoring with a standard Hund Wetzlar Wilovert S inverted light microscope (Helmut Hund Gmbh, Wetzlar, Germany). Already after two days formation of round spheroids was observed. To maintain spheroids half of the culture medium (15 to 25µl) was refreshed every other day.

Example 4: Fluorescent imaging of Spheroids in 384-tube coated inserts

HepG2 cells were cultured for two days in thermoformed 384-tube ULA-coated insert plates to obtain spheroids as described above. HepG2 spheroids were visualized by staining the DNA of the cells. To that end, Hoechst-33342 nuclear counterstain (H3570, Life Technologies) was diluted 500-fold in culture medium, and 15 to 25ul was added to the spheroids to obtain a final concentration of 16.2µM. Spheroids were incubated with Hoechst for two hours to overnight to ensure a homogeneous distribution of dye over the spheroid. Subsequently, spheroids were imaged at the Operetta imaging station (PerkinElmer, Waltham, MA, USA), by using 360-400nm excitation and 410-480nm emission filters. Images were generated with 2x or 10x long working distance (LWD) objectives and captured using the Harmony 4.1 software (PerkinElmer, Waltham, MA, USA). Representative images are shown in Figure 4.

Claims

1. An insert plate for detachably attaching to a microplate having dimensions established by the Society of Biomolecular Sciences (SBS Standards) and having a plurality of wells, the insert plate having a main body comprising a plurality of tubes for receiving a sample, each tube having a liquid impermeable optically clear bottom portion to allow for microscopic imaging and wherein at least part of said bottom portion has a concave arcuate surface, the plurality of tubes being positioned to fit into and align with a corresponding well of the microplate when the microplate and the insert plate are attached.

2. Insert plate according to claim 1, wherein the tubes have a circular cross section or square base tapered to round bottom.

3. Insert plate according to claim 1 or 2, wherein the concave arcuate surface comprises a hemi-spherical surface or a conical surface.

4. Insert plate according to any one of the preceding claims, wherein each of the tubes has a depth to position the concave arcuate surface at a desired distance from the base surface of the microplate when the microplate and the insert plate are attached.

5. Insert plate according to claim 4, wherein said desired distance is essentially the same for each of the tubes.

6. Insert plate according to claim 4 or 5, wherein said desired distance is 0 to 2 mm, preferably 0 to 1 mm.

7. Insert plate according to any one of the preceding claims, fabricated as a unitary piece from an optically transparent polymer, preferably selected from glycolyzed poly(ethylene terephthalate) (GPET and polyolefins.

8. Insert plate according to any one of the preceding claims, wherein the tubes comprise a low-adhesion or no-adhesion coating on the concave arcuate bottom surface

9. Insert plate according to any one of the preceding claims, wherein the insert plate comprises a plate orientation notch in the lower left corner

10. Insert plate according to any one of the preceding claims, comprising 96 or 384 tubes to fit into a microplate comprising, respectively, 96 or 384 wells.

11. Insert plate according to claim 10, comprising 96 tubes arranged in 8 rows and 12 columns.

12. Insert plate according to claim 10, comprising 384 tubes arranged in 16 rows and 24 columns.

13. Insert plate according to any one of the preceding claims, obtained by means of thermoforming plastic foil technology.

14. An assembly of (i) an insert plate according to any one of claims 1 to 13, detachably attached to (ii) a standardized microplate having a plurality of wells, each well having a flat, optically clear bottom portion and opaque side wall surfaces.

15. Assembly according to claim 14, wherein the number of tubes of the insert plate corresponds to the number of wells of the microplate

16. Assembly according to claim 14 or 15, wherein the bottom surface of the tubes fit into the bottom one-third of the corresponding wells of the microplate.

17. Assembly according to any one of claim 14 to 16, wherein each of the tubes has a length sized to position the concave arcuate bottom surface at a distance of about 0 to 2 mm from the flat, transparent bottom portion of

the well of the microplate when the microplate and the insert plate are attached.

18. Assembly according to any one of claim 14 to 17, wherein the footprint of said microplate is approximately 127.76 x 85.48 mm.

19. Assembly according to any one of claim 14 to 18, wherein said microplate is a 384-well SBS-standardized microplate.

20. Assembly according to any one of claim 14 to 19, wherein the clear bottom portion of the microplate is ultra-flat.

21. Assembly according to any one of claim 14 to 20, wherein said microplate is a Low bottom height (200±10 µm), black cyclic olefin microplate with optically clear, cyclic olefin foil bottom.

22. Assembly according to any one of claim 14 to 21, wherein at least one of the wells is provided with an optically clear matrix (liquid, gel, solidifying material) to fill at least part of the void volume formed by the interior space of the well not occupied by the tube.

23. Insert plate or assembly according to any one of the preceding claims, wherein at least one tube comprises a plurality of living cells.

24. Insert plate or assembly according to claim 23, wherein said cells are in suspension or wherein said cells form a 3D-structure, like aggregate or cluster or spheroid.

25. Insert plate or assembly according to any one of the preceding claims, further comprising a seal, lid or cover plate that is positioned over said plurality of tubes.

26. A method for providing an insert plate according to any one of claims 1 to 13, comprising subjecting a transparent polymer sheet to (vacuum) thermoforming technology.

27. Method according to claim 26, wherein said transparent polymer sheet has a thickness of between 0.3 and 1 mm, preferably between 0.5 and 0.8 mm.

28. Method according to claim 26 or 27, wherein said transparent polymer sheet is a glycolyzed poly(ethylene terephthalate) (GPET) sheet or a polyolefin sheet.

29. A method of spheroid handling, comprising:

a) inserting a plurality of cells into at least one tube of an assembly according to any one of claim 14-22 and allowing the formation of spheroid formation; and

b) performing on one or more of the spheroid handling methods selected from the group consisting of spheroid culturing, spheroid maintaining, spheroid analysis, spheroid testing, and combinations thereof.

30. Method according to claim 29, wherein said method comprises optical imaging, preferably using an automated microplate imager

31. Method according to claim 30, wherein said optical imaging involves high resolution fluorescence and/or brightfield microscopy in a high-throughput manner with a High Content Screening system.

32. Method according to claim 29 or 30, comprising contacting the spheroid(s) with a potential therapeutic agent.

33. Method according to claim 32, further comprising assaying for a marker indicative of modulation of a cellular target of said potential therapeutic agent or screening for activity in modulating the phenotype of a spheroid.

34. Method according to any one of claims 29-33, wherein the cells are neoplastic cells.

35. Method according to any one of claims 29-34, wherein the cells are human malignant tumor cells selected from the group consisting of breast cancer, lung cancer, prostate cancer, colon cancer, melanoma cancer, and cancer of the bone and connective tissues.

36. Method according to any one of claims 29-33, wherein the cells are stem cells selected from the group consisting of embryonic stem cells or adult stem cells, progenitor cells, bone marrow stromal cells macrophages, fibroblast cells, endothelial cells, epithelial cells, and mesenchymal cells

37. Use of an insert plate, assembly or method according to any one of claims 1-36 in a high throughput high throughput drug discovery program.

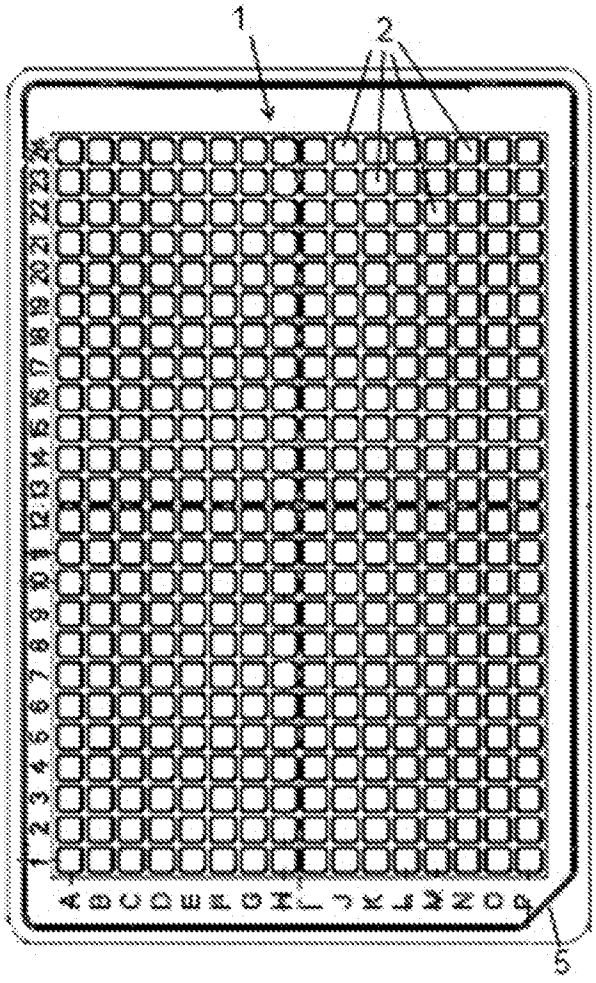

Fig. 1A

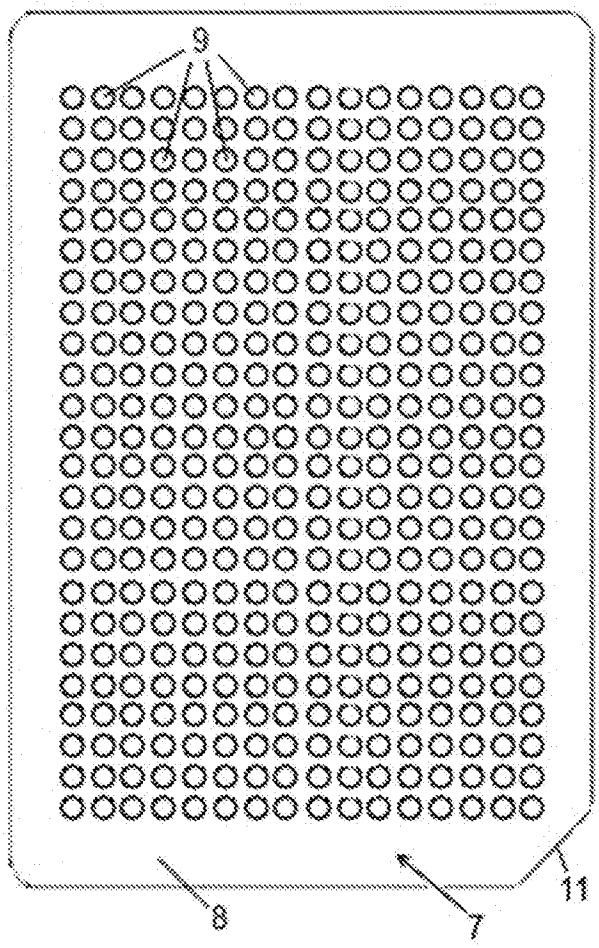

Fig. 2A

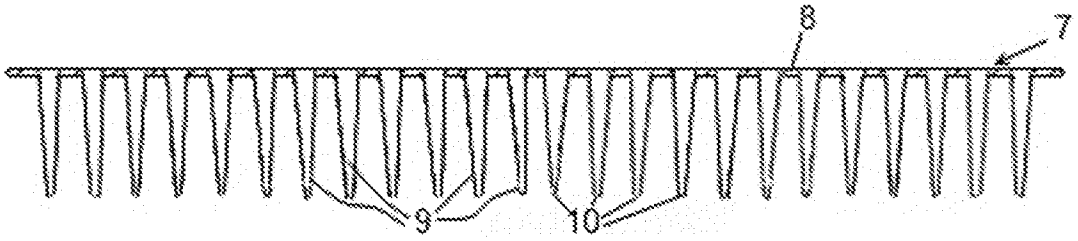

Fig. 2B

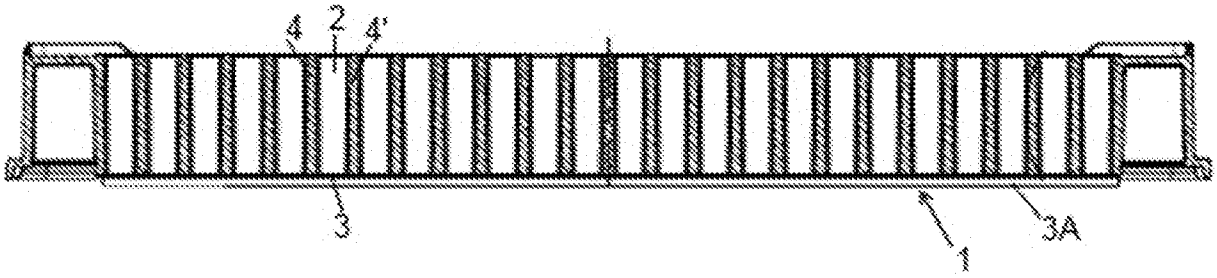

Fig. 1B

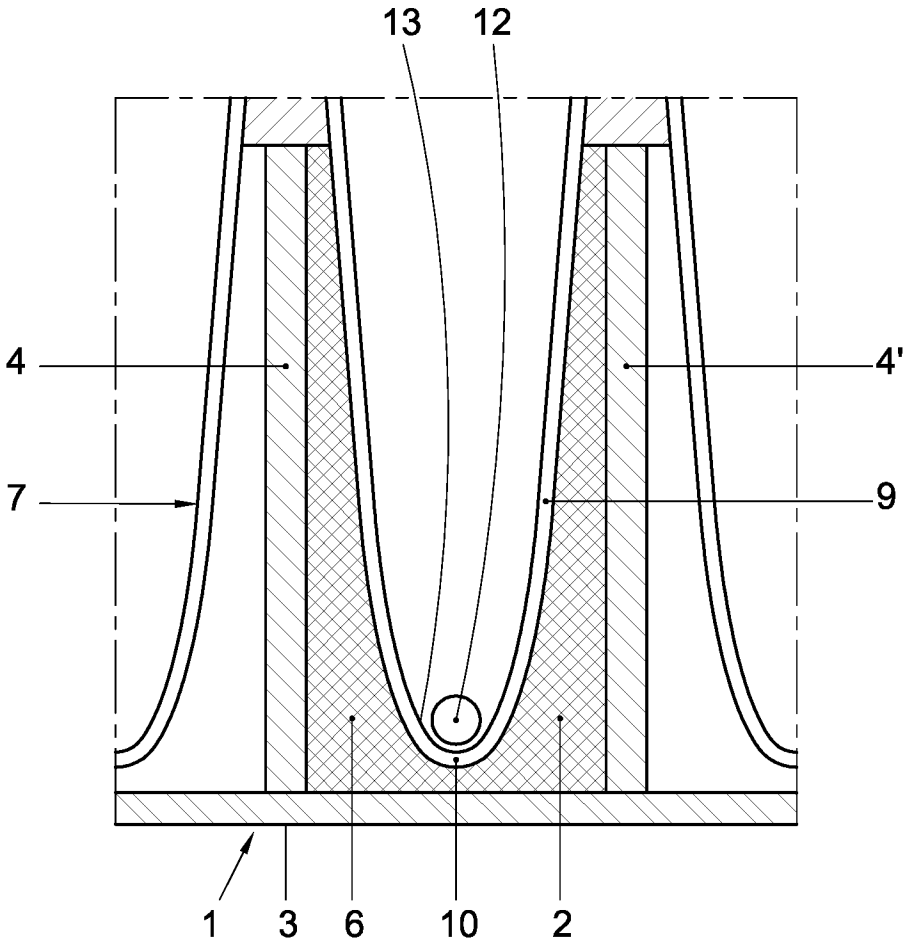

Fig. 3

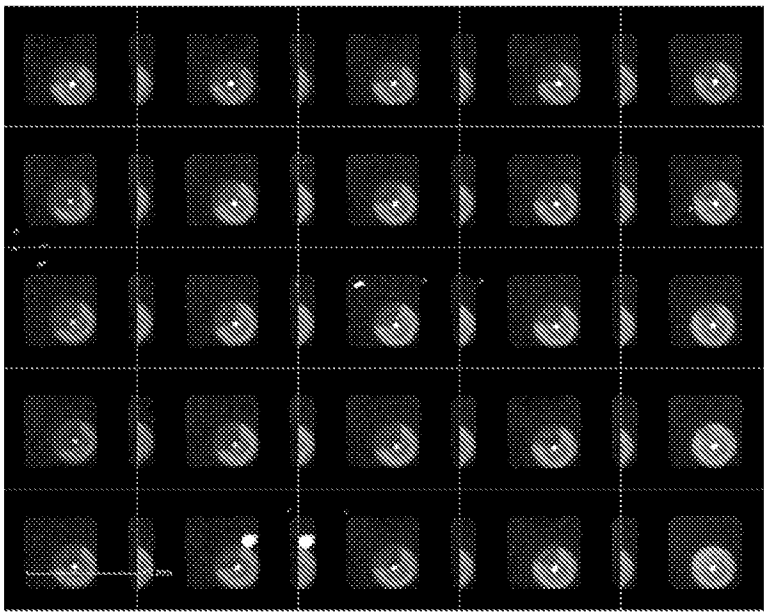

Fig. 4A

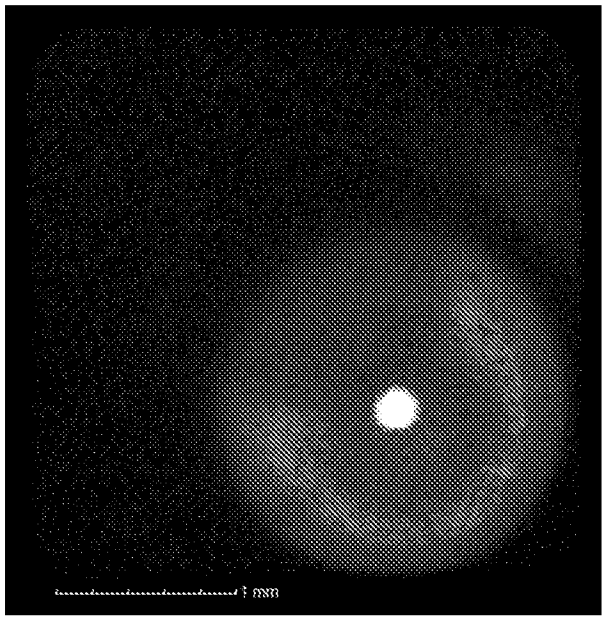

Fig. 4B

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2017/050098

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12M1/32 B01L3/00 C12M1/00 C12M1/12
ADD.

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)
C12M B01L G01N

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)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2014/233806 A1 (GOSSWEILER RICHARD [US] ET AL) 21 August 2014 (2014-08-21) cited in the application paragraphs [0003] - [0007], [0033], [0042] figures 3,4,6	1-37
Y	US 2007/237683 A1 (HO WINSTON Z [US] ET AL) 11 October 2007 (2007-10-11) paragraphs [0003], [0006] - [0009], [0013], [0018] - [0020], [0031] - [0033], [0035], [0038], [0039] figures 5a,5b	1-37

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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

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Date of the actual completion of the international search	Date of mailing of the international search report
27 April 2017	09/05/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Böhm, Ingo

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE 20 2011 110503 U1 (UNIV MICHIGAN [US]) 4 December 2014 (2014-12-04) paragraphs [0009] - [0011], [0018] - [0034] figures 1a,1d	1,14,26, 29,37
A	WO 2010/034013 A1 (HELIXIS INC [US]; SILBERGLEIT ARKADIY [US]; FAWCETT ADRIAN [US]; SWART) 25 March 2010 (2010-03-25) paragraphs [0005] - [0011], [0021] - [0028] figures 1-3	1,14,26, 29,37
A	US 2007/154357 A1 (SZLOSEK PAUL M [US]) 5 July 2007 (2007-07-05) figures 1,3 paragraphs [0010], [0016] - [0021]	1,14,26, 29,37
A	WO 2015/069742 A1 (UNIV JOHNS HOPKINS [US]) 14 May 2015 (2015-05-14) page 2, line 21 - page 3, line 25	1,14,26, 29,37
A	US 2005/112030 A1 (GAUS STEPHANIE E [US]) 26 May 2005 (2005-05-26) paragraphs [0004], [0022] - [0028] figures 4a,5	1

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2017/050098

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2014233806 A1	21-08-2014	NONE	
US 2007237683 A1	11-10-2007	CN 101046440 A	03-10-2007
		US 2007237683 A1	11-10-2007
DE 202011110503 U1	04-12-2014	CA 2788575 A1	04-08-2011
		CN 102947710 A	27-02-2013
		DE 202011110503 U1	04-12-2014
		EP 2529238 A2	05-12-2012
		JP 5847733 B2	27-01-2016
		JP 2013517809 A	20-05-2013
		US 2013040855 A1	14-02-2013
		US 2014179561 A1	26-06-2014
		WO 2011094572 A2	04-08-2011
WO 2010034013 A1	25-03-2010	US 2010103410 A1	29-04-2010
		WO 2010034013 A1	25-03-2010
US 2007154357 A1	05-07-2007	NONE	
WO 2015069742 A1	14-05-2015	US 2016281061 A1	29-09-2016
		WO 2015069742 A1	14-05-2015
US 2005112030 A1	26-05-2005	NONE	