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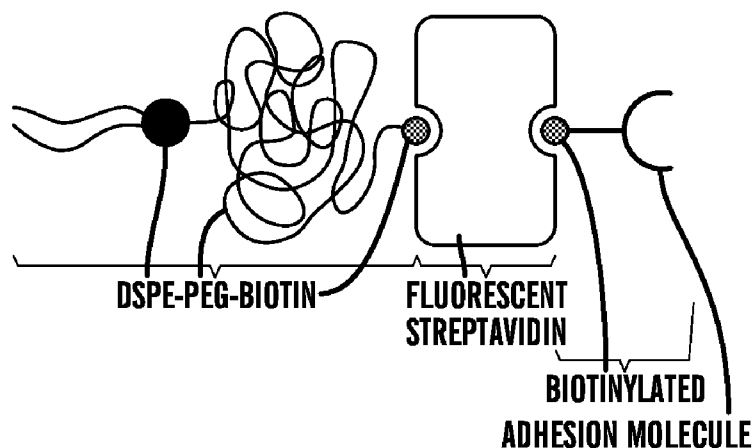
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(54) Title: COMPOSITIONS FOR DETECTING CELLULAR FORCES

**FIG. 1E**

(57) Abstract: Described herein are methods and compositions relating to the measurement of the forces exerted by and between cells. For example, the compositions can relate to droplets comprising an optically detectable molecule (e.g. a fluorescent molecule), an inner biocompatible oil layer, and an outer surfactant-transducer layer. The distortion of these droplets by cells can be detected and permits the measurement of the forces exerted by the cells.

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- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
- *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

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COMPOSITIONS FOR DETECTING CELLULAR FORCES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application Nos. 61/825,580 filed May 21, 2013 and 61/847,866 filed July 18, 2013, the contents of which are incorporated herein by reference in their entirety.

TECHNICAL FIELD

[0002] The technology described herein relates to methods, probes and compositions for detecting the forces exerted by and between cells.

BACKGROUND

[0003] Cell-generated mechanical forces play a critical role during tissue morphogenesis and organ formation in the embryo. However, little is known about how these forces shape embryonic organs, mainly because it has not been possible to measure cellular forces within developing three-dimensional (3D) tissues *in vivo*. Measuring cellular forces *in vivo* has proven very challenging. To date, the only available technique to probe cellular tension is Laser Ablation. While useful to qualitatively estimate the tension state at a cell junction and even in portions of a tissue (Rauzi, M et al. *Current Topics in Developmental Biology* 95, 93-144 (2011); Rauzi, M., et al. *Nat Cell Biol* 10, 1401-1410 (2008); Behrndt, M. et al. *Science* 338, 257-260 (2012); Hutson, M. S. *Science* 300, 145-149 (2003)), it does not provide a quantitative measure of cellular forces.

SUMMARY

[0004] Described herein are methods, probes and compositions which permit the quantitative measurement of forces exerted by and between cells. Importantly, the methods and compositions described herein permit such measurements *in vitro*, *ex vivo* and *in vivo*, including *in vitro* measurements with individual cells, cell monolayers and 3D cellular aggregates, as well as measurements *ex vivo* and *in vivo* with living tissues and developing organs. Embodiments of the technology described herein relate to fluorescent oil microdroplets which can bind to (or be bound by) cells. By measuring the deformation of the microdroplets and knowing the precise mechanical properties of the microdroplet, the stresses (force per unit surface) that cells apply at every point on the droplet surface can be determined. This permits the direct quantification of cellular stresses generated within living embryonic tissues.

BRIEF DESCRIPTION OF THE DRAWINGS

[0005] Figs. 1A-1E demonstrate the use of oil microdroplets as force transducers. Fig. 1A depicts a schematic of isolated spherical oil droplets in solution (left) and a droplet embedded in-between the cells forming an embryonic tissue (right); the deformation of the droplet is a consequence of local cellular forces. Fig. 1B depicts an image of a confocal section of an isolated fluorocarbon oil droplet coated as described in the main text. Droplet surface is fluorescently labeled with Cy5-streptavidin. Bar, 10 μm . Fig. 1C depicts a sketch of the interface between fluorocarbon oil and surrounding medium, indicating the different molecules involved in the coating (functionalization) of the droplets. Fig. 1D depicts a schematic of fluorocarbon-hydrocarbon (Krytox-DDA) diblocks used to vary the interfacial tension and Fig. 1E depicts a schematic of surfactant molecules (DSPE-PEG-biotin) used to stabilize and control the surface properties of the droplet.

[0006] Figs. 2A-2E depict cellular force measurements in epithelial and mesenchymal cell aggregates. Fig. 2A depicts a schematic of cells-droplets aggregate formation. Functionalized droplets and cells are mixed, compacted and cultured to form aggregates. Oil droplet shapes are obtained via high-resolution 3D confocal imaging and their surface coordinates are obtained from image analysis of the data. Fig. 2B depicts an image of a confocal section through an aggregate of GFP-positive tooth mesenchymal cells containing fluorocarbon droplets coated externally with ligands for integrin receptors. Fig. 2C depicts an example of 3D reconstruction of a droplet in a tooth mesenchymal cell aggregate with the values of the anisotropic stresses mapped on the droplet surface. Fig. 2D depicts an image of a confocal section through an aggregate of mammary epithelial cells (DNA is visible) and fluorocarbon droplets coated externally with ligands for E-cadherin receptors. Fig. 2E depicts an example of 3D reconstruction of a droplet in a mammary epithelial cell aggregate with the values of the anisotropic stresses mapped on the droplet surface. Gray arrows next to stress scales indicate the average values of the maximal anisotropic stresses obtained from statistics on 2D confocal sections of multiple droplets. Scale bars, 20 μm .

[0007] Figs. 3A-3H demonstrate ensemble statistics of droplet deformations in aggregates using 2D droplet confocal sections. Fig. 3A depicts an image of a confocal section of a droplet with the detected droplet contour overlaid. Fig. 3B depicts a schematic demonstrating that the contour is parametrized by its contour length normalized by the total contour length L . Fig. 3C depicts a graph of the calculated curvature along the contour. The average curvature, maximal curvature and the difference between the maximal and minimal values of

the curvature are defined as κ_0 , κ_p and $\Delta\kappa$, respectively. Fig. 3D depicts a schematic of undeformed and deformed confocal sections of a droplet with definitions of the droplet average radius of curvature $R_0 = 1/\kappa_0$ and the minimal radius of curvature along the contour $R_p = 1/\kappa_p$. Fig. 3E depicts a graph of normalized frequency of $\Delta\kappa$ for droplets in aggregates of mammary epithelial cells and tooth mesenchymal cells. Fig. 3F depicts a graph of relative droplet deformation $|R_p - R_0|/R_0$ as a function of the radius R_0 of the undeformed droplet section. Solid lines depict the envelope of maximal values of relative droplet deformation. The vertical bars indicate the measured values (mean (vertical dark gray line) $\pm$ standard deviation of the mean (light gray bar)) of cell size in the aggregates (see Example 2). Figs. 3G-3H depict images of the effects of inhibition of myosin II and actin polymerization on cellular forces, using (3G) Blebbistatin and (3H) Cytochalasin D, respectively. Confocal sections through mammary epithelial cell aggregates (DNA is visible) showing deformed droplets before addition of the drugs and 20 minutes after drugs were added. Droplets round up as a consequence on myosin II and actin polymerization inhibition, indicating a considerable reduction on the forces applied to the droplets.

[0008] Figs. 4A-4K demonstrate the use of oil droplets as force transducers in living tooth mandibles. Fig. 4A depicts an image of a mouse embryo at 11 days post fertilization (E11). Fig. 4B depicts an image of dissected, living tooth mandible (mandibular arch) at stage E11. Fig. 4C depicts an image of maximal intensity projection of a 3D reconstruction of a fluorescent reporter E13.5 embryonic mouse mandible. Epithelial cells express N-terminal membrane tagged version of EGFP and all other cells express an N-terminal membrane tagged version of tdTomato. Fig. 4D depicts an image which is the same as in Fig. 4C but showing only the epithelium. The thickening of the epithelium characteristic of tooth bud formation at E13.5 appears as localized increase in the EGFP signal (arrows indicate the location of epithelial thickening). Fig. 4E depicts an image of a confocal section of an incisor tooth bud. Fig. 4F depicts an image of an enlarged region of 4E showing the boundary between epithelial cells and mesenchymal cells. Fig. 4G depicts a schematic of functionalized droplet micro-injection in a dissected living mandible. Fig. 4H depicts an image of a confocal section of an incisor tooth bud with a fluorocarbon droplet (droplet surface labeled fluorescently) embedded in between cells of the dental mesenchyme. White arrow indicates the location of the droplet. Fig. 4I depicts an image of an enlarged region in 4H showing a close-up of the embedded droplet. Fig. 4J depicts an image of detected pixel-resolution contour of droplet in 4I. Fig. 4K depicts an image of detected pixel-resolution contour of a

droplet embedded in living tooth mesenchymal tissue showing the correspondence between higher curvature regions (arrows) on the droplet surface and cell-cell junctions contacting the droplet. Scale bars, 20 μm , except in 4C-4D, which are 200 μm .

[0009] Figs. 5A-5B depict the statistics of droplet deformations in living tooth mandibles. Fig. 5A depicts a graph of normalized frequency of $\Delta\kappa$ for confocal sections of multiple droplets in the dental mesenchyme of living mandibles at E11. Droplet interfacial tension is 4 mN/m. Fig. 4B depicts a graph of relative droplet deformation $|R_p - R_0|/R_0$ as a function of the radius R_0 of the undeformed droplet section ($R_0 = 1/\kappa_0$). Solid line (black) depicts the envelope for maximal values of relative droplet deformation in E11 living mandibles. The data for droplets in tooth mesenchymal cell aggregates (same as in Fig. 3F) is shown for comparison. The vertical bar indicates the measured value (mean (vertical dark gray line) $\pm$ standard deviation of the mean (light gray bar)) of mesenchymal cell size in living mandibles.

[0010] Figs. 6A-6E depict the characterization of cells and droplets. Fig. 6A depicts a schematic of the imaging setup. Fig. 6B depicts microscopy images of tooth mesenchymal cells. Fig. 6C depicts images of mammary epithelial cells. Fig. 6D depicts a schematic of the experimental approach to test adhesion of cells to droplets. Fig. 6E depicts microscopy images of the experiment shown in Fig. 6D.

[0011] Figs. 7A-7F depict the detection of droplet contour on confocal sections of droplets. Fig. 7A depict the original confocal section. Fig. 7B depicts the image after filtering with steerableJ filtering. Fig. 7C depicts the detection of the droplet contour. Fig. 7D depicts detection of the contour at pixel resolution. Figs. 7E-7F depict the generation of a closed B-spline curve, specifying a continuous curve for the droplet contour (Fig. 7E and Fig. 7F).

[0012] Figs. 8A-8D depict the detection of droplet contour from 3D confocal stacks. Fig. 8A depicts a schematic of confocal sections of the droplet. Fig. 8B depicts schematic of contour coordinates for each confocal section combined to obtain the coordinates of the droplet surface in 3D. Fig. 8C depicts a 2D B-Spline of the entire droplet surface. Fig. 8D depicts a graph of the mean curvature at each point of the droplet surface.

[0013] Figs. 9A-9J demonstrate the distribution of cellular sizes in cultured aggregates of mammary epithelial cells and tooth mesenchymal cells, and in living mandibles. Figs. 9A-9C demonstrate the measure of cellular sizes in aggregates of mammary epithelial cells. Fig. 9A depict an image of a confocal section through an aggregate of DNA-labeled mammary epithelial cells. Fig. 9B depicts a schematic of the nuclei in the aggregate with the definition

of the distance d between nearest neighbor nuclei. Fig. 9C depicts a graph of the distribution of d . Figs. 9D-9G depict the measure of cellular sizes in aggregates of GFP-positive tooth mesenchymal cells. Fig. 9D depicts an image of a confocal section through an aggregate. Fig. 9E depicts a schematic of the typical oblate cell shape and the definition of the lent and short axes, b and a respectively. Fig. 9F depicts a graph of measured values of a and b . Fig. 9G depicts a graph of distributions of a and b . Fig. 9H-9J depict the measure of cellular sizes in the dental mesenchyme of tooth mandibles at developmental stage E11. Fig. 9H depicts an image of a confocal section through the dental mesenchyme of a DNA-labeled mandible. Fig. 9I depicts an image of a confocal section of the dental mesenchyme of a mandible with a fluorescent membrane reporter for tooth mesenchymal cells. Fig. 9J depicts a graph of the distribution of tooth mesenchymal cell sizes in the dental mesenchyme of mouse mandibles.

[0014] Figs. 10A-10F are graphs demonstrating the time dependence of the surface/interfacial tension of FC70 oil at each step of the coating (functionalization) process. Fig. 10A depicts the surface tension of the FC70-air surface. Fig. 10B depicts the interfacial tension of FC70 and purified water. Fig. 10C depicts the interfacial tension of FC70 and a water solution containing DSPE-PEG2000-biotin surfactants at a concentration of 0.2 mM. The recordings of the interfacial tension started about two minutes after addition of the DSPE-PEG2000-biotin solution. Fig. 10D depicts the interfacial tension of FC70 containing a surface layer of DSPE-PEG2000-biotin surfactants with a water solution containing fluorescent streptavidin (FITC-streptavidin) at a concentration of 1 μ M. Fig. 10E depicts the interfacial tension of FC70 containing a surface layer of DSPE-PEG2000-biotin:streptavidin(FITC) with cell/tissue culture media at room temperature. The interfacial tension diminishes over time until it reaches its equilibrium value. Fig. 10F depicts the interfacial tension of the result in step at a temperature of 37°C.

DETAILED DESCRIPTION

[0015] In one aspect, described herein is a composition comprising a force measurement droplet, the droplet comprising a volume or layer of a biocompatible and/or incompressible oil, a surfactant-transducer layer; and an optically detectable molecule. As described herein, a “force measurement droplet” refers to any of the embodiments of microdroplets described herein, comprising at least an incompressible oil and a surfactant-transducer layer.

[0016] In some embodiments, the incompressible oil can be a biocompatible fluorocarbon or perfluorinated oil. In some embodiments, the oil can be Fluoroinert FC-70 fluorocarbon

oil (Perfluorotripropylamine). In some embodiments, the oil can be biocompatible silicon oil. In some embodiments, the oil can be biocompatible mineral oil.

[0017] As used herein, “a surfactant-transducer layer” refers to a layer comprising one or more types of molecules, comprising at least a surfactant and a molecule that can specifically bind to or be bound by a cell surface (e.g. a cell adhesion molecule).

[0018] In some embodiments, the surfactant-transducer layer comprises a layer comprising an amphiphilic-linker molecule bound to or attached to a cell adhesion molecule. In some embodiments, the cell adhesion molecule is directly adsorbed onto the oil droplet interface, acting itself as surfactant.

[0019] In some embodiments, the amphiphilic-linker molecule can be directly conjugated to the cell adhesion molecule, e.g. by a covalent bond. In some embodiments, the amphiphilic-linker molecule comprises an amphiphilic molecule conjugated to a linker molecule wherein the linker molecule can bind, or be bound by, a cell adhesion molecule. Examples of linker molecules are well known in the art, e.g. antibodies or biotin-streptavidin. In some embodiments, the amphiphilic-linker molecule comprises a linker molecule that binds a ligand molecule on the cell adhesion molecule. In some embodiments, the amphiphilic-linker molecule comprises a ligand molecule that is bound by a linker molecule on the cell adhesion molecule.

[0020] In some embodiments, the amphiphilic molecule is a phospholipid. In some embodiments, the amphiphilic molecule is 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE). In some embodiments, the surfactant-linker molecule can be 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) with a polyethylene glycol spacer linked to biotin (PEG-biotin) attached to its head group. In some embodiments, the amphiphilic molecule can be a phospholipid molecule. In some embodiments, the amphiphilic molecule can be a phospholipid molecule with modified headgroup, e.g. containing biotin or PEG-biotin groups.

[0021] In some embodiments, the cell adhesion molecule can be selected from the group consisting of an integrin ligand; RGD peptide; a cadherin ligand; anti-E-cadherin antibody. Non-limiting examples of integrin ligands can include RGD peptide, osteopontin, BSP, MGF-E8, vitronectin, vWF, tenascin, LAP-TGF-beta, fibrillin, fibrinogen, factor X, iC3b, E-cadherin, iCAM, VCAM-1, collagen, fibronectin, thrombospondin, laminin, LDV peptide, and fragments thereof. Non-limiting examples of cadherin ligands can include other cadherins, including both homophilic and heterophilic interactions.

[0022] In some embodiments, the surfactant-transducer layer and/or the oil layer comprises an optically detectable molecule. An optically detectable molecule can be, e.g. a fluorescently detectable molecule and a luminescently detectable molecule. In some embodiments, the optically detectable molecule can be bound to, e.g. conjugated to the amphiphilic molecule or the cell adhesion molecule. In some embodiments, the optically detectable molecule is an optically detectable streptavidin molecule conjugated to the cell adhesion molecule via a biotin group. In some embodiments, the optically detectable molecule is a fluorescently detectable streptavidin molecule conjugated to the cell adhesion molecule via a biotin group. In some embodiments, the amphiphilic surfactant is itself a fluorescent molecule. In some embodiments, the ligand for cell adhesion receptors is itself a fluorescent molecule. In some embodiments, the oil layer of the force measurement droplet is labeled fluorescently.

[0023] In some embodiments, e.g. when the microdroplet is to be used in a tissue environment, the oil layer (or phase) of the droplet can further comprise a specific soluble co-surfactant. Non-limiting examples of co-surfactants can include fluorocarbon-hydrocarbon molecules; fluorocarbon-hydrocarbon diblocks; Krytox-Dodecylamine.

[0024] In some embodiments, the droplet can be from about 0.5 to about 500 μm in radius. In some embodiments, the droplet can be from 0.5 to 500 μm in radius. In some embodiments, the droplet can be from about 2 to about 40 μm in radius. In some embodiments, the droplet can be from 2 to 40 μm in radius.

[0025] In one aspect, described herein is a method of measuring the mechanical forces exerted by cells on their surrounding environment, the method comprising contacting a cell or group of cells with a composition comprising a force measurement droplet as described herein; binding a droplet of the composition to one or more of the cells and measuring the strength and distribution of a signal from the optically detectable molecule wherein the signal from the optically detectable molecule indicates the location of the droplet surface. The magnitude of the cellular forces contacting the force measurement droplet is obtained from the detected droplet surface shape as described herein. In some embodiments, the contacting step can comprise contacting a cell suspension with the composition, centrifuging the mixture to pellet the cells into an aggregate, and maintain the cells in culture for at least 6 hours. In some embodiments, the contacting step can comprise injecting the composition into a living tissue.

[0026] In some embodiments, measuring the strength and distribution of the signal comprises imaging one or more droplets by epifluorescence microscopy, confocal microscopy, multi-photon microscopy or light-sheet microscopy.

[0027] In some embodiments, cellular stresses (forces per unit surface) at the force measurement droplet surface can be determined using Eq. 1.

$$p'_i = p'_e + 2\gamma H(\theta, \phi) - \sigma_{nn}(\theta, \phi) \quad (1)$$

wherein γ is the interfacial tension of the droplet and the surrounding medium, σ_{nn} is the cellular forces per unit area, H is the local mean curvature of the droplet surface, θ and ϕ are angular spherical coordinates, p'_i and p'_e are, respectively, the droplet internal and external hydrostatic pressures.

[0028] In some embodiments, the anisotropic cellular stresses are mapped to the droplet surface using Eq. 2:

$$\delta\sigma_{nn}(\theta, \phi) = 2\gamma \delta H(\theta, \phi) = 2\gamma \left(H(\theta, \phi) - \frac{1}{R} \right) \quad (2)$$

wherein R is the radius of the initial undeformed spherical droplet and the isotropic component H_i is $1/R$ and wherein the tissue pressure $P_i = (p'_i - p_i) - (p'_e - p_e)$.

[0029] In some embodiments, the average maximal anisotropic stresses can be determined using Eq. 3:

$$\overline{\delta\sigma_{nn}^M} = \gamma \overline{\Delta\kappa} \quad (3)$$

wherein $\overline{\delta\sigma_{nn}^M}$ is the average value of the maximal anisotropic stresses and $\overline{\Delta\kappa}$ is the average value of the maximal curvature difference.

[0030] For convenience, the meaning of some terms and phrases used in the specification, examples, and appended claims, are provided below. Unless stated otherwise, or implicit from context, the following terms and phrases include the meanings provided below. The definitions are provided to aid in describing particular embodiments, and are not intended to limit the claimed invention, because the scope of the invention is limited only by the claims. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention

belongs. If there is an apparent discrepancy between the usage of a term in the art and its definition provided herein, the definition provided within the specification shall prevail.

[0031] For convenience, certain terms employed herein, in the specification, examples and appended claims are collected here.

[0032] The terms “decrease”, “reduced”, “reduction”, or “inhibit” are all used herein to mean a decrease by a statistically significant amount. In some embodiments, “reduce,” “reduction” or “decrease” or “inhibit” typically means a decrease by at least 10% as compared to a reference level (e.g. the absence of a given treatment) and can include, for example, a decrease by at least about 10%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, at least about 99% , or more. As used herein, “reduction” or “inhibition” does not encompass a complete inhibition or reduction as compared to a reference level.

“Complete inhibition” is a 100% inhibition as compared to a reference level. A decrease can be preferably down to a level accepted as within the range of normal for an individual without a given disorder.

[0033] The terms “increased”, “increase”, “enhance”, or “activate” are all used herein to mean an increase by a statically significant amount. In some embodiments, the terms “increased”, “increase”, “enhance”, or “activate” can mean an increase of at least 10% as compared to a reference level, for example an increase of at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100% as compared to a reference level, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level. In the context of a marker or symptom, a “increase” is a statistically significant increase in such level.

[0034] As used herein, a "subject" means a human or animal. Usually the animal is a vertebrate such as a primate, rodent, domestic animal or game animal. Primates include chimpanzees, cynomologous monkeys, spider monkeys, and macaques, e.g., Rhesus. Rodents include mice, rats, woodchucks, ferrets, rabbits and hamsters. Domestic and game animals include cows, horses, pigs, deer, bison, buffalo, feline species, e.g., domestic cat,

canine species, e.g., dog, fox, wolf, avian species, e.g., chicken, emu, ostrich, and fish, e.g., trout, catfish and salmon. In some embodiments, the subject is a mammal, e.g., a primate, e.g., a human. The terms, "individual," "patient" and "subject" are used interchangeably herein.

[0035] As used herein, the terms "protein" and "polypeptide" are used interchangeably herein to designate a series of amino acid residues, connected to each other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. The terms "protein", and "polypeptide" refer to a polymer of amino acids, including modified amino acids (e.g., phosphorylated, glycosylated, etc.) and amino acid analogs, regardless of its size or function. "Protein" and "polypeptide" are often used in reference to relatively large polypeptides, whereas the term "peptide" is often used in reference to small polypeptides, but usage of these terms in the art overlaps. The terms "protein" and "polypeptide" are used interchangeably herein when referring to a gene product and fragments thereof. Thus, exemplary polypeptides or proteins include gene products, naturally occurring proteins, homologs, orthologs, paralogs, fragments and other equivalents, variants, fragments, and analogs of the foregoing.

[0036] As used herein, the term "specific binding" refers to a chemical interaction between two molecules, compounds, cells and/or particles wherein the first entity binds to the second, target entity with greater specificity and affinity than it binds to a third entity which is a non-target. In some embodiments, specific binding can refer to an affinity of the first entity for the second target entity which is at least 10 times, at least 50 times, at least 100 times, at least 500 times, at least 1000 times or greater than the affinity for the third nontarget entity.

[0037] As used herein, the term "biocompatible" refers to substances that are not toxic to cells. In some embodiments, a substance is considered to be "biocompatible" if its addition to cells in vitro results in less than or equal to approximately 20% cell death. In some embodiments, a substance is considered to be "biocompatible" if its addition to cells in vivo does not induce major adverse effects, e.g. inflammation.

[0038] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about." The term "about" when used in connection with percentages can mean $\pm 1\%$.

[0039] As used herein the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the method or composition, yet open to the inclusion of unspecified elements, whether essential or not.

[0040] The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[0041] As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment.

[0042] The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The abbreviation, "e.g." is derived from the Latin *exempli gratia*, and is used herein to indicate a non-limiting example. Thus, the abbreviation "e.g." is synonymous with the term "for example."

[0043] Definitions of common terms in cell biology and molecular biology can be found in *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); Benjamin Lewin, *Genes X*, published by Jones & Bartlett Publishing, 2009 (ISBN-10: 0763766321); Kendrew et al. (eds.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8) and *Current Protocols in Protein Sciences 2009*, Wiley Intersciences, Coligan et al., eds.

[0044] Unless otherwise stated, the present invention was performed using standard procedures, as described, for example in Sambrook et al., *Molecular Cloning: A Laboratory Manual* (3 ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (2001); Davis et al., *Basic Methods in Molecular Biology*, Elsevier Science Publishing, Inc., New York, USA (1995); *Current Protocols in Protein Science (CPPS)* (John E. Coligan, et. al., ed., John Wiley and Sons, Inc.), *Current Protocols in Cell Biology (CPCB)* (Juan S. Bonifacino et. al. ed., John Wiley and Sons, Inc.), and *Culture of Animal Cells: A Manual of Basic Technique* by R. Ian Freshney, Publisher: Wiley-Liss; 5th edition (2005), *Animal Cell Culture Methods* (Methods in Cell Biology, Vol. 57, Jennie P. Mather and David Barnes

editors, Academic Press, 1st edition, 1998) which are all incorporated by reference herein in their entirety.

[0045] Other terms are defined herein within the description of the various aspects of the invention.

[0046] All patents and other publications; including literature references, issued patents, published patent applications, and co-pending patent applications; cited throughout this application are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the technology described herein. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

[0047] The description of embodiments of the disclosure is not intended to be exhaustive or to limit the disclosure to the precise form disclosed. While specific embodiments of, and examples for, the disclosure are described herein for illustrative purposes, various equivalent modifications are possible within the scope of the disclosure, as those skilled in the relevant art will recognize. For example, while method steps or functions are presented in a given order, alternative embodiments may perform functions in a different order, or functions may be performed substantially concurrently. The teachings of the disclosure provided herein can be applied to other procedures or methods as appropriate. The various embodiments described herein can be combined to provide further embodiments. Aspects of the disclosure can be modified, if necessary, to employ the compositions, functions and concepts of the above references and application to provide yet further embodiments of the disclosure. Moreover, due to biological functional equivalency considerations, some changes can be made in protein structure without affecting the biological or chemical action in kind or amount. These and other changes can be made to the disclosure in light of the detailed description. All such modifications are intended to be included within the scope of the appended claims.

[0048] Specific elements of any of the foregoing embodiments can be combined or substituted for elements in other embodiments. Furthermore, while advantages associated

with certain embodiments of the disclosure have been described in the context of these embodiments, other embodiments may also exhibit such advantages, and not all embodiments need necessarily exhibit such advantages to fall within the scope of the disclosure.

[0049] The technology described herein is further illustrated by the following examples which in no way should be construed as being further limiting.

[0050] Some embodiments of the technology described herein can be defined according to any of the following numbered paragraphs:

1. A composition comprising a force measurement droplet, the droplet comprising:
 - a biocompatible oil layer;
 - a surfactant-transducer layer; and
 - an optically detectable molecule.
2. The composition of paragraph 1, wherein the optically detectable molecule is located in the surfactant-transducer layer.
3. The composition of paragraph 1, wherein the optically detectable molecule is located in the biocompatible oil layer.
4. The composition of any of paragraphs 1-3, wherein the biocompatible oil is selected from the group consisting of:
 - fluorocarbon oil; perfluorinated oil; silicone oil; and mineral oil.
5. The composition of paragraph 4, wherein the fluorocarbon oil is Fluoroinert FC-70 fluorocarbon oil (Perfluorotripentylamine).
6. The composition of any of paragraphs 1-5, wherein the surfactant-transducer layer comprises a layer comprising an amphiphilic-linker molecule bound to a cell adhesion molecule;
 - wherein the amphiphilic-linker molecule comprises an amphiphilic molecule conjugated to a linker molecule;
 - wherein the linker molecule can bind, or be bound by, a cell adhesion molecule.
7. The composition of paragraph 6, wherein the amphiphilic molecule is selected from the group consisting of:
 - a phospholipid molecule and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE).

8. The composition of any of paragraphs 1-7, wherein the surfactant-linker molecule is a phospholipid molecule with a polyethylene glycol spacer linked to biotin (PEG-biotin) attached to its head group.
9. The composition of paragraph 8, wherein the phospholipid molecule is 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE).
10. The composition of any of paragraphs 1-9, wherein the cell adhesion molecule is selected from the group consisting of:
 - an integrin ligand; RGD peptide; a cadherin ligand; anti-E-cadherin antibody
11. The composition of any of paragraphs 1-10, wherein the optically detectable molecule is selected from the group consisting of:
 - a fluorescently detectable molecule and a luminescently detectable molecule.
12. The composition of any of paragraphs 1-11, wherein the optically detectable molecule is an optically detectable streptavidin molecule conjugated to the cell adhesion molecule.
13. The composition of any of paragraphs 1-12, wherein the optically detectable molecule is a fluorescently detectable streptavidin molecule conjugated to the cell adhesion molecule.
14. The composition of any of paragraphs 1-13, wherein the oil layer further comprises a co-surfactant.
15. The composition of paragraph 14, wherein the co-surfactant is selected from the group consisting of:
 - fluorocarbon-hydrocarbons; fluorocarbon-hydrocarbon diblocks; and Krytox-Dodecylamine.
16. The composition of any of paragraphs 1-15, wherein the droplet is from about 0.5 to about 500 μm in radius.
17. The composition of any of paragraphs 1-16, wherein the droplet is from about 2 to about 40 μm in radius.
18. A method of measuring the mechanical forces exerted by cells on their surrounding environment, the method comprising;
 - contacting a cell or group of cells with a composition of any of paragraphs 1-17;
 - binding a droplet of the composition to one or more of the cells;

and measuring the strength and distribution of a signal from the optically detectable molecule;

wherein the signal from the optically detectable molecule indicates the location and magnitude of forces exerted by the cells.

19. The method of paragraph 18, wherein the contacting step comprises contacting a cell suspension with the composition, centrifuging the mixture to pellet the cells into an aggregate, and maintain the cells in culture for at least 6 hours.
20. The method of paragraph 18, wherein the contacting step comprises injecting the composition into a tissue.
21. The method of any of paragraphs 18-20, wherein measuring the strength and distribution of the signal comprises imaging one or more droplets by confocal microscopy.
22. The method of any of paragraphs 18-20, wherein cellular stresses (forces per unit area) on the droplet is determined using Eq. 1:

$$p'_i = p'_e + 2\gamma H(\theta, \phi) - \sigma_{nn}(\theta, \phi) \quad (1)$$

wherein γ is the interfacial tension of the droplet and the surrounding medium, σ_{nn} is the forces per unit area, H is the local mean curvature of the droplet surface, θ and ϕ are angular spherical coordinates, p'_i and p'_e are, respectively, the droplet internal and external hydrostatic pressures.

23. The method of any of paragraphs 18-22, wherein anisotropic stresses are mapped to the droplet surface using Eq. 2:

$$\delta\sigma_{nn}(\theta, \phi) = 2\gamma \delta H(\theta, \phi) = 2\gamma \left(H(\theta, \phi) - \frac{1}{R} \right) \quad (2)$$

wherein R is the radius of the initial undeformed spherical droplet and the isotropic component H_i is $1/R$; and

wherein the tissue pressure $P_i = (p'_i - p_i) - (p'_e - p_e)$.

24. The method of any of paragraphs 18-23, wherein the average maximal anisotropic stresses is determined using Eq. 3:

$$\overline{\delta\sigma_{nn}^M} = \gamma \overline{\Delta\kappa} \quad (3)$$

wherein $\delta\sigma^M$ is the average value of the maximal anisotropic stresses and $\Delta\kappa$ is the average value of the maximal curvature difference.

EXAMPLES

[0051] **EXAMPLE 1: Quantifying cell-generated mechanical forces within living embryonic tissues**

[0052] Cell-generated mechanical forces play a critical role during tissue morphogenesis and organ formation in the embryo. However, little is known about how these forces shape embryonic organs, mainly because it has not been possible to measure cellular forces within developing three-dimensional (3D) tissues *in vivo*. Described herein is a method to quantify cell-generated mechanical stresses that are exerted locally within living embryonic tissues using fluorescent, cell-sized, oil microdroplets with defined mechanical properties and coated with surface integrin or cadherin receptor ligands. After introducing a droplet between cells in a tissue, local stresses are determined from the droplet shape deformations, which are obtained via fluorescence microscopy and computerized image analysis. Using this method, the stresses generated by individual embryonic epithelial cells cultured within 3D aggregates are quantified (3.5 nN/ μm^2). These stresses are demonstrated to be dependent on myosin II activity and more than two-fold larger than the stresses generated by cells of embryonic tooth mesenchyme when analyzed within cultured aggregates or in developing mouse mandibles.

[0053] Mechanical forces have been known to sculpt embryonic structures for over a century¹ and their influence on cell behavior is now well established²⁻⁸. *In vitro* studies with cultured cells have shown that cellular forces affect their directionality, intensity and coherence of movement⁹⁻¹¹, orientation of division^{12,13}, rate of proliferation¹⁴⁻¹⁷ and even differentiation^{16,18}. Furthermore, the application of external forces to living embryonic tissues has proved that mechanical forces can induce the expression of key developmental genes^{19,20}. Despite abundant evidence that cell behavior depends critically on mechanical forces, the precise mechanisms by which these forces influence cell behavior *in vivo* and drive developmental processes that shape whole embryonic tissues and organs remain unknown.

[0054] Most of our current knowledge on how mechanical forces alter cell behavior was enabled by the development of techniques that permit either the measurement of cellular forces or the application of controlled mechanical force on cultured cells. Atomic Force

Microscopy^{21,22}, Micropipette Aspiration^{23,24} and Magnetic Cytometry²⁵ have been applied to measure cell mechanics and adhesion forces, and, more recently, FRET-based molecular force sensors have been developed to measure molecular tension in cultured cells^{26,27}. These approaches have been complemented by *in vitro* experiments using soft gel substrates (Traction force microscopy^{9,28,29}), elastic micro-pillars^{31,31} and gel matrices^{32,33} to quantify traction forces generated by cultured cells, individually and collectively in 2D and 3D geometries. However, none of these techniques can be used to measure mechanical forces generated by individual cells within the physiological context of living tissues and organs *in vivo*.

[0055] Measuring cellular forces *in vivo* has proven very challenging. To date, the only available technique to probe cellular tension is Laser Ablation³⁴. Using a femtosecond pulsed laser to ablate cell-cell junctions in the living embryo and quantifying the retraction speed of the cut cell junction, this technique permits one to qualitatively infer relative differences in cell tension in different tissue contexts. While useful to qualitatively estimate the tension state at a cell junction^{34,35} and even in portions of a tissue^{36,37}, it does not provide a quantitative measure of cellular forces. This is because the material properties of the cells and tissue surrounding the ablation site are unknown, which makes it impossible to determine the quantitative relation between cell tension and retraction speed at the ablated site.

[0056] Described herein is a new technique that permits direct quantification of endogenous cellular forces *in situ* within living tissues and developing organs. The technique consists of using oil microdroplets similar in size to individual cells, with defined mechanical properties and displaying ligands for cell surface adhesion receptors, as force transducers in living embryonic tissues (Fig. 1A). When a fluorescently-labeled microdroplet is injected in the intercellular space of a living embryonic tissue, adjacent cells adhere to the surface receptor ligands on the microdroplet and exert forces on it, causing its deformation from the equilibrium spherical shape. By reconstructing the shape of the deformed droplet in 3D using confocal microscopy, and knowing its precise mechanical properties, it is possible to obtain the stresses (force per unit surface) that cells apply at every point on the droplet surface. This permits the direct quantification of cellular stresses generated within living embryonic tissues on scales comparable to cell size.

[0057] RESULTS

[0058] Oil microdroplets as force transducers. Vegetable oil droplets with defined mechanical properties have been previously used to successfully measure forces generated by

growing actin networks *in vitro* by measuring their deformation from a spherical form^{38,39}. Unlike experiments with isolated molecules, force measurements involving living cells or tissues cannot be performed with vegetable oils as the lipids composing the droplets easily transfer to cell membranes, potentially causing toxicity or complicated side effects.

[0059] To overcome this problem, droplets were developed, as described herein, that utilize biocompatible fluorocarbon oils⁴⁰⁻⁴² that are immiscible in vegetable oils. In order to use fluorocarbon oil droplets as force transducers, it is necessary to: (1) stabilize them and control their interfacial tension (which determines the droplet's resistance to deformation), (2) modify their surface chemistry to promote specific adhesion of adjacent living cells, and (3) fluorescently label the droplet (or its surface) to visualize its deformation *in situ* in real-time.

[0060] These goals were achieved by stabilizing droplets (ranging from about 2 to 40 μm in radius) composed of Fluoroinert FC-70 fluorocarbon oil (Perfluorotripentylamine) using a biocompatible surfactant consisting of an amphiphilic molecule, 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), with a polyethylene glycol spacer linked to biotin (PEG-biotin) attached to its head group (Figs. 1C, 1E). The PEG spacer of the DSPE-PEG-biotin surfactant prevents non-specific interactions at the droplet surface, while the biotin group enables specific coating of the droplet with biotinylated ligands for integrins (RGD peptide) or cadherins (anti-E-cadherin antibody) using intervening bifunctional fluorescent streptavidin molecules, which also enable microscopic visualization of the droplets (Figs. 1B, 1C). The interfacial tension of the fluorocarbon droplets needs to be adjusted to allow the measurement of the stresses applied by different types of cells. We accomplished this by adding fluorocarbon-hydrocarbon (Krytox-Dodecylamine or Krytox-DDA) co-surfactants⁴³ that are soluble in fluorinated solvents (Figs. 1C, 1D) and permitted the lowering of the interfacial tension 6-fold.

[0061] To quantify the mechanical forces that cells apply on the surface of an oil droplet, it is necessary to relate the geometry of the droplet to the local cellular forces responsible for its deformation. Such a relation is provided by Laplace's Law⁴⁴, which accounts for the local normal force balance at every point of the droplet interface. The internal pressure, p_i , of an oil droplet with radius R suspended in aqueous medium is given by $p_i = p_e + 2\gamma/R$, where p_e is the external pressure and γ the interfacial tension of the droplet and the surrounding medium. In absence of external forces other than the isotropic hydrostatic pressure, the equilibrium droplet shape is a sphere (Fig. 1B). However, when large enough anisotropic

forces are applied on the droplet, its shape deviates from the sphere. Specifically, when placed in spaces between cells within living tissues (Fig. 1A), droplets will be deformed if the stresses generated by the cells are greater than the resisting stresses ($2\gamma/R$) of the droplet interfacial tension. Consider the forces per unit area, or stresses σ_{nn} , applied by cells in the outward normal direction at each point on the droplet surface (positive values of σ_{nn} indicate cells pulling on the droplet and vice versa). In general, as the cells surrounding a droplet apply different forces at different points, the normal stresses σ_{nn} vary across the droplet surface. Accounting for the normal stresses σ_{nn} that cells apply on the droplet, local normal force balance (Laplace's Law) on the droplet surface reads³⁸

$$p'_i = p'_e + 2\gamma H(\theta, \varphi) - \sigma_{nn}(\theta, \varphi), \quad (1)$$

where H is the local mean curvature of the droplet surface⁴⁵, parameterized using angular spherical coordinates θ and φ , and p'_i and p'_e are, respectively, the droplet internal and external hydrostatic pressures, which are in general different from the initial values (p_i and p_e) of the spherical droplet in solution. It is convenient to decompose the isotropic and anisotropic contributions to H and σ_{nn} by defining $H = H_i + \delta H$ and $\sigma_{nn} = -P_i + \delta\sigma_{nn}$, where H_i and $-P_i$ are the isotropic contributions to the droplet mean curvature and normal stress respectively, and $\delta H(\theta, \varphi)$ and $\delta\sigma_{nn}(\theta, \varphi)$ are their anisotropic components (hence the explicit dependence on the position on the droplet surface). The isotropic component of the stress, $-P_i$, is independent of the fluid hydrostatic pressure and is generated by cells in the tissue^{15,46}; it corresponds to an effective tissue pressure P_i due to cellular crowding. Given that oil is essentially incompressible, the isotropic component H_i is given by $H_i = 1/R$, with R being the radius of the initial undeformed spherical droplet, while the tissue pressure reads $P_i = (p'_i - p_i) - (p'_e - p_e)$. This implies that the measure of the local tissue pressure P_i requires monitoring of the droplet internal pressure, which cannot be done by simply observing the droplet shape if fully surrounded by cells. Therefore, it is only the anisotropic stresses on the droplet, arising from the local non-homogenous and/or anisotropic mechanics of tissues, that will induce deviations of the droplet shape from its isotropic spherical state. Writing Eq. 1 for the anisotropic components, leads to

$$\delta\sigma_{nn}(\theta, \varphi) = 2\gamma\delta H(\theta, \varphi) = 2\gamma (H(\theta, \varphi) - 1/R), \quad (2)$$

which provides a direct relation between the droplet shape in 3D and the (anisotropic)

stresses inducing that deformation. Eq. 2 is the 3D analog of the force-extension relation for a linear spring, with the droplet interfacial tension and the local mean curvature playing the roles of the spring constant and the spring extension, respectively.

[0062] Quantification of cellular stresses in cultured 3D aggregates

[0063] To explore the utility of this method, fluorocarbon droplets (ranging from about 2 to 40 μm in radius) lacking Krytox-DDA co-surfactant but functionalized with ligands for either integrin or E-cadherin receptors were respectively combined with suspensions of mesenchymal cells (isolated from day 10 embryonic tooth rudiments) or premalignant mammary epithelial cells (isolated from mammary glands of 8 week-old transgenic mice), compacted into 3D cell aggregates via centrifugation, and maintained in culture for 2-5 days depending on cell type (Fig. 2A). Cell-droplet attachment was confirmed using confocal microscopy in sparse mixtures of cells and droplets. Confocal imaging of the compact 3D cellular aggregates confirmed that both epithelial and mesenchymal cells were able to apply large enough forces to induce moderate droplet deformations (Fig. 2B, 2D). The 3D shape of a given droplet was obtained through computerized reconstruction using confocal stacks through the sample, allowing the calculation of the local mean curvature $H(\theta, \phi)$ at the droplet surface. The interfacial tension of the droplets in contact with cell culture medium was determined to be $\gamma = 26 \pm 2 \text{ mN/m}$ under cell aggregate culture conditions. Using Eq. 2 and the measured local mean curvature and interfacial tension, the anisotropic stresses applied by cells were mapped on the droplet surface (Fig. 2C, 2E). Regions on the droplet surface with positive anisotropic stresses ($\delta\sigma_{nn} > 0$) are associated with cells either pushing the droplet less strongly than the isotropic pressure P_i or directly pulling on the droplet (tensional stresses), whereas regions with $\delta\sigma_{nn} < 0$ (compressive stresses) are associated with cells pushing stronger on the droplet than the isotropic tissue pressure P_i , either directly or indirectly by pulling on surrounding regions. The values of the anisotropic stresses measured *in situ* within cell aggregates are in the range of several $\text{nN}/\mu\text{m}^2$ for both cell types, in agreement with previous *in vitro* measurements^{32,47}.

[0064] While *in situ* measurements of the stresses acting on a droplet require 3D reconstruction of its shape, statistical values of cellular stresses within a tissue can be obtained from ensemble statistics on 2D confocal sections of different droplets in the cellular aggregate or whole tissue. Specifically, the average value of the maximal anisotropic stresses,

$\delta\sigma^M$, can be obtained from the average value of the maximal curvature difference, $\Delta\kappa$, along the contour of droplet sections (Fig. 2B, 2D and Figs. 3A-3D), namely

$$\overline{\delta\sigma_{nn}^M} = \gamma \overline{\Delta\kappa} \quad (3)$$

Measuring the curvature along 2D confocal sections (Figs. 3A-3C) on multiple droplets embedded in cell aggregates allowed us to build the distribution of maximal curvature differences $\Delta\kappa$ for both mammary epithelial cells and tooth mesenchymal cells (Fig. 3E). The average value of the maximal curvature differences, $\Delta\kappa$, is obtained directly from their distribution. Using Eq. 3, the measured average values of maximal curvature differences and the value of the interfacial tension, we obtained the average value of maximal anisotropic stresses to be $3.5 \pm 0.4 \text{ nN}/\mu\text{m}^2$ and $1.5 \pm 0.2 \text{ nN}/\mu\text{m}^2$ for mammary epithelial cells and tooth mesenchymal cells, respectively. These values are in good agreement with our *in situ* 3D measurements of anisotropic stresses (Figs. 2C, 2E) and also with recent 3D traction force microscopy measurements³², which reported that fibroblasts generate maximal stresses of about $2 \text{ nN}/\mu\text{m}^2$. Analysis of the relative droplet deformations with varying cross-section sizes shows the existence of a characteristic length scale, different for epithelial and mesenchymal cells, for which relative droplet deformations are maximal (Fig. 3F). Measuring the average cell size in the aggregates shows that the observed length scale of largest relative droplet deformations corresponds to the average cell size in each aggregate (Fig. 3F), indicating that the largest departures from the isotropic mechanical state occur at cellular scales.

[0065] Importantly, inhibition of myosin II activity and actin polymerization, using blebbistatin (Fig. 3G) and cytochalasin D (Fig. 3H), respectively, in mammary epithelial cell aggregates led, in both cases, to complete rounding of the droplets, confirming that the measured stresses are actively generated by cells via an actomyosin-based contraction mechanism. Moreover, disruption of cells with the detergent sodium dodecyl sulfate led to the disassembly of cell aggregates and complete rounding of the droplets.

[0066] Quantification of cellular stresses in living embryonic tissue. Based on the successful measurements of stresses in cultured cell aggregates, it was determined whether this new method can be used to directly quantify cell-generated forces within living embryonic tissues (Fig. 4A). To do this, the cellular stresses exerted by tooth mesenchymal cells in living tooth mandible explants (Fig. 4B-4F), which contain the same tooth

mesenchymal cells as in the 3D cell aggregates studied *in vitro* was determined. Between 10 to 30 microdroplets coated with ligands for integrin receptors (RGD peptide) were microinjected into the dental mesenchyme of living dissected mouse mandibles at either stage E11 or E13.5 (Fig. 4G). The droplets were placed as close as possible to the thickened epithelium that overlies the tooth buds during these early developmental stages. Interestingly, negligible droplet deformations were observed when droplets identical to those used previously in the 3D cell aggregate experiments were utilized. I was hypothesized that the stresses generated by cells within these developing tissues were not large enough to deform the droplets, and Krytox-DDA co-surfactant was developed (Fig. 1C-1D) to lower the interfacial tension to 4 ± 3 mN/m, thereby making the droplets more easily deformable.

[0067] Microdroplets of similar size with this lower interfacial tension showed substantial deformations when embedded between cells of the dense dental mesenchymal tissue (Fig. 4H). Statistical analysis of confocal sections of multiple droplets in the tissue permitted the determination of the distribution of maximal curvature differences $\Delta\kappa$ (Fig. 5A), as well as its average value $\Delta\kappa = 0.41 \pm 0.04 \mu\text{m}^{-1}$. Using Eq. 3, the measured average value of the maximal curvature differences and the interfacial tension, the average value of the maximal anisotropic stresses generated by mesenchymal cells in living dental mesenchyme at embryonic stage E11 was determined to be $1.6 \pm 0.8 \text{ nN}/\mu\text{m}^2$. This value corresponds, within the experimental error, to the value measured for tooth mesenchymal cells in cultured aggregates. The relative droplet deformations are maximal at a length scale of about $10 \mu\text{m}$ (Fig. 5B), which corresponds to the average size ($10 \pm 2 \mu\text{m}$) of tooth mesenchymal cells that were measured in the dental mesenchyme (Fig. 5B). As the stresses necessary to deform a droplet with interfacial tension γ at cell size l are of order γ/l (for droplets larger than cell size), the smaller size that tooth mesenchymal cells exhibit within whole tissues *in vivo* prevents them from deforming the original droplets with higher interfacial tension, despite the fact that they can generate the same stresses *in vivo* and *in vitro*. Indeed, knowing the average size of tooth mesenchymal cells *in vivo*, the minimal cell-generated stresses necessary to deform the original droplets (with $\gamma = 26 \pm 3 \text{ mN/m}$) at the cell scale would be $\gamma/l \approx 2.6 \text{ nN}/\mu\text{m}^2$, which are larger than the measured average values of maximal stresses for tooth mesenchymal cells, explaining why no deformations were observed on the original droplets with higher interfacial tension.

[0068] **DISCUSSION**

[0069] The data described herein demonstrate that fluorescent oil microdroplets coated with specific ligands for cellular adhesion receptors allow quantitative measurements of cellular stresses within the local 3D microenvironment of both cultured dense cell aggregates and whole living embryonic tissues. Using surface-functionalized oil microdroplets as force transducers, cell-generated anisotropic stresses were measured in cultured 3D aggregates of epithelial and tooth mesenchymal cells as well as in the dental mesenchyme of living mouse mandibles. It was confirmed that the stresses generated by mammary epithelial cells are myosin II-dependent and more than 2-fold larger than those generated by tooth mesenchymal cells, whether these are measured in cultured aggregates or in their native tissue environment. This finding suggests that epithelial tissues require stronger mechanical contacts between cells than mesenchymal tissues, which is consistent with the typical cell packing densities observed in these tissues (i.e., higher in epithelium than mesenchyme), as well as the presence of a loose interstitial extracellular matrix only within the mesenchyme.

[0070] Importantly, the measured average values of maximal anisotropic stresses generated by tooth mesenchymal cells in cultured aggregates and in living tissue (dental mesenchyme) were very similar to each other, and to values measured *in vitro* for cultured fibroblasts both in 2D⁴⁷ and 3D³² geometries. However, cell shape and size varied strongly between cultured 3D cell aggregates and living 3D embryonic tissues, suggesting an important role of cell shape in directing cell-generated stresses during tissue growth and remodeling.

Measurement of spatial patterns of cellular forces *in vivo* requires the injection of multiple droplets in the embryonic tissue of interest. To be sure not to interfere with normal tissue development, the droplets need to be administered sparsely between the cells forming the tissue, separated by several cell lengths. Although a single measurement of this type can only provide a low spatial resolution cellular force map in a tissue, stereotypical patterns of cellular forces (force fields) with cellular resolution may be obtained from statistics over several samples at the same developmental stage.

[0071] In the embodiments described above herein, quantification of the isotropic component of the stresses is not possible using incompressible droplets unless the internal droplet pressure is monitored or there is a region of the droplet surface where no cellular forces are applied. If droplets are only partially embedded in tissues or in contact with cells in culture and a region of their surface remains free of cell contact, then the isotropic stresses can be directly measured from the shape of the droplets³⁸. However, when droplets are fully

embedded in between the cells in a whole tissue, the isotropic component of the stresses cannot be determined from the droplet shape. This limitation can be overcome by monitoring the internal droplet pressure using pressure-controlled oil microinjection techniques developed originally for other systems⁴⁸.

[0072] It is specifically contemplated that the technique also can be applied to quantify stresses generated by single cells or cells grown in standard monolayer cultures. The combination of 3D droplet reconstruction and time-lapse fluorescence microscopy allows quantitative measurements of both tensional and compressional cellular stresses surrounding the droplet as well as their temporal changes. In addition, the ability to control the type and concentration of ligands on the surface of the droplet, as well as its interfacial tension, allows these force transducers to be adapted to a wide variety of experimental conditions. Therefore, the characteristics of this technique are well suited for any study that requires quantification of stresses generated by individual living cells or groups of cells whether in culture, forming embryonic tissues or adult organs. This technique can therefore permit quantitative analysis of the role of cellular forces in embryonic development, and potentially, in disease processes as well.

[0073] METHODS

[0074] Formation and functionalization of oil droplets. All oil droplets used in these experiments consist of FC70 fluorocarbon oil. Purified, deionized water for droplet preparation and functionalization was obtained by reverse osmosis (Milli-Q Purification System, Millipore) and autoclaved thereafter. FC70 oil was filtered using a syringe filter (Pall Life Sciences) with a 0.2 μm pore size before preparing the droplets. A stable emulsion of polydispersed droplets was obtained by mixing 150 μL of filtered FC70 with 1 mL of purified water solution containing biocompatible surfactants, namely 1,2-distearoyl-sn-glycero-3-phosphoethanolamine- N-[biotinyl(polyethylene glycol)-2000] (DSPE-PEG-biotin; Avanti Polar Lipids, Inc), at a concentration of 0.2 mM. The surfactant concentration used is above the critical micelle concentration of DSPE-PEG[2000] (about 1 μM ⁴⁹), ensuring an excess of surfactant is solution. The mix was shaken vigorously to achieve droplets with radii ranging from about 1 μm to 40 μm . The resultant stable emulsion was positioned on a polycarbonate membrane (Transwell, Corning Inc.) with 3 μm size holes and a water flow was imposed through the membrane to eliminate droplets smaller or about 3 μm in diameter. The droplets remaining on the porous membrane were rinsed three times with purified water, where they remain stable for several days. The resulting stabilized droplets were further

coated with fluorescent streptavidin (Cy5-Streptavidin, Alexa488- Streptavidin, Alexa555-Streptavidin from Invitrogen were used depending on the experiment) by slowly pouring 50 μ L of highly concentrated DSPE-PEG-biotin coated FC70 droplets into a 30 mL solution of fluorescent streptavidin at a concentration of 1 μ M while constantly stirring. A large excess of fluorescent streptavidin molecules in solution allowed fast and high density coating of the droplets via biotin-streptavidin linkages. The resulting droplets were rinsed three times with purified water. Lastly, 25 μ L of high density emulsion of the droplets obtained in the previous step were poured into 0.5 mL of purified water solution of a biotinylated adhesion molecule of choice at a 10 μ M concentration. Droplets used to measure mechanical stresses in mouse mammary epithelial cell aggregates were coated with biotinylated mE-cadherin Antibodies (R&D systems).

[0075] Droplets used to measure mechanical stresses in tooth mesenchymal cell aggregates and in the dental mesenchyme of mouse mandibles were coated with RGD peptide, the integrin-binding domain of fibronectin. Specifically cyclo [Arg-Gly-Asp-d-Phe-Lys(Biotin-PEG-PEG)] (Peptides International), which is composed of a biotinylated PEG group bound to the RGD peptide was used. The resulting droplets were then rinsed three times in purified water and stored in 1 mL of purified water in a glass vial. Functionalized droplets were always used within a week from their preparation.

[0076] Synthesis of fluorocarbon-hydrocarbon diblocks. In order to obtain fluorocarbon-hydrocarbon diblocks, a Krytox fluorinated molecule was coupled to a hydrocarbon dodecylamine (DDA) molecule. For the coupling reaction, 32 g of perfluoropolyether Krytox 157 FSH (DuPont) is diluted with an equal volume of HFE-7100 (3M Co.) in a round flask and the carboxylic groups activated with a 10x molar excess of oxalyl chloride (4.2 mL; Sigma-Aldrich). The mixture became hazy and slightly yellow and was stirred overnight. Then, the solvent and unreacted oxalyl chloride were distilled off and neutralized by bubbling the vapors through 2M KOH. To remove all possible oxalyl chloride, the mixture was kept on a rotation evaporator under vacuum and heating the flask to 70°C. The activated Krytox was allowed to cool down before diluting it in 50 mL HFE-7100, and a 5x molar excess of DDA (4.6 g, Sigma-Aldrich), dissolved in 50 mL anhydrous dichloromethane, was added to the flask. The sample was briefly placed in a heating bath (65°C) under stirring, until strong evaporation was observed. The flask was then left stirring at room temperature overnight to avoid complete evaporation of the solvents. A milky-white sample was obtained, and the mixture of HFE-7100 and dichloromethane was removed on a

rotation evaporator. The sample was re-diluted in a small quantity of HFE-7100 and equally distributed into 50 mL plastic centrifuge tube. After centrifugation at 15000g for 1 hour, the sample separated into a clear bottom phase and a white top layer consisting of the excess of unreacted DDA. With a sharp razor, a cut was made into the bottom of the plastic tube and the clear fluorinated bottom fraction collected into a new tube. After evaporation of the solvent at 65°C over two days, the sample was viscous and still turbid. The product was extracted with 3 x 40 mL hexane to remove all residual DDA that was not coupled to Krytox. After drying the sample, a clear product was obtained. After several weeks of storage in a closed tube, the sample became opaque again. This was attributed to the molecules re-organizing into large micellar structures that cause strong scattering of light.

[0077] Measure of droplet interfacial tension. Interfacial tension was measured using the DuNouÿ ring technique (Sigma 700, Biolin Scientific). An interface of FC70 and purified water was prepared and the interfacial tension was measured at every step of the coating procedure explained above. After the final coating step, purified water was substituted by the culture media used to grow the cells and tissue used in these experiments. Interfacial tension between the coated interface and the culture media was measured at 37°C to be 26 ± 2 mN/m. The value of the interfacial tension was further checked by measuring it with the pendant drop method (homemade experimental set-up and Matlab analysis software), obtaining a value of 28 ± 3 mN/m. The interfacial tension of FC70 oil, containing 1% w/w Krytox-DDA diblocks and coated with the previously described protocol, with culture media at 37°C was measured to be 4 ± 3 mN/m.

[0078] Formation of mammary epithelial cell aggregates. Premalignant mammary epithelial cells, M28, isolated from 8 week-old FVB/C3(1)/SV40 T-antigen transgenic mice were cultured in Dulbeccos Modified Eagles Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS), and 1% penicillin and streptomycin (PenStrep), and maintained at 37°C and 5% CO₂. Mammary epithelial cell aggregates containing functionalized droplets were prepared as follows. Mammary epithelial cells, M28, from two T75 flasks at 80% confluence were centrifuged (720g for 5 min) and the obtained cell pellet was resuspended in 0.25 mL of cell culture media. Between 4 – 10 µL of concentrated functionalized droplet emulsion (prepared as described above) were added to this high density cell suspension and carefully stirred for 5 minutes. The suspension was then centrifuged again (720g for 7 min) to obtain a high density cells-droplets pellet. Portions of this pellet containing microdroplets in-between cells were added to a glass-bottom dish (MatTek Co.) containing 3 mL of cell

culture media. Cell pellets containing droplets were cultured for 48h-72h, until cells formed a compact cell aggregate. Culture media was replaced every 24h.

[0079] Formation of tooth mesenchymal cell aggregates. Tooth mesenchymal cells were obtained from the dental mesenchyme of mouse embryos. Specifically, the first pharyngeal arch was dissected from E10-11 embryos using a sterile technique. For isolation of tooth mesenchymal cells, the tissues were treated with Dispase II (2.4 U/ml; Roche) and DNase I (QIAGEN) at 37°C for 23 min. After separating epithelium and mesenchyme using fine forceps, the presumptive dental mesenchyme (DM) was dissected out and physically triturated several times using a fire-polished Pasteur pipette before being cultured on fibronectin (Becton Dickinson) coated glass-bottom dishes (MatTek Co.) in DMEM supplemented with 10% FBS. The purity of the isolated dental epithelium (DE) was confirmed for cell culture and DM overlay studies using GFP-labeled DE cells isolated from keratin (K)-14/GFP transgenic mice from The Jackson Laboratory. The DM cells were passaged using fibronectin-coated microcarrier beads for the first several passages (Thermo Scientific). Tooth mesenchymal cells were GFP labeled with retroviral transduction. All cell aggregates used in these experiments were prepared with these GFP-positive tooth mesenchymal cells and all studies utilized cells at passage less than eight. Cell aggregates of GFP-positive tooth mesenchymal cells were prepared as follows. High-density pellets of tooth mesenchymal cells containing functionalized droplets (prepared as those of mammary epithelial cells described above) were carefully positioned on a porous polycarbonate membrane (Whatman Nucleopore track-etched membrane; 0.2 μ m pore size) lying on top of a sterile metal mesh (mesh size of 1mm) inside a well of a 6-well plate. Sterile metal supports 3 – 4 mm tall were used to keep the metal mesh elevated from the bottom of the well. The gap between the bottom of the well and the porous polycarbonate membrane was filled with cell culture media (DMEM supplemented with 10% FBS and 1% PenStrep). The pellets lying on the top of the polycarbonate membrane were covered with a very thin film of culture media. Surface tension sustained the pellets on the membrane. Cell culture media was kept in contact with the porous polycarbonate membrane at its lower side, allowing the transfer of nutrients from the culture media reservoir under the membrane and the pellets, which can be cultured in these conditions for over 7 days. Pellets were cultured for 3 – 4 days, changing cell culture media every 24h, until they became compact cell aggregates. The cell aggregates were transferred to glass-bottom dishes (MatTek Co.) for imaging.

[0080] Adhesion of cells on functionalized droplets. Microdroplets used to measure forces in mammary epithelial cell aggregates and GFP-positive tooth mesenchymal cell aggregates were respectively coated with mE-Cadherin antibodies and RGD peptide to allow cells to adhere on the droplet surface. Cells from a single T25 flask at 80% confluence were resuspended in 0.25 mL of culture media (DMEM supplemented with 10% FBS and 1% PenStrep) and 10 μ L of functionalized droplets were added to the suspension. Cells and droplets were carefully stirred for 5 min and placed on a 35mm diameter glass-bottom dish containing 2 mL of culture media (both cells and droplets sedimented on the glass coverslip; FC70 oil density is 1940 kg/m³), incubated for 1h (37°C and 5% CO₂) and imaged using confocal microscopy. By imaging the droplets 20 – 30 μ m away from the coverslip, it was possible to see if cells were attached at the droplet surface (Fig. 6A). Tooth mesenchymal cells localized at the surface of RGD-coated droplets even far away from the coverslip (Fig. 6B), indicating attachment of cells on the droplets. 3-dimensional reconstruction of the sample shows cells localized on the droplet surface. If cells were not adhered to the droplets, they would have been found on the coverslip surface instead. In order to be able to observe localization of mammary epithelial cells with respect to E-cadherin antibody coated droplets, their culture media was supplemented with the DNA dye Hoechst 33342 (Invitrogen) at a concentration of 3 μ M, 30 min before imaging. Nuclei of mammary epithelial cells were observed to surround E-cadherin antibody coated droplets even far away from the coverslip (Fig. 6C), suggesting their adhesion to the droplets. However, as contacts could not be observed directly, it was decided to test adhesion further using a different method. A large excess of E-cadherin antibody coated droplets were deposited on mammary epithelial cells at 50% confluence cultured on a glass-bottom dish, making a thin layer of droplets covering the dish bottom surface (Fig. 6D, left panel). The entire dish was then filled with culture media (approx. 5 mL) and the top was sealed with a thin plastic plate. The glass-bottom dish was then turned upside-down and the cells imaged using an upright fluorescence microscope (Fig. 6D, right panel). Droplets not attached to cells fell to the plastic cover because of gravity (a droplet of 30 μ m in diameter weights approx. 100 pN in culture media). E-cadherin antibody coated droplets were observed to localize perfectly with regions of the coverslip containing cells (Fig. 6E), indicating that droplets were attached to cells, which were preventing the droplets from falling by their own weight.

[0081] Perturbation of cellular forces with drugs. Drug addition was done directly to the glass-bottom culture dish containing mammary epithelial cell aggregates while imaging

the samples and the culture media was mixed using a pipette to achieve a homogeneous distribution of the drug in the culture dish. Myosin II inhibition was achieved by addition of blebbistatin at a 50 μ M final concentration. Inhibition of actin polymerization was achieved by addition of cytochalasin D at a 4 μ M final concentration. Disruption of cell membranes was achieved with detergent (Sodium dodecyl sulfate, SDS) at 1% v/v final concentration.

[0082] Mouse mandible dissection. Keratin14-Cre recombinase mice, STOCK Tg(KRT14-cre)1Amc/J (#004782), were purchased from Jackson laboratories (<http://jaxmice.jax.org/strain/004782.html>). Double-Fluorescent Cre reporter mice, STOCK Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J (#007576), were purchased from Jackson Laboratories (<http://jaxmice.jax.org/strain/007576.html>). Male mice containing a Cre recombinase transgene under the regulation of an epithelial keratin enhancer element, Keratin 14, were mated with a double-fluorescent Cre reporter mouse, that expresses membrane-targeted tandem dimer Tomato prior to Cre-mediated excision and membrane-targeted GFP after excision. This mating combination permitted the generation of embryos in which the plasma membranes of mesenchymal cells (red) and epithelial cells (green) could be distinguished (Fig. 4D). Wild type CD1 mouse embryos (Charles River) were used in some cases. Embryos were harvested from pregnant females 11 or 13 days post detection of a copulation plug and were kept at room temperature in phosphate buffered saline. Embryonic heads were immediately decapitated. Embryonic mandibles were dissected using Dumont #5 forceps and the associated tongue was removed for optimal imaging. Dissected mandibles were kept on ice, in a petri dish containing tissue culture media (DMEM supplemented with 10% FBS and 1% PenStrep), and immediately prepared for droplet micro-injections.

[0083] Micro-injection of functionalized oil droplets in living tooth mandibles. Tissue microinjection of previously functionalized oil microdroplets was accomplished by positioning freshly dissected mouse mandibles (E10.5 and E13.5 depending on experiment) dorsal surface up in a droplet of tissue culture media (DMEM supplemented with 10% FBS and 1% PenStrep) stabilized against a PDMS (SYLGARD 184 silicone elastomer from Dow Corning) block located on a petri dish surface. Surface tension of the tissue culture media droplet with air was sufficient to immobilize both the PDMS block and tissue on the surface during injections. Between 5 – 10 μ L of functionalized oil microdroplets emulsion were back-filled into microinjection needles pulled from glass capillaries with 0.75/1 mm inner/outer diameters (World Precision Instruments). Droplets were injected using a pressure-controlled PLI-100 Pico-Injector (Harvard Apparatus). Six to eight injections, each releasing

from 1 to 5 droplets, were done along the mandible and into the dental mesenchyme, as close as possible to the boundary with the epithelium. All injections were performed on a standard epifluorescence stereo dissection microscope (Nikon SMZ1500) to visualize injection sites and the fluorescent microdroplets upon injection. After injections, mandibles containing oil droplets were transferred to glass-bottom dishes with tissue culture media and maintained at 37°C and 5% CO₂ for 7 – 10 hours before imaging, allowing the tissue to repair the injection sites.

[0084] Measuring average maximal stresses from droplet confocal sections. Given two principal directions on the surface of a droplet, with principal curvatures κ_1 and κ_2 and mean curvature $H = (\kappa_1 + \kappa_2)/2$, the anisotropic stress on the droplet surface (Eq.2) can be written as $\delta\sigma_{nn} = \gamma((\kappa_1 - 1/R) + (\kappa_2 - 1/R))$. The maximal and minimal possible values of the anisotropic stresses are given by

$$\delta\sigma_{nn}^{max} = \gamma((\kappa_1^{max} - 1/R) + (\kappa_2^{max} - 1/R)) \quad \text{and}$$

$$\delta\sigma_{nn}^{min} = \gamma((\kappa_1^{min} - 1/R) + (\kappa_2^{min} - 1/R)) \quad \text{respectively. The}$$

maximal amplitude of anisotropic stresses is given by $\delta\sigma_{nn}^{max} - \delta\sigma_{nn}^{min}$, which reads

$$\delta\sigma_{nn}^{max} - \delta\sigma_{nn}^{min} = 2\gamma((\kappa_1^{max} - \kappa_1^{min}) + (\kappa_2^{max} - \kappa_2^{min}))$$

Defining the curvature amplitude along a principal direction as $\Delta\kappa = \kappa^{max} - \kappa^{min}$, the maximal anisotropic stress amplitude reads

$$\delta\sigma_{nn}^{max} - \delta\sigma_{nn}^{min} = \gamma(\Delta\kappa_1 + \Delta\kappa_2). \quad \text{The average value of the}$$

anisotropic stress amplitude many different droplets reads

$$\overline{\delta\sigma_{nn}^{max} - \delta\sigma_{nn}^{min}} = \gamma(\overline{\Delta\kappa_1} + \overline{\Delta\kappa_2}).$$

While the specific values of $\Delta\kappa_1$ and $\Delta\kappa_2$ are different for different droplets, their average values are equal, i.e.,

$$\overline{\Delta\kappa_1} = \overline{\Delta\kappa_2} \equiv \overline{\Delta\kappa},$$

if no major anisotropies are present in the tissue. In this case, defining the average value of the maximal anisotropic stresses,

$$\overline{\delta\sigma_{nn}^M}, \text{ as } \overline{\delta\sigma_{nn}^M} = \overline{\delta\sigma_{nn}^{max} - \delta\sigma_{nn}^{min}}/2,$$

we obtain

$$\overline{\delta\sigma_{nn}^M} = \gamma \overline{\Delta\kappa}$$

which corresponds to Eq. 3.

[0085] Imaging of cell aggregates and tissue containing droplets. Glass-bottom dishes containing the samples were imaged with a laser scanning confocal microscope (Zeiss LSM 710) equipped with incubation chamber (XL1 heating chamber, PeCon GmbH) and environmental control. Both cell aggregates and living mandible tissue were imaged under the same incubation conditions (37°C and 5% CO₂). Samples were mostly imaged using a (LD) C-Apochromat 40x water-immersion objective with 1.1 NA and, in some cases, using dry 20x and 10x objectives. Confocal imaging parameters were optimized for maximal resolution and minimal noise in each experiment.

[0086] Image analysis. When visualized using confocal microscopy, a 2D confocal section of a surface- labeled droplet appears as a closed curve (Figs. 7A-7F). In order to detect the 2D con- tour of the droplet, the image was first filtered using Steerable filters. While these filters can be implemented in Matlab or other programming languages, the SteerableJ plugin for ImageJ was used. These filters convolve a kernel optimized to detect specific image patterns with the original image to obtain an image where patterns similar to the kernel pattern are enhanced. Using a specific kernel for edge detection (kernel shown in Fig. 7B, left panel) we were able to obtain enhanced images of the droplet confocal section with substantially reduced noise (Fig. 7B, right panel). The filtered image was then processed in Matlab™ (Mathworks) to obtain the coordinates of the droplet contour. To do so, a linear path was defined from a point close to the droplet center to the outside of the droplet (Fig. 7C, left panel) and the intensity profile measured along that path (Fig. 7C, right panel).

[0087] By fitting a gaussian profile to the measured intensity profile along the linear path, the coordinates of the location along the droplet contour intersecting the defined linear path were obtained. These coordinates are given by the maximum (mean) of the gaussian fit. To obtain the coordinates of the entire contour, the defined linear path was rotated around the starting point (Fig. 7C, left panel) and the procedure described above repeated to determine the contour coordinates at each angle. Using this method, the contour of the confocal section of the droplet can be detected at pixel resolution (Fig. 7D). Once the coordinates of the

contour were known, a closed B-Spline curve was obtained (using Wolfram Mathematica™ 8) from the droplet contour coordinates. The B-Spline curve specifies a continuous curve for the droplet contour (Fig. 7E and Fig. 7F) and eliminates high frequency noise at the pixel level that would otherwise make the calculation of the curvature very complicated. The curvature along the droplet contour was obtained from the continuous droplet contour (B-Spline) using standard differential geometry⁴⁵.

[0088] To obtain the 3D shape of a droplet the droplet contour coordinates are detected for each of the confocal sections of the droplet in a 3D confocal stack using the procedure just described for confocal sections (Fig. 8A). The obtained contour coordinates for each confocal section are combined to obtain the coordinates of the droplet surface in 3D (Fig. 8B). Once the coordinates of the droplet surface are known, a 2D B-Spline of the entire droplet surface is obtained (using Wolfram Mathematica™ 8). In this case, the B-Spline specifies a continuous surface for the droplet shape (Fig. 8C) and eliminates high frequency noise at the pixel level that would otherwise make the calculation of surface curvatures very complicated. The mean curvature at each point of the droplet surface (Fig. 8D) was obtained from the continuous droplet shape (B-Spline) using standard differential geometry⁴⁵.

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EXAMPLE 2: Measure of cell size in cultured cellular aggregates and living mouse mandibles

[0090] This Example relates to the methods used to measure the cell size both in cultured cellular aggregates and living mouse mandibles.

[0091] Measure of cell size in cultured aggregates of mammary epithelial cells. In order to measure cell size in aggregates of mammary epithelial (M28) cells, cell aggregates were incubated with cell culture media (DMEM supplemented with 10% FBS and 1% PenStrep) containing the DNA dye Hoechst 33342 (Invitrogen) at a concentration of 3 μ M. The aggregates were incubated for about 12 hours to allow the dye to penetrate within the aggregates. The aggregates were imaged as explained in Example 1 and the distance d between nearest neighbor nuclei measured (Figs. 9A, 9B). For these measurement only nuclei from nearest neighboring cells that appear to be in the same focal plane were used. These measurements permitted the determination of the distribution of the distance d between nearest neighbor nuclei (Fig. 9C). The average cellular size corresponds to the average distance between nearest neighbor nuclei, which is obtained directly from the distribution of d to be 12 ± 2 μ m.

[0092] Measure of cell size in cultured aggregates of tooth mesenchymal cells. Cellular aggregates of GFP-positive tooth mesenchymal cells allow a direct measure of cellular size as the fluorescent levants for each cell differ slightly, allowing a direct identification of the cell contour (Fig. 9D). Tooth mesenchymal cells in dense aggregates tend to acquire an oblate shape with long and short axes, b and a respectively (Figs. 9D, 9E). Cellular size was measured along both axis and their corresponding distributions obtained (Fig. 9G). The size of the cell along its longest axis shows a wide distribution, indicating a large variation in cellular sizes. The average cellular sizes along the short and long axis were obtained from their corresponding distributions to be 10 ± 3 μ m and 24 ± 4 μ m respectively. Cells were never observed to contact droplets along the short axis. Instead, their long axis was essentially perpendicular to the droplet normal at the contact point. The fact that the maximal relative droplet deformations occur at a length scale of 23 μ m (Fig. 3F), essentially the same as the average cell size along their long axis, and that the relative droplet deformations are spread over a wide range of length scales (Fig. 3F), similar to the spread of cell sizes along both axes, indicates that the mechanical inhomogeneities in the cell aggregate are dominated by the largest cellular sizes.

[0093] Measure of cell size in living mouse mandibles. The average size of tooth mesenchymal cells in the dental mesenchyme of mouse mandibles was measured in two different ways. First, dissected mouse mandibles were incubated for 7 hours in culture media (as described in Example 1) containing the DNA dye Hoechst 33342 (Invitrogen) at a concentration of 3 μ M. The distance d between nearest neighbor nuclei was measured as

described above for mammary epithelial cells (Fig. 9H and Fig. 9B). The obtained distribution for distance d between nearest neighbor nuclei is shown in Fig. 9J. The average cellular size corresponds to the average distance between nearest neighbor nuclei, which is obtained directly from the distribution of d to be $10 \pm 2 \mu\text{m}$. The value of the average cellular size was checked by estimating the size of tooth mesenchymal cells in mouse mandibles with fluorescent membrane reporters (Fig. 9I).

EXAMPLE 3: Measure of droplet interfacial tension

[0094] This Example relates to the measurements of the interfacial tension between FC70 oil, coated as described in the main text, and the cell/tissue culture media.

[0095] The interfacial tension was measured using the Du Noüy ring technique (Sigma 700, Biolin Scientific). All experiments were conducted at room temperature if not stated otherwise. Surface and interfacial tension were measured over time to make sure equilibrium values were measured. All the standard deviations provided for the surface/interfacial tensions values correspond to statistics on 5 different samples. First, filtered FC70 was poured into an open glass container creating an FC70 surface exposed to air, and the surface tension of FC70 was measured to be $18 \pm 2 \text{ mN/m}$, which coincides with the surface tension value (18 mN/m) provided by the vendor (3M Co.). Then, purified, deionized water was poured on the top of the FC70 oil layer. As FC70 is nearly twice as dense as water (FC70 density is 1970 kg/m^3), the poured water created a layer on top of FC70. The interfacial tension of FC70 and purified water was measured to be $46 \pm 2 \text{ mN/m}$.

[0096] The water layer was pipetted out and the interfacial tension measured at every step of the oil coating (functionalization) procedure described in Example 1. The water layer was pipetted out on top of FC70 and substituted with a water solution containing DSPE-PEG2000-biotin surfactants at a concentration of 0.2 mM . The surface tension in this case was measured to be $32 \pm 2 \text{ mN/m}$.

[0097] The DSPE-PEG2000-biotin solution was almost completely removed and purified water was added and subsequently removed 3 times, leaving only a DSPE-PEG2000-biotin layer at the FC70-water interface. The interfacial tension of the FC70-water interface with the DSPE-PEG2000-biotin surfactant layer was measured to be $35 \pm 2 \text{ mN/m}$, slightly larger than the previous measurement due to desorption of some of the surfactant.

[0098] The water layer was then substituted with a water solution containing fluorescent streptavidin (FITC-streptavidin) at a concentration of $1 \mu\text{M}$. This concentration and the volume

of solution used (50 mL) ensured a large excess of streptavidin molecules to quickly, and fully, coat the surface. The interfacial tension in this case remained unchanged (35 ± 2 mN/m). The fluorescent streptavidin solution was almost completely removed and purified water was added and subsequently removed 3 times, leaving only a DSPE-PEG2000-biotin:streptavidin (FITC) layer at the FC70-water interface, which appeared colored thanks to the high concentration of fluorescent streptavidin at the interface. The water layer on top of the DSPE-PEG2000-biotin:streptavidin(FITC) coated FC70 layer was then substituted with the cell/tissue culture media used in the experiments described in the main text (DMEM supplemented with 10% FBS and 1%PenStrep). While in all previous steps the interfacial tension reached a constant value in less than a minute, when cell/tissue culture media was added, the interfacial tension took about 40 minutes to equilibrate, as indicated by the time evolution of the interfacial tension (Figs 10A-10F). This is due to the fact that several chemical species (mainly BSA proteins, a large component of FBS) adsorb on the interface, lowering the interfacial tension. The equilibrium interfacial tension of the DSPE-PEG2000-biotin:streptavidin(FITC) coated FC70 with the cell/tissue culture media was measured to be 27 ± 2 mN/m.

[0099] The temperature of the system was changed to 37°C by putting it in contact with a thermal bath at this temperature, and measured the interfacial tension at this temperature to be 26 ± 2 mN/m.

[00100] Due to limitations in the quantity of available Krytox-DDA diblocks, the interfacial tension of FC70 containing Krytox-DDA diblocks, coated as described above and in Example 1, was measured using the pendant drop method because it required a much smaller quantity of reagents for the measurement. For these measurements a custom-built pendant drop apparatus was utilized. In order to check that meaningful values of interfacial tensions were possible, already known values of interfacial tensions in the absence of Krytox-DDA diblocks were first determined. The interfacial tension of FC70 and purified water was measured to be 49 ± 3 mN/m, and the interfacial tension of FC70 with a water solution containing DSPE-PEG2000-biotin surfactants at a concentration of 0.2 mM was measured to be 31 ± 3 mN/m. Therefore, the values of the interfacial tension measured with the pendant drop apparatus agree within the experimental error with the values measured using the Du Noüy ring technique.

[00101] In order to check if the Krytox-DDA diblocks were functional, the interfacial tension of FC70 containing Krytox-DDA diblocks at a 1% w/w concentration with a water

solution containing DSPE-PEG2000-biotin surfactants at a concentration of 0.2 mM was measured. It is expected that the DDA block will interact with the DSPE hydrocarbon tail, thereby stabilizing further the DSPE-PEG2000-biotin surfactants on the interface thanks to the Krytox block (Figs. 1A-1E). The interfacial tension of a droplet of FC70 containing Krytox-DDA diblocks at a 1% w/w concentration with a water solution containing DSPE-PEG2000-biotin surfactants at a concentration of 0.2 mM, was measured to be 14 ± 3 mN/m. This value is substantially smaller than in the absence of Krytox-DDA diblocks, indicating that the diblocks are active and stabilize further the DSPE-PEG2000-biotin surfactants at the interface. Finally, the interfacial tension of FC70 containing Krytox-DDA diblocks at a 1% w/w concentration and coated with DSPE-PEG2000-biotin:streptavidin(FITC) with cell/tissue culture media at 37°C was measured to be 4 ± 3 mN/m.

What is claimed herein is:

1. A composition comprising a force measurement droplet, the droplet comprising:
 - a biocompatible oil layer;
 - a surfactant-transducer layer; and
 - an optically detectable molecule.
2. The composition of claim 1, wherein the optically detectable molecule is located in the surfactant-transducer layer.
3. The composition of claim 1, wherein the optically detectable molecule is located in the biocompatible oil layer.
4. The composition of any of claims 1-3, wherein the biocompatible oil is selected from the group consisting of:
 - fluorocarbon oil; perfluorinated oil; silicone oil; and mineral oil.
5. The composition of claim 4, wherein the fluorocarbon oil is Fluoroinert FC-70 fluorocarbon oil (Perfluorotripentylamine).
6. The composition of any of claims 1-5, wherein the surfactant-transducer layer comprises a layer comprising a amphiphilic-linker molecule bound to a cell adhesion molecule;
 - wherein the amphiphilic-linker molecule comprises an amphiphilic molecule conjugated to a linker molecule;
 - wherein the linker molecule can bind, or be bound by, a cell adhesion molecule.
7. The composition of claim 6, wherein the amphiphilic molecule is selected from the group consisting of:
 - a phospholipid molecule and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE).
8. The composition of any of claims 1-7, wherein the surfactant-linker molecule is a phospholipid molecule with a polyethylene glycol spacer linked to biotin (PEG-biotin) attached to its head group.
9. The composition of claim 8, wherein the phospholipid molecule is 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE).
10. The composition of any of claims 1-9, wherein the cell adhesion molecule is selected from the group consisting of:

- an integrin ligand; RGD peptide; a cadherin ligand; anti-E-cadherin antibody
11. The composition of any of claims 1-10, wherein the optically detectable molecule is selected from the group consisting of:
- a fluorescently detectable molecule and a luminescently detectable molecule.
12. The composition of any of claims 1-11, wherein the optically detectable molecule is an optically detectable streptavidin molecule conjugated to the cell adhesion molecule.
13. The composition of any of claims 1-12, wherein the optically detectable molecule is a fluorescently detectable streptavidin molecule conjugated to the cell adhesion molecule.
14. The composition of any of claims 1-13, wherein the oil layer further comprises a co-surfactant.
15. The composition of claim 14, wherein the co-surfactant is selected from the group consisting of:
- fluorocarbon-hydrocarbons; fluorocarbon-hydrocarbon diblocks; and Krytox-Dodecylamine.
16. The composition of any of claims 1-15, wherein the droplet is from about 0.5 to about 500 μm in radius.
17. The composition of any of claims 1-16, wherein the droplet is from about 2 to about 40 μm in radius.
18. A method of measuring the mechanical forces exerted by cells on their surrounding environment, the method comprising;
- contacting a cell or group of cells with a composition of any of claims 1-17;
- binding a droplet of the composition to one or more of the cells;
- and measuring the strength and distribution of a signal from the optically detectable molecule;
- wherein the signal from the optically detectable molecule indicates the location and magnitude of forces exerted by the cells.
19. The method of claim 18, wherein the contacting step comprises contacting a cell suspension with the composition, centrifuging the mixture to pellet the cells into an aggregate, and maintain the cells in culture for at least 6 hours.
20. The method of claim 18, wherein the contacting step comprises injecting the composition into a tissue.

21. The method of any of claims 18-20, wherein measuring the strength and distribution of the signal comprises imaging one or more droplets by confocal microscopy.
22. The method of any of claims 18-20, wherein cellular stresses (forces per unit area) on the droplet is determined using Eq. 1:

$$p'_i = p'_e + 2\gamma H(\theta, \phi) - \sigma_{nn}(\theta, \phi) \quad (1)$$

wherein γ is the interfacial tension of the droplet and the surrounding medium, σ_{nn} is the forces per unit area, H is the local mean curvature of the droplet surface, θ and ϕ are angular spherical coordinates, p'_i and p'_e are, respectively, the droplet internal and external hydrostatic pressures.

23. The method of any of claims 18-22, wherein anisotropic stresses are mapped to the droplet surface using Eq. 2:

$$\delta\sigma_{nn}(\theta, \phi) = 2\gamma \delta H(\theta, \phi) = 2\gamma \left(H(\theta, \phi) - \frac{1}{R} \right) \quad (2)$$

wherein R is the radius of the initial undeformed spherical droplet and the isotropic component H_i is $1/R$; and

wherein the tissue pressure $P_i = (p'_i - p_i) - (p'_e - p_e)$.

24. The method of any of claims 18-23, wherein the average maximal anisotropic stresses is determined using Eq. 3:

$$\overline{\delta\sigma_{nn}^M} = \gamma \overline{\Delta\kappa} \quad (3)$$

wherein $\delta\sigma^M$ is the average value of the maximal anisotropic stresses and $\Delta\kappa$ is the average value of the maximal curvature difference.

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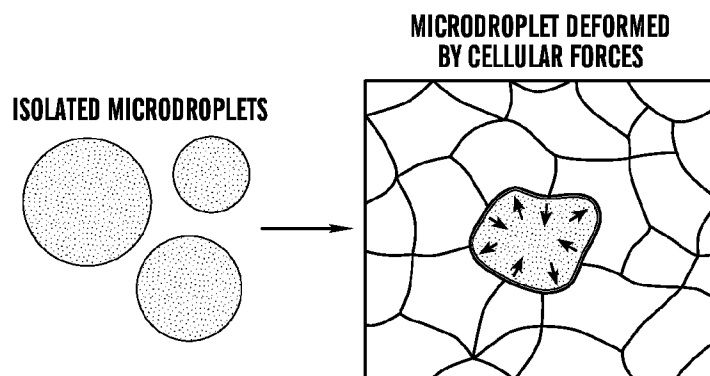


FIG. 1A

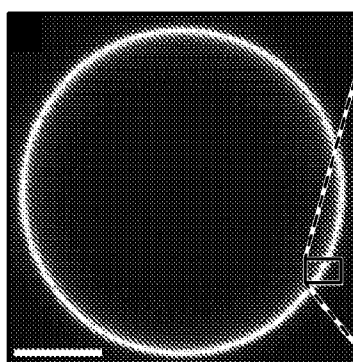


FIG. 1B

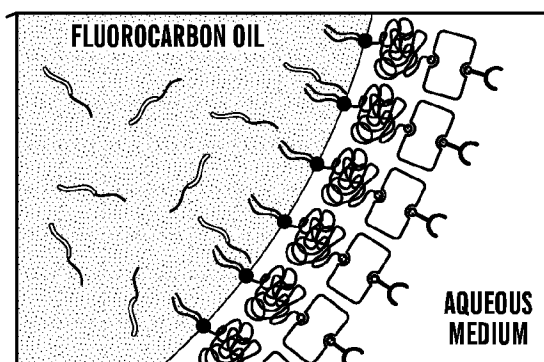


FIG. 1C

KRYTOX-DODECYLAMINE DIBLOCK

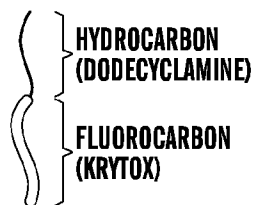


FIG. 1D

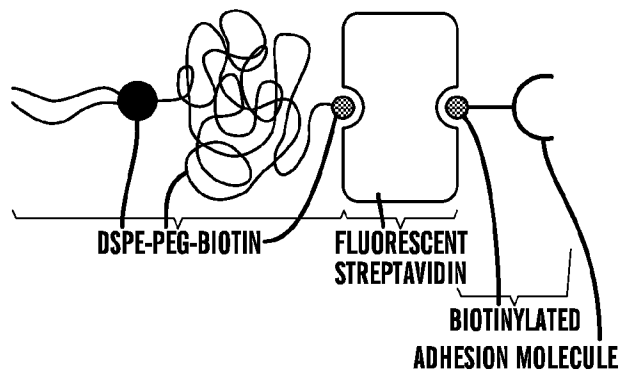


FIG. 1E

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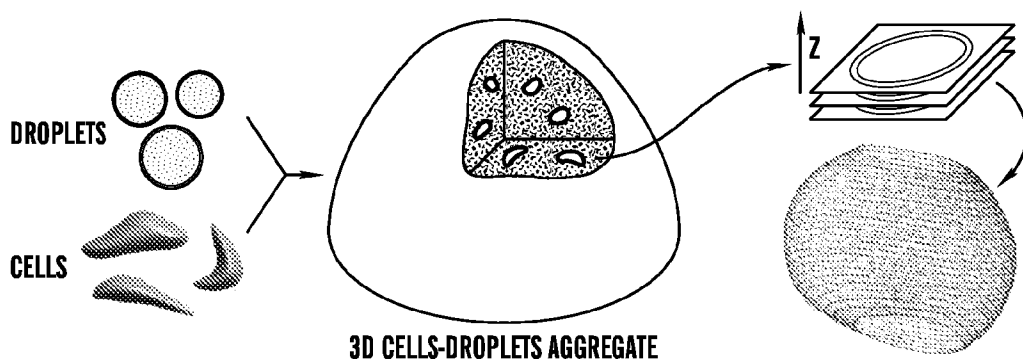


FIG. 2A

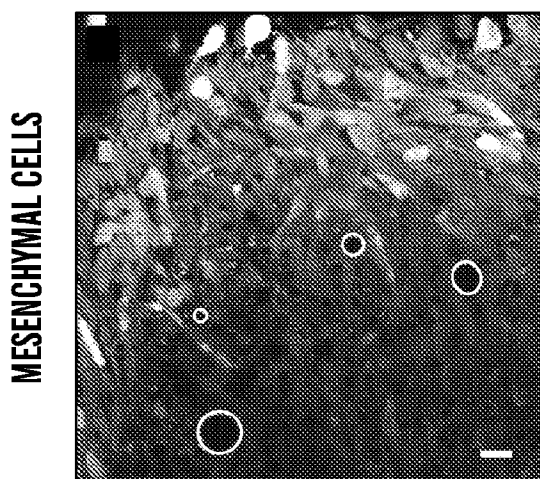


FIG. 2B

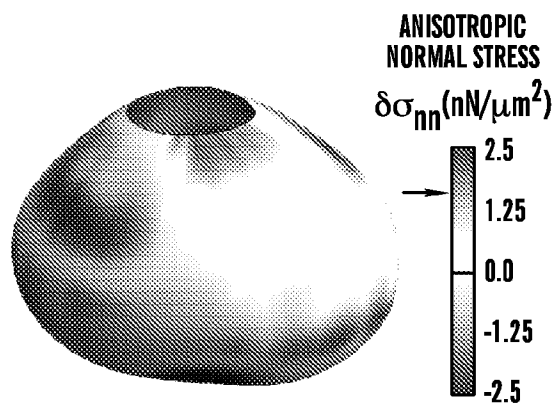


FIG. 2C

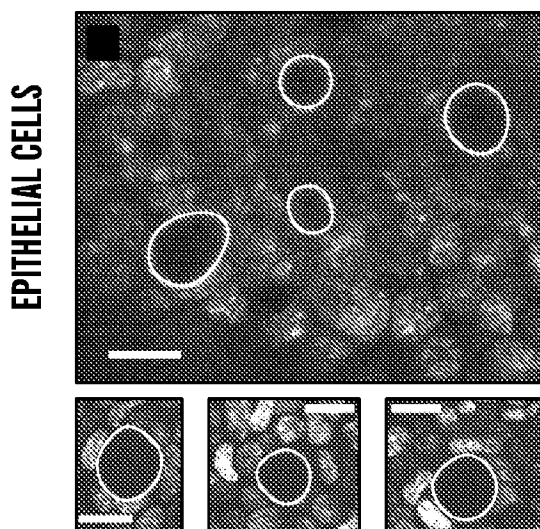


FIG. 2D

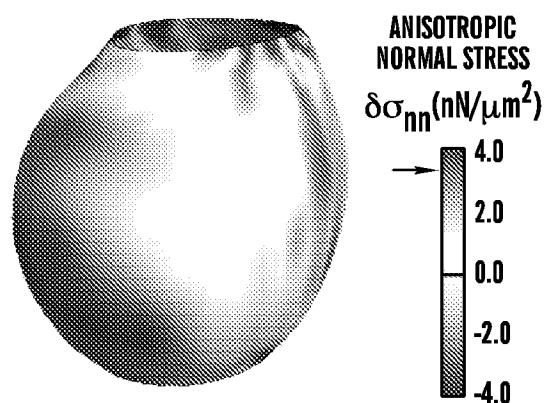


FIG. 2E

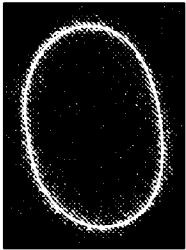


FIG. 3A

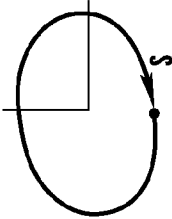


FIG. 3B

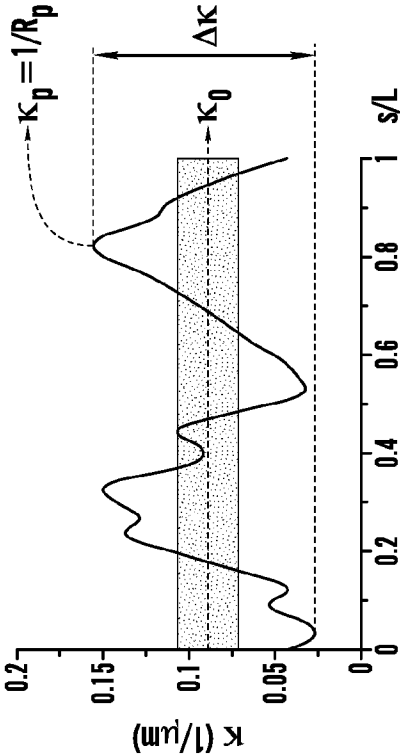


FIG. 3C

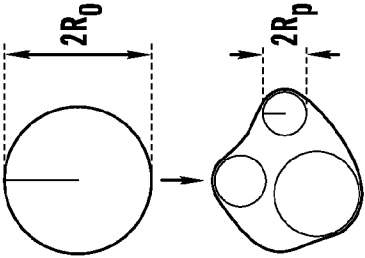
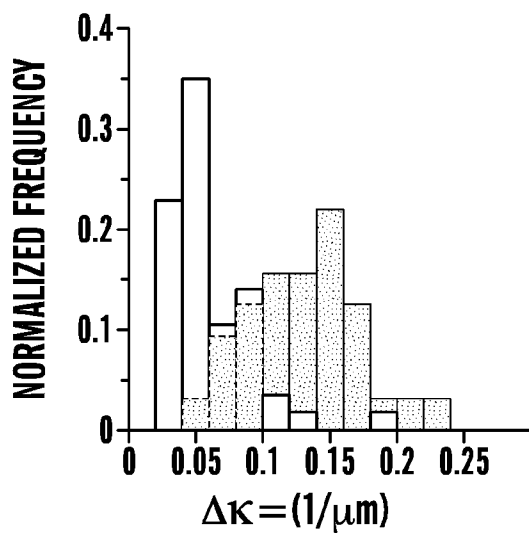
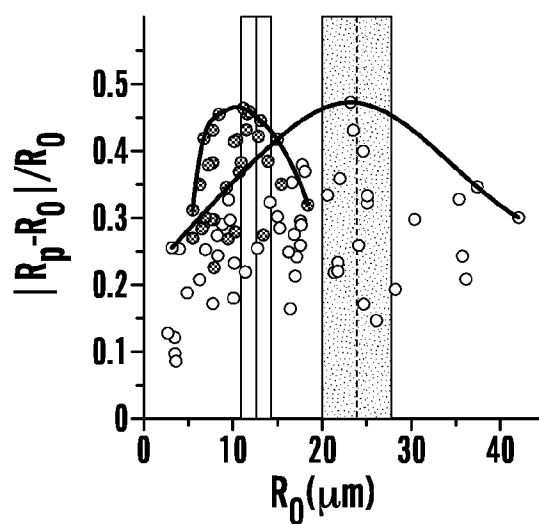
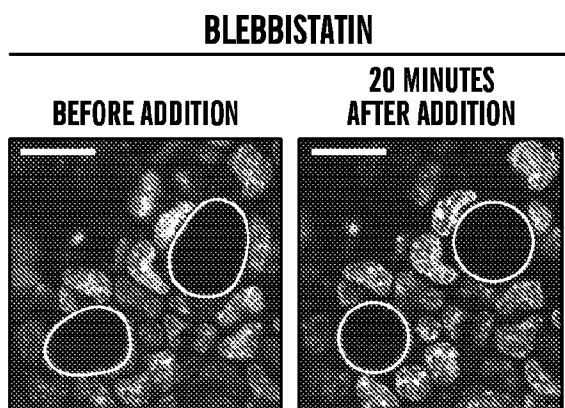
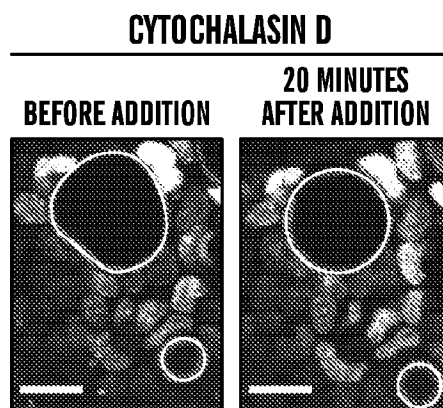


FIG. 3D

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**FIG. 3E****FIG. 3F****FIG. 3G****FIG. 3H**

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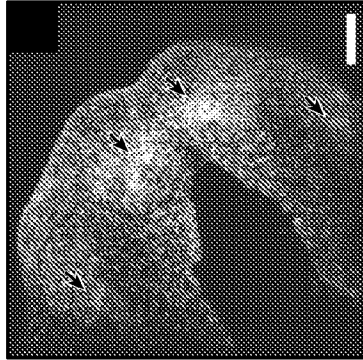


FIG. 4D

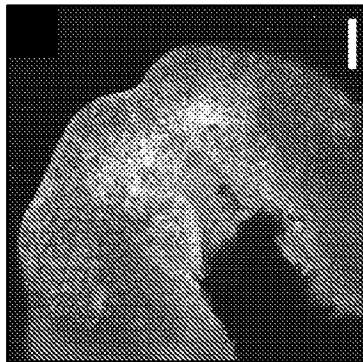


FIG. 4C

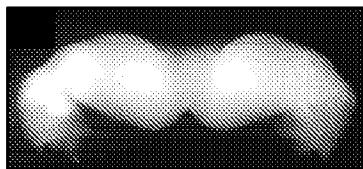


FIG. 4B

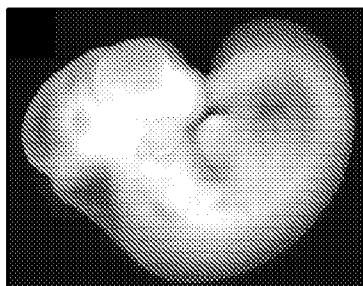


FIG. 4A

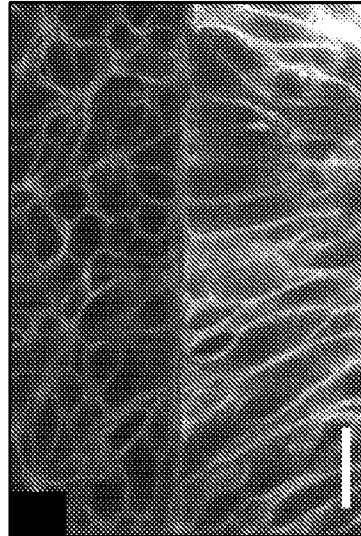


FIG. 4F

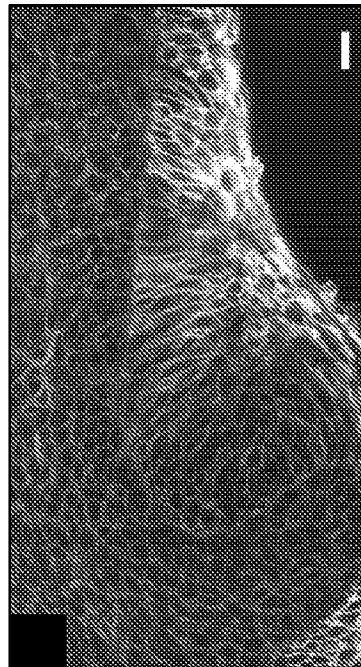


FIG. 4E

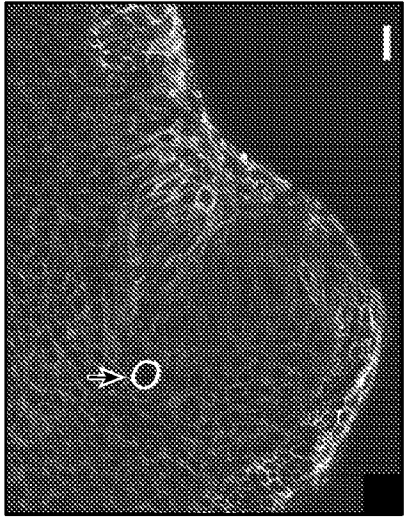


FIG. 4H

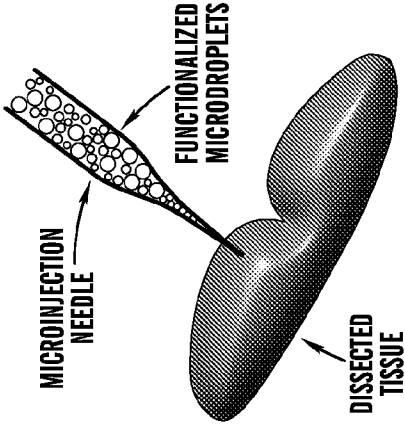


FIG. 4G

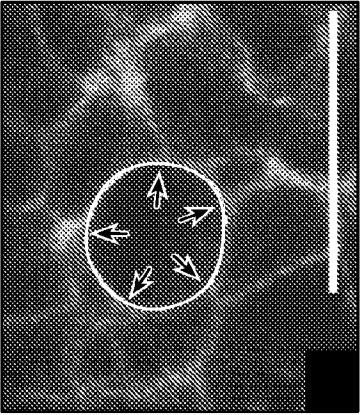


FIG. 4K

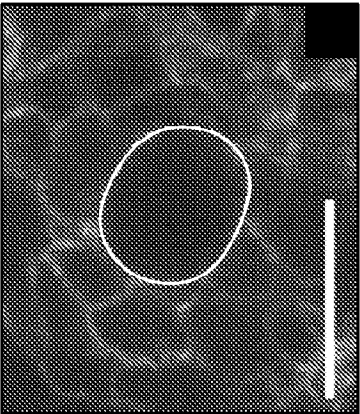


FIG. 4J

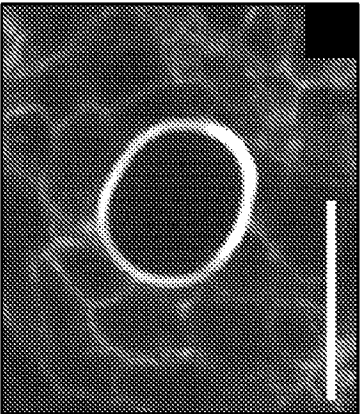
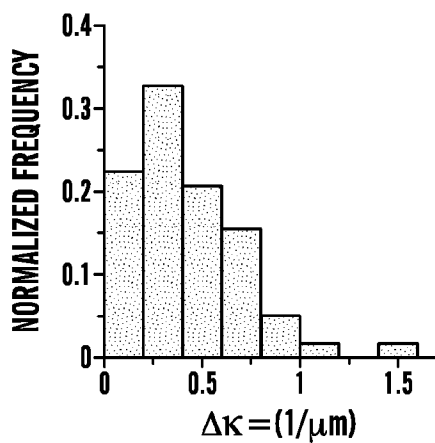
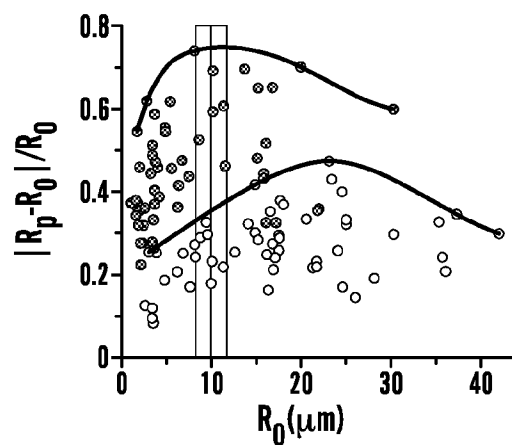


FIG. 4I

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**FIG. 5A****FIG. 5B**

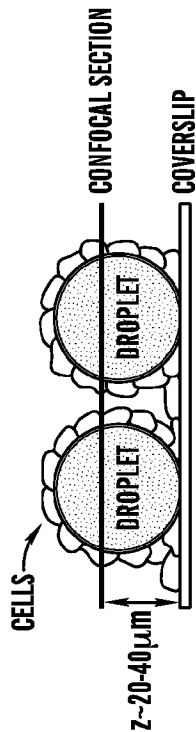


FIG. 6A

- MAMMARY EPITHELIAL CELLS (M28)
DNA LABELED WITH HOECHST 33342
- FC70 DROPLETS COATED WITH mE-CADHERIN ANTIBODY

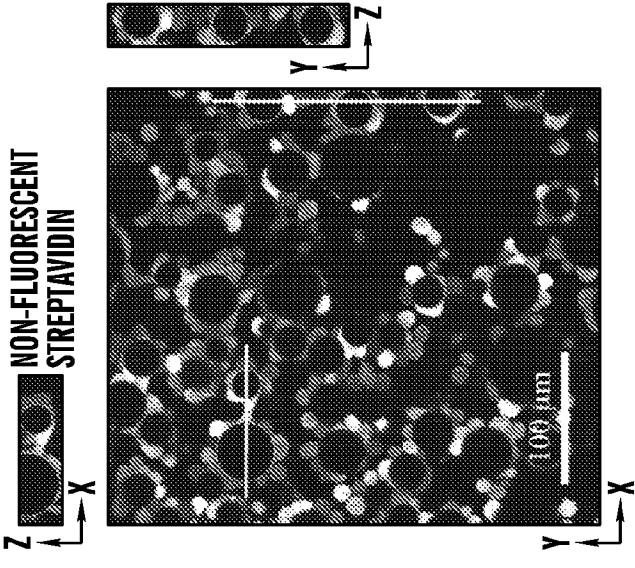


FIG. 6B

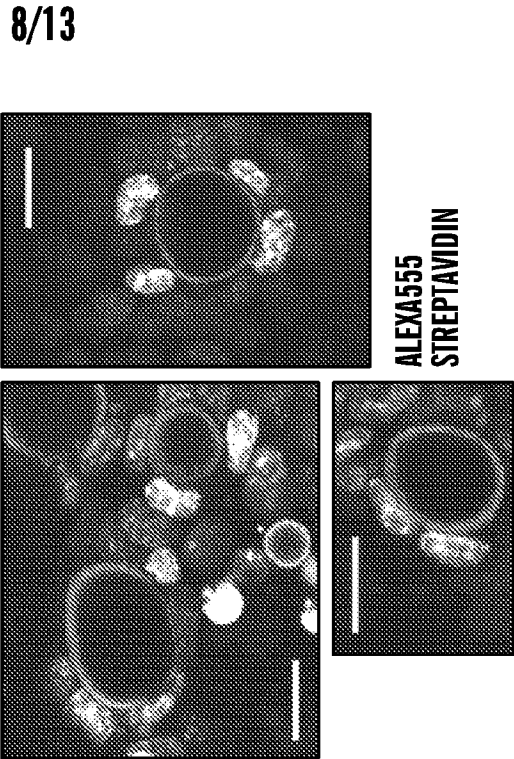


FIG. 6C

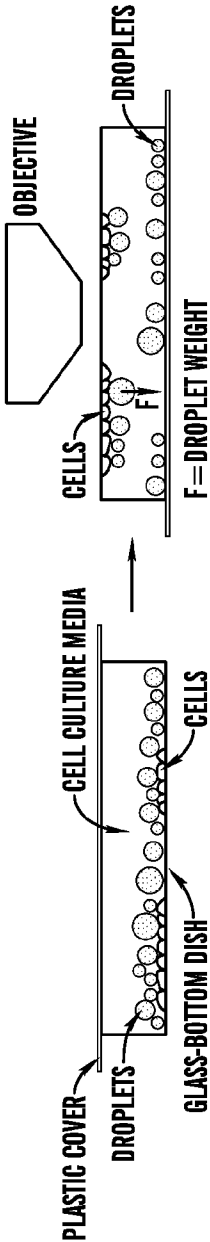


FIG. 6D

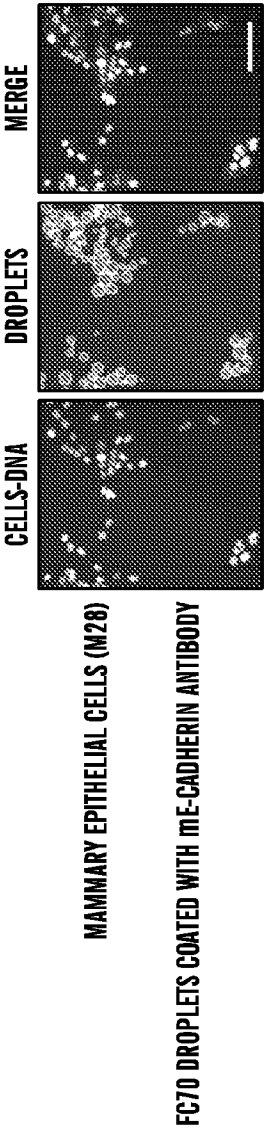
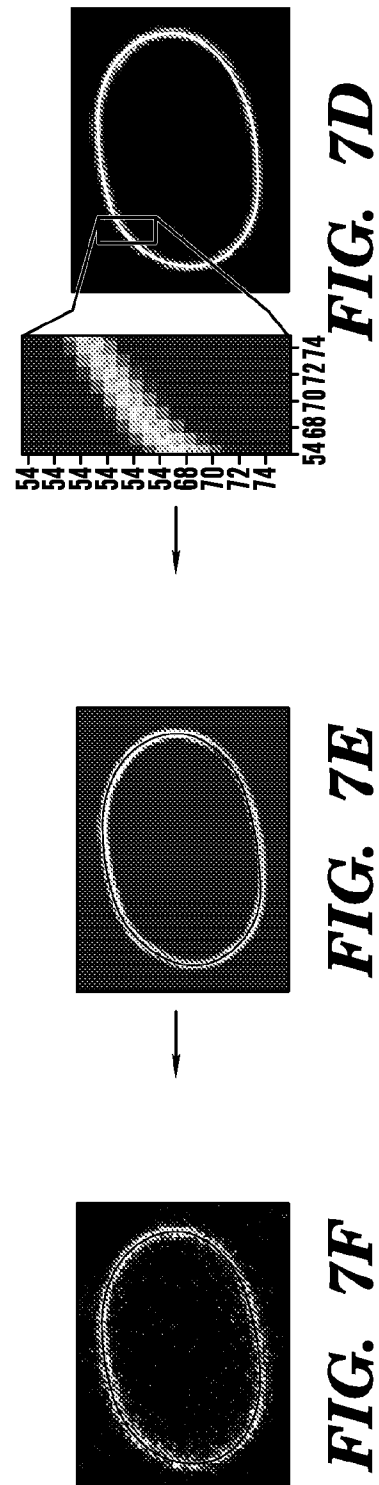
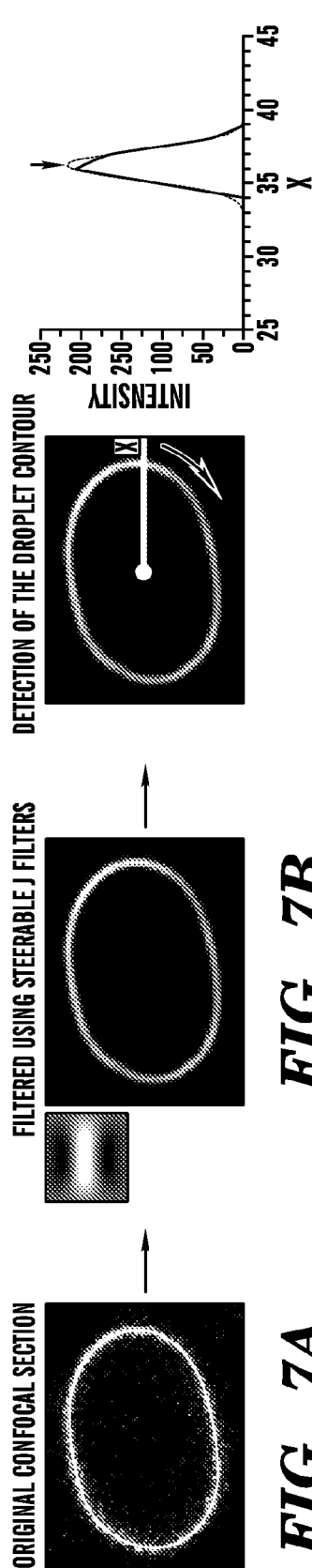


FIG. 6E

DETECTION OF DROPLET CONTOUR ON CONFOCAL SECTIONS OF DROPLETS



DETECTION OF DROPLET CONTOUR FROM 3D CONFOCAL STACKS

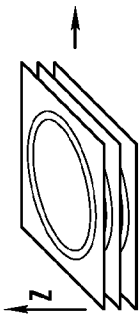


FIG. 8A

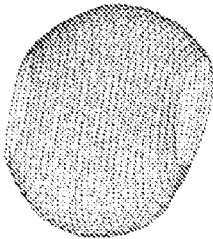


FIG. 8B

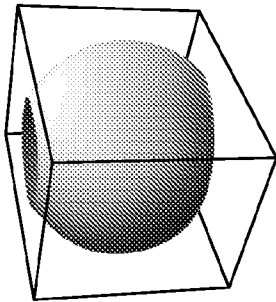


FIG. 8C

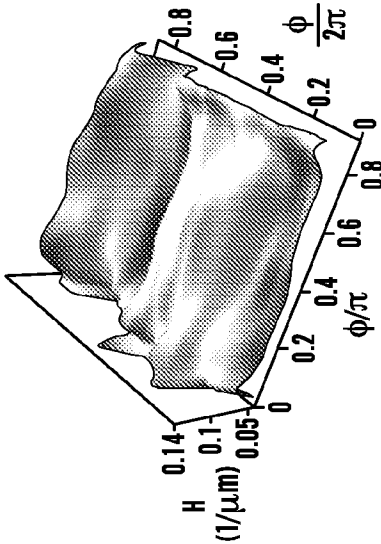
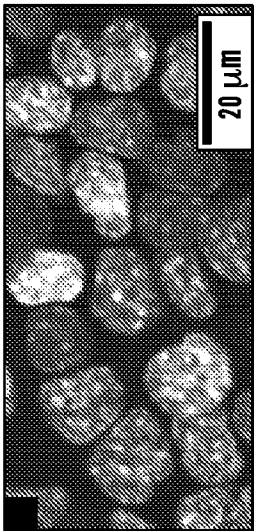


FIG. 8D



EPITHELIAL M28 CELLS
(CELL AGGREGATE)

FIG. 9A

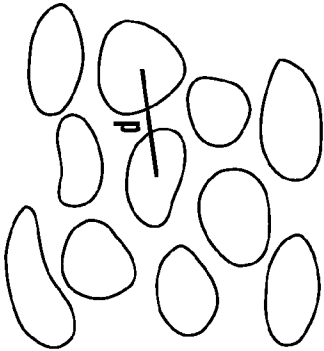


FIG. 9B

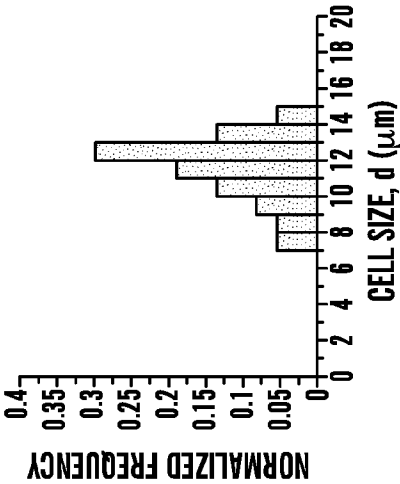


FIG. 9C



FIG. 9D

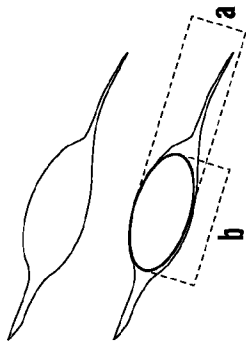


FIG. 9E

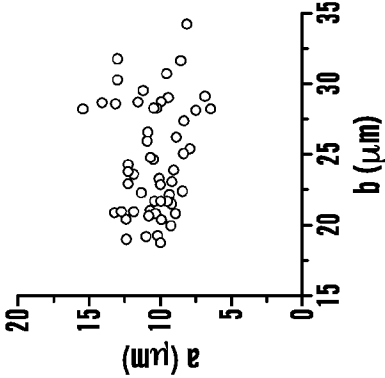


FIG. 9F

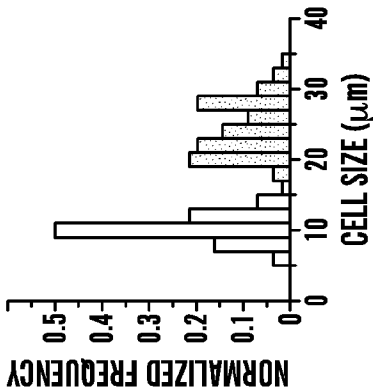


FIG. 9G

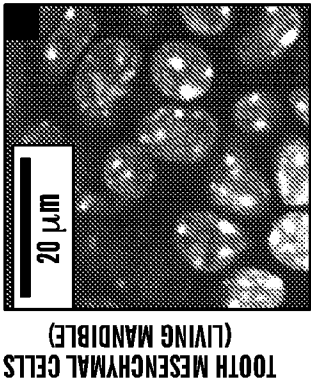


FIG. 9H

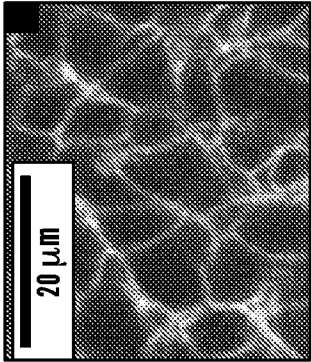


FIG. 9I

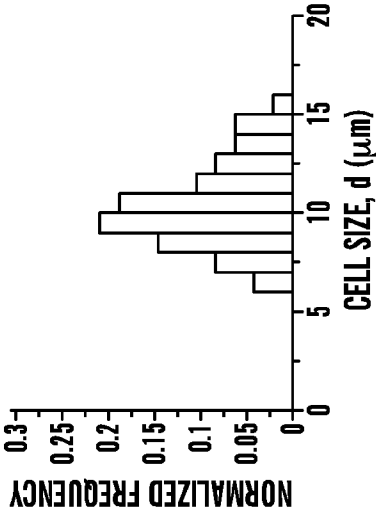
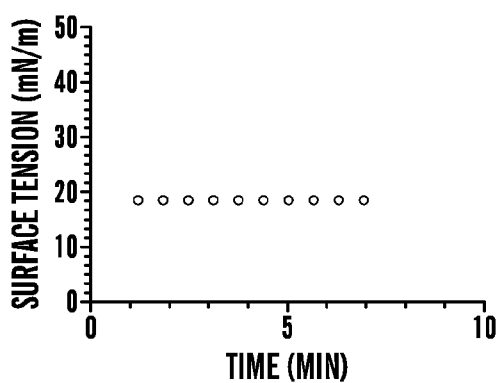
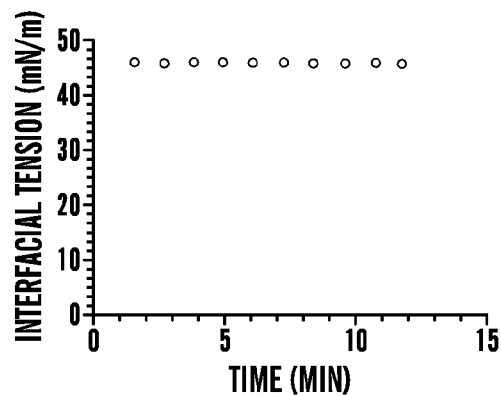
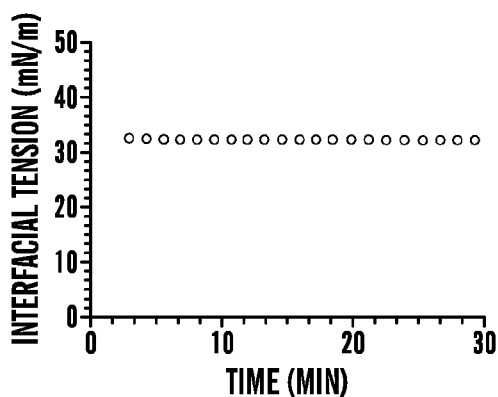
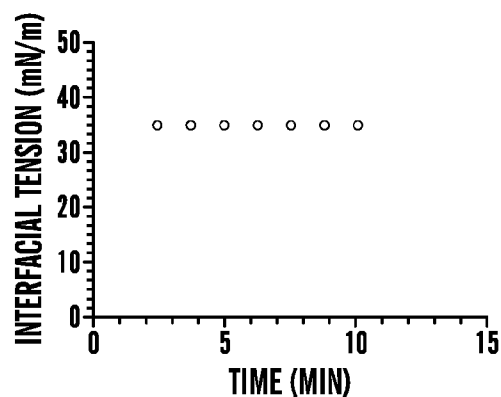
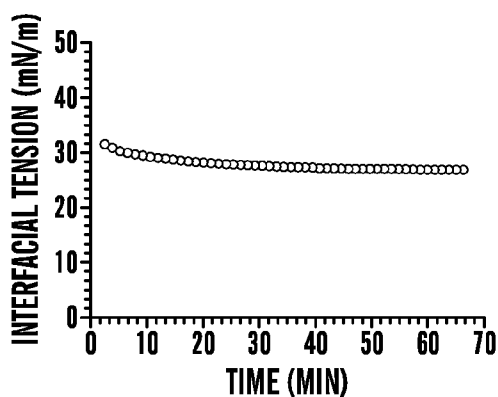
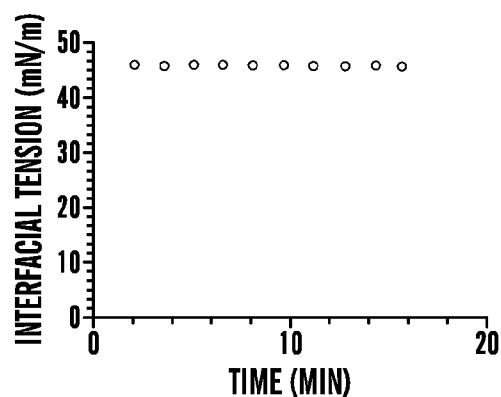


FIG. 9J

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**FIG. 10A****FIG. 10B****FIG. 10C****FIG. 10D****FIG. 10E****FIG. 10F**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 14/38744

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 15/02 (2014.01)

CPC - G01N 15/0205; G01N 15/0211

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8)-G01N15/02 (2014.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 356/336,338,342,341
CPC- G01N 15/0205; G01N 15/0211

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Patbase, Google patents, Google Scholar (without Patents)
Keywords: Cellular intercellular forces biocompatible layer oil surfactant fluorocarbon perfluorotripentylamine fluorescent optical detection measurement morphogenesis silicone moneral perfluorinated transducer droplet detectable molecule

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2010/0022414 A1 (Link et al.) 28 January 2010 (28.01.2010) para[0006], para [0007], para [0015], para [0045]	1-5
Y	US 2011/0256577 A1 (Himmelhaus et al.) 20 October 2011 (20.10.2011) para [0079], para [0082], para [0170]	1-5
Y	Choi et al, High throughput analysis of protein-protein interactions; Anal. Chem. Vol 84 pp 3849-3854 (2012) 25 March 2012 (25.03.2012) pp 3849-3850	5
Y,P	Campas et al., Quantifying cell-generated mechanical forces within living embryonic tissue; Nature Methods vol 11 pp 183-189 (2014) available online 08 December 2013 (08.12.2013)	1-5
Y	US 2010/0105112 A1 (Holtze et al.) 29 April 2010 (29.04.2010) entire document	1-5
Y	US 2008/0102036 A1 (Poss et al.) 1 May 2008 (01.05.2008) entire document	1-5



Further documents are listed in the continuation of Box C.



* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

25 August 2014 (25.08.2014)

Date of mailing of the international search report

10 SEP 2014

Name and mailing address of the ISA/US

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

International application No.

PCT/US 14/38744

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 6-24
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

Remark on Protest

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