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(71) Applicant (for all designated States except US): **UNIVERSITÉ PARIS DIDEROT - PARIS 7** [FR/FR]; 5, rue Thomas Mann, F-75013 Paris (FR).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **ASNACIOS, Atef** [FR/FR]; 90, avenue Victor Hugo, F-94100 Saint Maur (FR). **MITROSSILIS, Démosthène** [FR/FR]; 266, rue des Pyrénées, F-75020 Paris (FR). **BROWAEYS, Julien** [FR/FR]; 6, rue Boulle, F-75011 Paris (FR).

(74) Agents: **VIGNESOULT, Serge** et al.; Cabinet Plasseraud, 52 rue de la Victoire, F-75440 Paris Cedex 09 (FR).

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(54) Title: METHOD FOR DETERMINING THE RESPONSE OF A CONTRACTILE OR EXPANSIBLE BIOLOGICAL MATERIAL

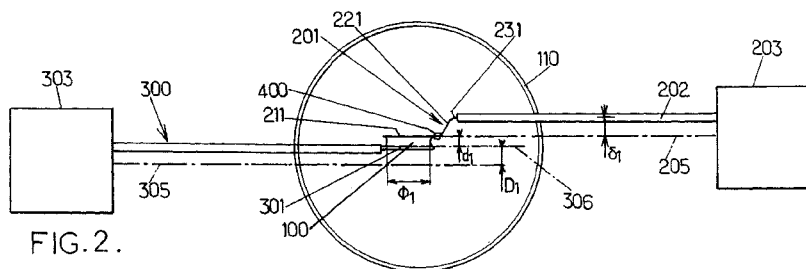


FIG. 2.

(57) Abstract: A method for determining the response of a contractile or expansible biological material to a variation of the rigidity of its environment comprising the steps of: a) placing a sample (100) of the biological material between a force measurement device (200, 500) and a rigid support (300, 600); b) choosing an effective rigidity value, k_{eff} , of the environment of the sample (100); c) maintaining the contacting area in a constant position by applying a force, $F(t)$, where $F(t) = k_e \cdot \delta(t)$ and where $\delta(t)$ is the stretching of the elastic probe; - simultaneously translating the rigid support of a distance $D(t)$, where $D(t) = \delta(t) \cdot (k_e / k_{eff})$; - determining the response of the biological material sample (100); d) repeating steps b) and c) when choosing an effective rigidity value different from former effective rigidity value when the sample (100) remains in situ.

**METHOD FOR DETERMINING THE RESPONSE OF A CONTRACTILE OR
EXPANSIBLE BIOLOGICAL MATERIAL**

The present invention relates to a method for
5 determining the response of a contractile or expansible
biological material to variation of the rigidity (also
called stiffness) of its environment.

There is a growing piece of evidence that passive
mechanical properties, especially the stiffness of the
10 local environment, influence cellular functions. In
particular, substrate rigidity appears to be a key factor
controlling cell migration and differentiation and may thus
play a major role in the development and maintenance of
tissues in vivo, as well as in tissues engineering in
15 vitro.

The same applies to other biological material such as
roots or stems.

In order to study the influence of substrate rigidity
on biological samples, experiments are performed up to now
20 by using a plurality of substrates of different rigidities.

These experiments are time consuming and expensive
because the whole experimental process has to be carried on
for each of the substrates, i.e. for each rigidity or
stiffness value tested (including tricky biological samples
25 preparations and micromanipulations). Moreover, while
current methods allow one to compare the behaviour of
different samples facing different rigidities, it is not
possible, up to now, to determine the response of a given
sample to a sudden change in the stiffness of its
30 environment.

The aim of the present invention is to overcome said disadvantages and to offer a method for determining the response of a contractile or expansible biological material by using a simple, efficient and time saving method.

5 The object of the present invention is reached thanks to a method for determining the response of a contractile or expansible biological material according to a variable rigidity environment comprising the steps of:

10 a) placing a sample of the biological material between a force measurement device and a rigid support, wherein:

15 - the force measurement device measures the force applied by the sample to a contacting area of an elastic probe of the force measurement device and said elastic probe has an elastic response to the said applied force which spring constant is k_0 ;

 - the rigid support is translatable movable;

 b) choosing an effective rigidity value, k_{eff} , of the environment of the sample;

20 c) maintaining the contacting area in a constant position by applying at the time, t , a force, $F(t)$, where $F(t) = k_0 \cdot \delta(t)$ and where $\delta(t)$ is the stretching of the elastic probe;

 - simultaneously translating the rigid support of a distance $D(t)$, where $D(t) = \delta(t) \cdot (k_0 / k_{eff})$;

25 - determining the response of the biological material sample ;

 d) repeating steps b) and c) when choosing an effective rigidity value different from former effective rigidity value when the sample remains in situ.

According to the present invention following wording have to be understood as following:

- "biological material" refers to a biological system consisting of one or a plurality of cells;

5 - "contractile" refers to the ability to contract or causing contraction, i.e. to decrease in size and to generate force. Contractility of cells or tissues is a well known characteristic for biologists. Muscle is an example of a contractile tissue. The ability of a muscle tissue to
10 contract relates namely in the ability to produce force resulting from different degrees of binding between myosin (thick) and acting (thin) filaments and depends on the concentration of Ca^{++} ions;

 - "expansible" refers to the ability to expand or
15 causing expansion, i.e. to increase in size and to generate force. Expansibility of roots or stems is a well known characteristic for biologists;

 - "elastic response" refers to the ability of a material to act as a spring of stiffness (also called
20 "spring constant") k . According to an embodiment, a material is characterized by a linear stress-strain relationship, and thus by a Young modulus E . The stiffness k is determined by the value of E and the geometric features (size, shape) of the material. In the frame of the
25 present invention and according to preferred embodiments, the value of the spring constant k_0 of the elastic probe is chosen so that the typical forces generated by the biological sample, when contracting or expanding, leads to measurable bending or deflection. For instance, when
30 working with single living cells, typical length scales are in the μm range and forces are in the nano-Newton range;

- "rigid" refers to a material whose elastic modulus and geometric characteristics are such that it can be regarded as non deformable according to the forces generated by the biological sample when contracting,
5 expanding or being deformed;

- "maintaining the contacting area in a constant position by applying at the time, t , a force, $F(t)$ " has to be understood as maintaining said position identical to former position within a given range of admissible error;
10 as for an example, admissible errors are from about 100 nm to about 500 nm; thanks to simultaneously applying a force, $F(t)$, and translating the rigid support such a result can be achieved.

According to the present invention, it has to be
15 understood that the meaning of the wording "environment" relates to the environment, the rigidity of which may affect the response of the biological material. Therefore, the liquid media around the sample is not the "environment" according to the present invention.

20 Said "environment" corresponds explicitly and unambiguously to the system consisting of the force measurement device and the rigid support which are interrelated. The force measurement device has an elastic response and the rigid support is substantially infinitely
25 stiff (or rigid). The rigidity of the system consisting of the interrelated force measurement device and rigid support is thus the effective rigidity perceived by the biological material.

30 Interrelation between the force measurement device and the rigid support can be implemented according to every means known by a man skilled in the art so that the

effective rigidity perceived by the biological material is variable. According to embodiments, the link suitable to let the force measurement device and the rigid support be interrelated is obtained thanks to electro-mechanical and/or electro-optical means. According to an embodiment, said link comprises regulation means.

Thanks to the present invention, it is now possible to study continuously the behaviour of a biological material according to different rigidity environment, namely of the system consisting of the interrelated force measurement device and rigid support, when the biological material remains in situ. The effective rigidity of the environment can vary on a very wide range.

It is then possible to study the biological material behaviour in a situation corresponding to different rigidities that can simulate the rigidity of different living tissues the biological material may meet. One can then simulate the behaviour of the biological material when moving within an organism and meeting different types of living tissues.

The "choosing" step b) thus relates to the choice by the experimenter of a rigidity corresponding to this of a tissue of interest. This choice can be for example decided step by step by the experimenter or according to predefined series of values in order to study a range or ranges of effective rigidity.

Thanks to the present invention, the environment of the biological material may have a variable and/or tunable rigidity.

Experiments can thus be performed on the same biological material sample for numerous, continuously

tunable, values of the environment rigidity. The present method is thus time and/or cost effective. Moreover, according to the embodiment of the present invention, it becomes now possible to follow, in real-time, the
5 biological sample response/adaptation to a sudden change in the effective stiffness of its environment.

According to various embodiments that can be combined according to all the possible combinations:

- step c) is performed thanks to a regulation loop;
- 10 - the value of k_{eff} is continuously changed over time; according to another embodiment the value of k_{eff} is changed step by step over time;
- the determined response of the sample is the variation of the size, namely the diameter ($\emptyset$) of said
15 sample and/or the force generated by the said sample;
- the determined response of the sample is obtained by simultaneous optical observation;
- the rigid support is a rigid plate rigidly fixed to a movable holder.

20 According to an embodiment, the elastic probe of the measurement device is an elastically bendable plate comprising a contacting area, an intermediate part and a basis part rigidly fixed to a movable holder; according to said embodiment the plates may be glass plates where the
25 bendable plate is thin and the rigid plate is thick.

According to another embodiment, the elastic probe of the measurement device consists of a dielectric particle actuated by optical tweezers; according to said embodiment the dielectric particle may be a sphere.

The present invention is also directed to a computer program product comprising one or more stored sequence of instruction that is accessible to a processor and which, when executed by the processor, causes the processor to

5 carry out of the steps of the previously mentioned process of the invention and of its various embodiments, and to a computer-readable medium carrying one or more sequences of instructions of said computer program product.

The invention will be better understood from the

10 accompanying non limiting drawings and examples, not necessarily drawn at scale, taken in conjunction with the accompanying description, in which like reference characters refer to similar parts, and in which:

Figures 1 to 4 illustrate schematic top views of

15 embodiments where the force measurement device comprises a two plates mechanical testing device, for given steps of the method according to the present invention.

Figure 5 illustrates a schematic view where the force measurement device comprises optical tweezers.

20 Figure 1 illustrates the initial step of an experiment performed according to the present invention. In this particular example the biological material sample is a single cell 100. The cell 100 has previously been captured and stuck between an elastically bendable plate (first

25 plate) 201 and a rigid plate (second plate) 301. The cell and first and second plates are placed in a chamber 110 filled with essential medium 115 suitable to keep the cell 100 alive.

According to an example the size of the cell 100 is

30 about 10 μm .

The force measurement device is an elastically bendable plate 201 comprising a contacting area 211, an intermediate part 221 and a basis part rigidly fixed to a movable holder 202. The rigid support is a rigid plate 301
5 rigidly fixed to a movable holder 302.

A first plane surface 102 of the cell 100 contacts the internal surface of a first part 211 of the first plate 201. According to an example the contacting area 211 surface is about $100\text{ }\mu\text{m}^2$.

10 A second plane surface 101 of the cell 100 contacts the internal surface of the second plate 301.

The first and second plates are oriented parallel to each other.

In this example the plates are composed of two parts:
15 a shaped glass plate melted down with a capillary tube. The plates are pulled from a borosilicate glass plate of 10 cm long x 1 mm large x 0.1 to 0.3 mm thick. After being pulled, the plates are cut to remove the tapering tip as well as the most of the unstretched plate portion. Removing
20 the tapering tip allows to stick the cells on a wide rectangular extremity.

Said rectangular extremity is used as the first and second plate of the present invention, according to the thickness of the pulled glass.

25 Typical size of the first plate 201 which is elastically bendable is $30\text{ }\mu\text{m}$ large x $5\text{ }\mu\text{m}$ thick x 6 mm long.

Typical size of the second plate 301 which is rigid is $60\text{ }\mu\text{m}$ large x $30\text{ }\mu\text{m}$ thick x 1 mm long.

The first and second plates 201, 202 are rigidly fixed to movable first and second holders 202, 302. Said holders are arms made of stainless steel rods and are movable thanks to piezoelectric devices 203, 303.

5 Chamber 110 is placed horizontally.

A microscope is placed to observe the cell 100 and the optical axis of said microscope is vertical.

First and second holders 202, 302 are tilted with an angle of about 30° to the microscope stage (60° to the
10 optical axis) so that the glass plates can be surrounded by medium 115.

To ensure that the plates 201, 301 can be settled parallel to the focal plan of the microscope during the experiments, the pulled plates and the capillary tubes are
15 fused with a tilt angle of about 25° .

According to this example the two arms 202, 302 are fixed symmetrically on each side of the optical axis of a Leica DMIRB inverted microscope from Leica Microsystems. Each arm 202, 302 is mounted on a M-UTR46_A precision
20 manual rotation stage from MicroControle company. The latter is fixed by a pivot pin on a piezoelectric stage 203, 303 referred as "100 x 100 x 100 μm^3 travel NanoCube XYZ" from OptoSigna-Photonetics Company. The two manual rotations allow the microplates 201, 301 to be parallel to
25 each other and to the optical axis. Translation in the three space directions are achieved by XYZ (three directional) stages for rough (micrometer precision) positioning of the plates before the beginning of the assays, while piezoelectric stages allow fine computer
30 controlled displacements (with a sub-micronic precision).

The chamber 110 is a stainless steel box with a glass window allowing optical visualization.

The whole setup is enclosed in a Plexiglas box maintained at 37° plus/minus 0.2° , and lies on an active
5 anti-vibration table which itself is supported by a stiff optical table.

The micro-plates 201, 301 with the cell 100 in between are visualized under bright light illumination with a Plan
Fluctar L 63 x /0.70 objective and a Micromax digital
10 charge-coupled device (CCD) camera from Princeton Instruments.

The position of the first part 211 of the first plate 201, said first part 211 contacting the biological sample, is determined thanks to a Photo Sensitive Device (PSD),
15 S3979 from Hamamatsu, placed in zone 400.

Other movements and/or applied loads are determined thanks to the piezoelectric devices 203, 303.

The initial distance, Δ , between the first part 211 of the first plate and the second plate 301 may be measured.
20 The initial position of the internal surface of the first part 211 defines a vertical plane comprising an axis 205. The initial position of the internal surface of the rigid plate 301 defines a vertical plane, parallel to preceding plane, comprising an axis 305. The axis 205 and 305 are
25 disposed in a same horizontal plane.

The cell thickness d is given by the distance between said two axes 205 and 305.

The initial diameter of the cell 100 is $\varnothing_0$.

A rigidly index α is chosen, where α is defined as
30 $\alpha = k_{\text{eff}}/(k_0 + k_{\text{eff}})$ and where $0 \leq \alpha \leq 1$, and is entered in

a computer system suitable to provide a regulation loop on the experimental device.

The cell 100 contracts over the time, t , as shown on figure 2. Figure 2 relates to a step consecutive to a prior
5 initial step as illustrated on figure 1. The cell pulls on both plates and the cell thickness is $d(t)$.

Following displacements are measured over the time:

$p(t)$ is a distance corresponding to the position variation of the internal surface of the first part 211 of
10 the first plate 201 (i.e. the variation of the position of axis 205) ; $p(t)$ is measured thanks to the PSD in zone 400;

$D(t)$ is a distance corresponding to the position variation of the internal surface of the rigid plate 301 (i.e. that of axis 305); $D(t)$ is measured thanks to
15 position variation of arm holder 302 captured by piezoelectric stage 303;

Stretching of the elastic probe, $\delta(t)$, is the position variation of the basis part 231 of the first plate; $\delta(t)$ is measured thanks to position variation of arm holder 202
20 captured by piezoelectric stage 203.

It has to be understood that the wording "internal" is used to designate the side of a plate which is contacting the cell 100.

Displacements are applied to the first and second
25 holders 202, 302 so that the first part 211 of the first plate 201 is maintained at its initial position, i.e. $p = 0$.

According to a first embodiment shown on figure 2,
 $\alpha \neq 0$ and $\alpha \neq 1$.

At a given step of the process, after measuring the actual $p(t)$ value, a feedback loop correction $C(t)$ is applied to the system so as a displacement, $\delta_1(t) = \alpha \cdot C(t)$, is applied to the basis part 231 of the first plate 201, and so as a displacement $D_1(t) = (1 - \alpha) \cdot C(t)$ is applied to the rigid plate 301.

The system then acts as if the cell was facing an elastically bendable plate of an effective stiffness:

$$k_{\text{eff}} = \frac{\alpha}{1 - \alpha} \cdot k_0$$

One can also determine corresponding cell thickness d_1 and diameter ϕ_1 .

According to a first example, one chooses $k_{\text{eff}} = k_0$. The applied force is $F(t) = k_0 \cdot \delta_1(t)$ and $D_1(t) = \delta_1(t)$. According to this embodiment $\alpha = 0.5$ and $C(t) = 2 \cdot \delta_1(t)$.

According to a second example, one chooses $k_{\text{eff}} = 5 \cdot k_0$. The applied force is $F(t) = k_0 \cdot \delta_1(t)$ and $D_1(t) = \delta_1(t)/5$. According to this embodiment $\alpha = 0.83$ and $C(t) = 1.2 \cdot \delta_1(t)$.

According to a second embodiment shown on figure 3, $\alpha = 1$. Figure 3 relates to a step consecutive to a prior initial step as illustrated on figure 1. The position of the rigid plate 301 remains constant so as the cell thickness remains equal to Δ .

The position of the basis part 231 of the first plate 201 is submitted to a displacement $\delta_2(t) = C(t)$

According to this second embodiment, the system acts as an effective rigidity environment for the cell where the substrate rigidity is infinite.

According to a third embodiment shown on figure 4,

5 $\alpha = 0$. Figure 4 relates to a step consecutive to a prior initial step as illustrated on figure 1. The position of the rigid plate 301 is moved so as the position of the basis part 231 of the first plate 201 remains constant, thus $\delta_3(t) = 0$.

10 Corresponding displacement of the rigid plate 301 is $D_3(t) = C(t)$.

According to this third embodiment, the system acts as an effective rigidity environment for the cell where the substrate rigidity is nil.

15 It is thus possible to study the cell behaviour over the whole effective rigidity range.

The embodiment of figure 1 to 4 is a non limiting example regarding embodiments where the force measurement device comprises a two plates mechanical testing device.

20 According to another embodiment of the invention, the first plate which is elastically bendable is a cantilever of a single cell force spectrometer such as CellHesion® apparatus commercialized by JPK Instruments, for example a CellHesion® 200 apparatus.

25 According to another embodiment of the invention, the first plate which is elastically bendable is a cantilever of an atomic force microscope (AFM). The elastically bendable plate may be a metallic plate, as for example a 50 μm thick plate which length may be about 0.5 mm.

The cantilever of the AFM may optionally comprise an indenter, such as a silica sphere.

Local tips of AFM may also be used.

Figure 5 illustrates an embodiment of the present invention where the elastic probe of the measurement device, 500, consists of a dielectric spherical particle, 501, actuated by optical tweezers.

Optical tweezers are capable of manipulating nanometer and micrometer-sized dielectric particles by exerting extremely small forces via a highly focused laser beam. The beam is typically focused by sending it through a microscope objective. The narrowest point of the focused beam, known as the beam waist, contains a very strong electric field gradient. It turns out that dielectric particles are attracted along the gradient to the regions of strongest electric field, which is the centre of the beam. The laser light also tends to apply a force on particles in the beam along the direction of beam propagation. It is easy to understand why if one considers light to be a group of particles, each impinging on the tiny dielectric particle in its path. This is known as the scattering force and results in the particle being displaced slightly downstream from the exact position of the beam waist.

Optical traps are very sensitive instruments and are capable of the manipulation and detection of sub-nanometer displacements for sub-micrometre dielectric particles. For quantitative scientific measurements, most optical traps are operated in such a way that the dielectric particle rarely moves far from the trap centre. The reason for this is that the force applied to the particle is linear with

respect to its displacement from the centre of the trap as long as the displacement is small. In this way, an optical trap can be compared to a simple spring, which follows Hooke's law and has a spring constant k_0 .

5 Said dielectric particle displaced thanks to optical tweezers can be used as elastic probe and be a component of a measurement device.

 Detailed embodiments of optical tweezers are disclosed for example in following publications which are hereby
10 enclosed by reference:

 - "*A revolution in optical manipulation*" - David G. Grier - Nature / Vol 424 / 14 August 2003;

 - "*Optical trapping*" - Keir C. Neuman and Steven M. Block - Review of Scientific Instruments, Volume 75, n°9,
15 September 2004.

 As shown on the embodiment of Figure 5, a laser beam 521 is reflected on a tiltable mirror 530 and the reflected beam 522 is focused thanks to an optical lens device 535 so as the focus beam 523 converges on a dielectric spherical
20 particle 501.

 According to an example, the dielectric spherical particle 501 has a 2 μm diameter.

 Said particle 501 contacts a biological sample 100 in a contacting area 511. According to an example, the
25 contacting surface is about 1 μm^2 .

 The biological sample 100 is surrounded by a biological medium 115, such as salted culture medium. Said sample 100 is arranged on a rigid support 600. The rigid support 600 consists of a rigid plate 601, as for example a
30 thick glass plate, placed on a rigid annular spacer 603.

The rigid spacer may be arranged on a piezoelectric stage, as those previously exemplified.

The rigid support 600 is translatable movable, in a direction perpendicular and/or parallel to the main surface
5 of the rigid plate 601.

The tilt of the mirror 530 induces a displacement of the trap centre and then elastically reacts to the force applied by the sample 100 in the contacting area 511.

The mirror 530 can be for example a galvanometric
10 mirror which rotation, i.e. tilt, can be fine tuned and be actuated as a component of a regulation loop.

Experiments can thus be performed in situ on the same biological material sample for numerous, continuously tunable, values of the environment rigidity. Following, in
15 real-time, the biological sample response/adaptation to a sudden change in the effective stiffness of its environment is then possible.

According to other embodiments of the invention, one can arrange a microscope so as to observe the cell through
20 the plates and where the plates are for example disposed in a horizontal plane perpendicular to the optical axis.

Furthermore it is possible to carry on fluorescence studies monitoring cellular functions while performing simultaneously the process of the invention.

25 The exemplified embodiments are non limiting embodiments and the method of the invention can be implemented on a wide number of systems so as to study contractile or expansible biological materials.

CLAIMS

1. A method for determining the response of a
5 contractile or expansible biological material, arranged
between a force measurement device (200, 500) and a rigid
support (300, 600) which are interrelated, to a variation
of the rigidity of the system consisting of the
interrelated force measurement device and rigid support,
10 the said method comprising the steps of:

a) placing a sample (100) of the biological material
between the force measurement device (200, 500) and the
rigid support (300, 600), wherein:

- the force measurement device (200, 500) measures the
15 force applied by the sample (100) to a contacting area
(211, 511) of an elastic probe (201, 501) of the force
measurement device (200, 500) and said elastic probe (201,
501) has an elastic response to the said applied force
which spring constant is k_0 ;

20 - the rigid support (300, 600) is translatable;

b) choosing an effective rigidity value, k_{eff} , of the
system consisting of the interrelated force measurement
device and rigid support of the sample (100);

c) maintaining the contacting area (211, 511) in a
25 constant position by applying at the time, t , a force,
 $F(t)$, where $F(t) = k_0 \cdot \delta(t)$ and where $\delta(t)$ is the stretching
of the elastic probe (201, 501);

- simultaneously translating the rigid support (300,
600) of a distance $D(t)$, where $D(t) = \delta(t) \cdot (k_0 / k_{eff})$;

- determining the response of the biological material sample (100);

d) repeating steps b) and c) when choosing an effective rigidity value different from former effective
5 rigidity value when the sample (100) remains in situ.

2. The method of claim 1 wherein the contractile material is a cell, an aggregate of cells or a living tissue.

3. The method of claim 1 wherein the expansible
10 material is a root or a stem.

4. The method of any of preceding claims wherein step c) is performed thanks to a regulation loop.

5. The method of any of preceding claims wherein the value of k_{eff} is continuously changed over time.

15 6. The method of any of claims 1 to 4 wherein the value of k_{eff} is changed step by step over time.

7. The method of any of preceding claims wherein the determined response of the sample (100) is the variation of the size, namely the diameter ($\emptyset$) of said sample (100)
20 and/or the force, $F(t)$, generated by the said sample.

8. The method of any of preceding claims wherein the determined response of the sample (100) is obtained by simultaneous optical observation.

9. The method of any of preceding claims wherein the
25 rigid support is a rigid plate (301) rigidly fixed to a movable holder (302).

10. The method of any of preceding claims wherein the elastic probe of the measurement device is an elastically bendable plate (201) comprising a contacting area (211), an
30 intermediate part (221) and a basis part (231) rigidly fixed to a movable holder (202).

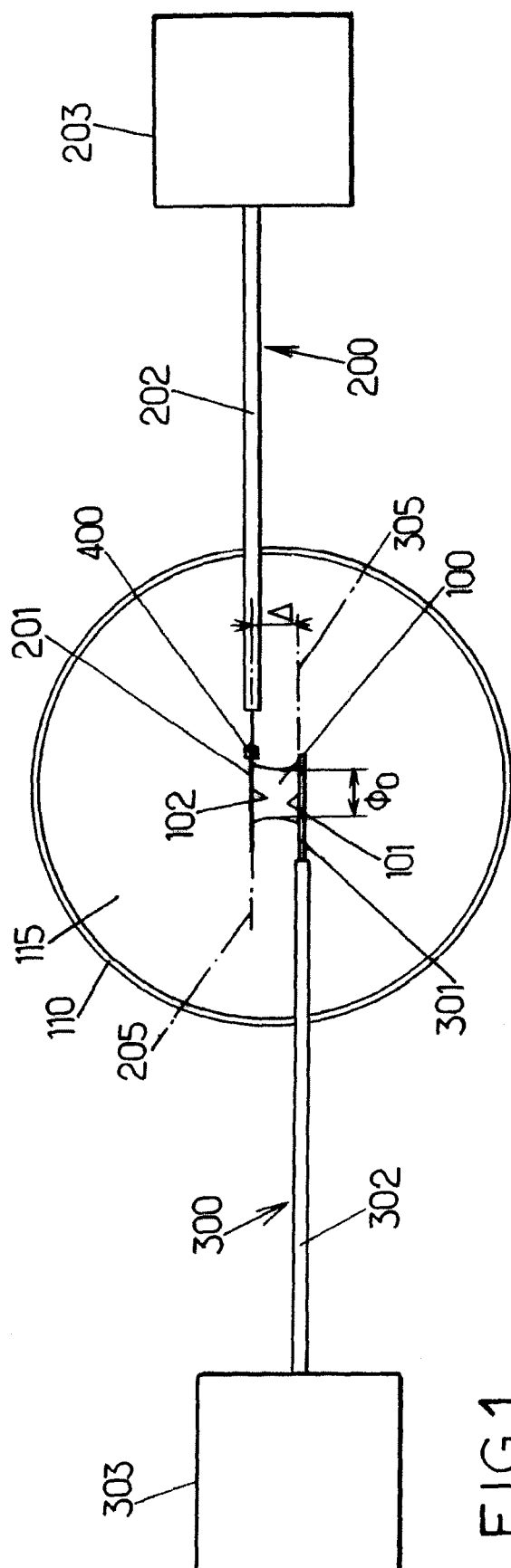
11. The method of claims 9 and 10 combined wherein the plates (201, 301) are glass plates where the bendable plate (201) is thin and the rigid plate (301) is thick.

12. The method of any of claims 1 to 9 wherein the
5 elastic probe of the measurement device consists of a dielectric particle actuated by optical tweezers.

13. The method of preceding claim where the dielectric particle is a sphere.

14. A computer program product comprising one or more
10 stored sequence of instruction that is accessible to a processor and which, when executed by the processor, causes the processor to carry out the steps of any of claims 1 to 13.

15. A computer-readable medium carrying one or more
15 sequences of instructions of the computer program product of claim 14.



161.

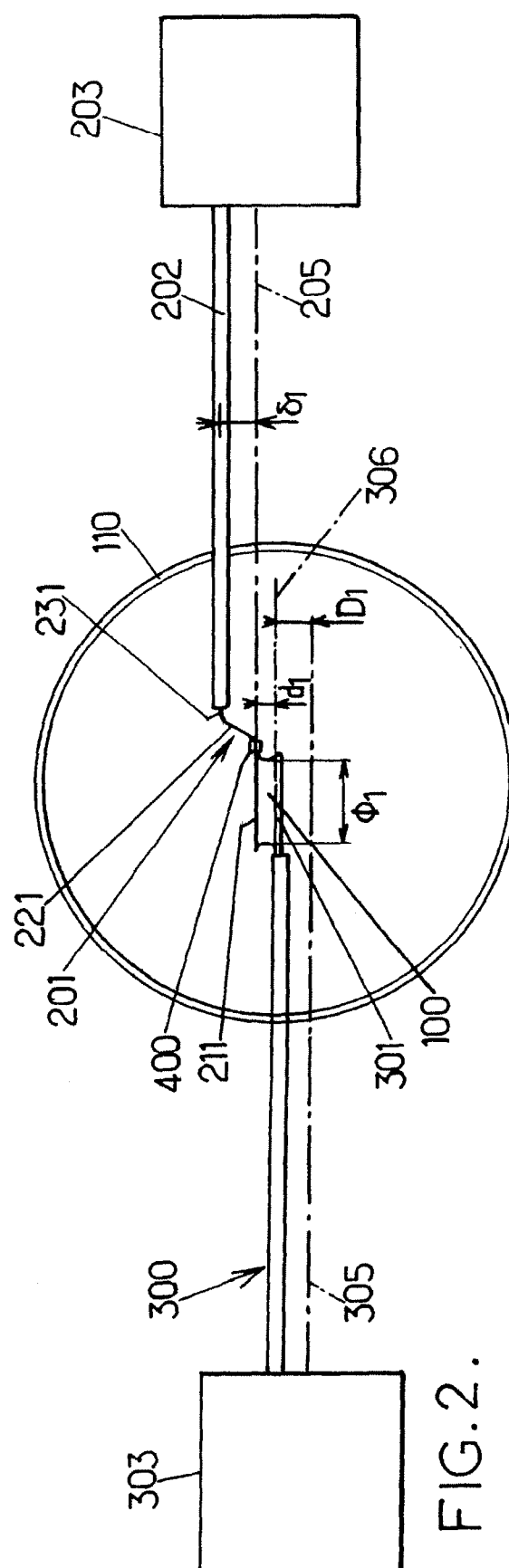
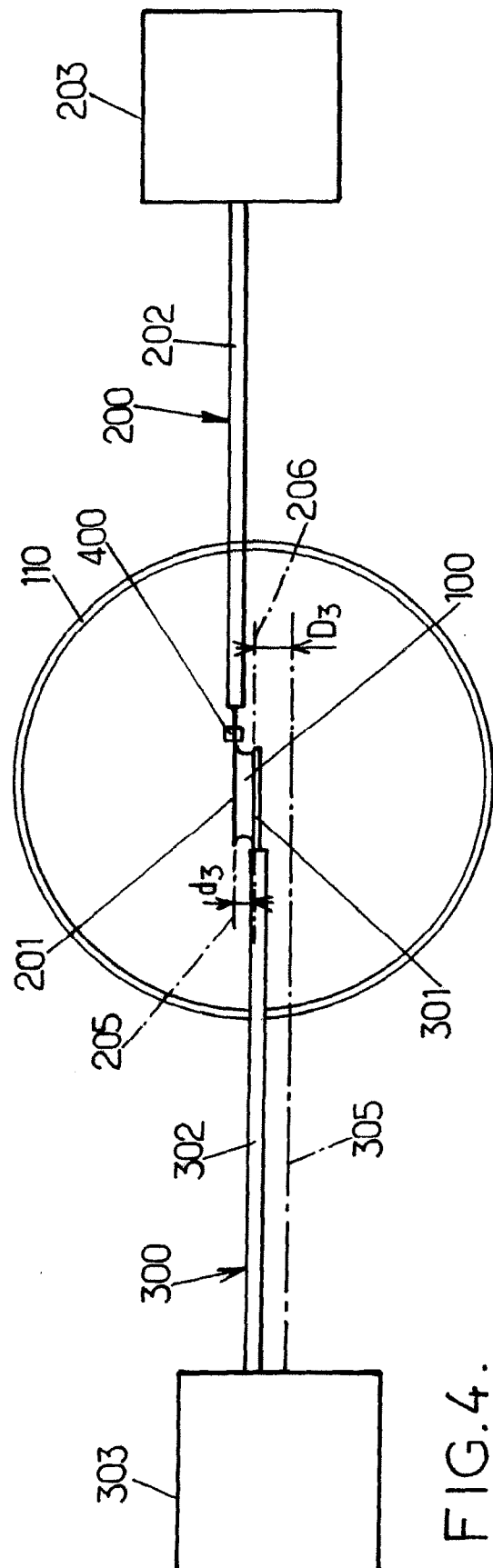
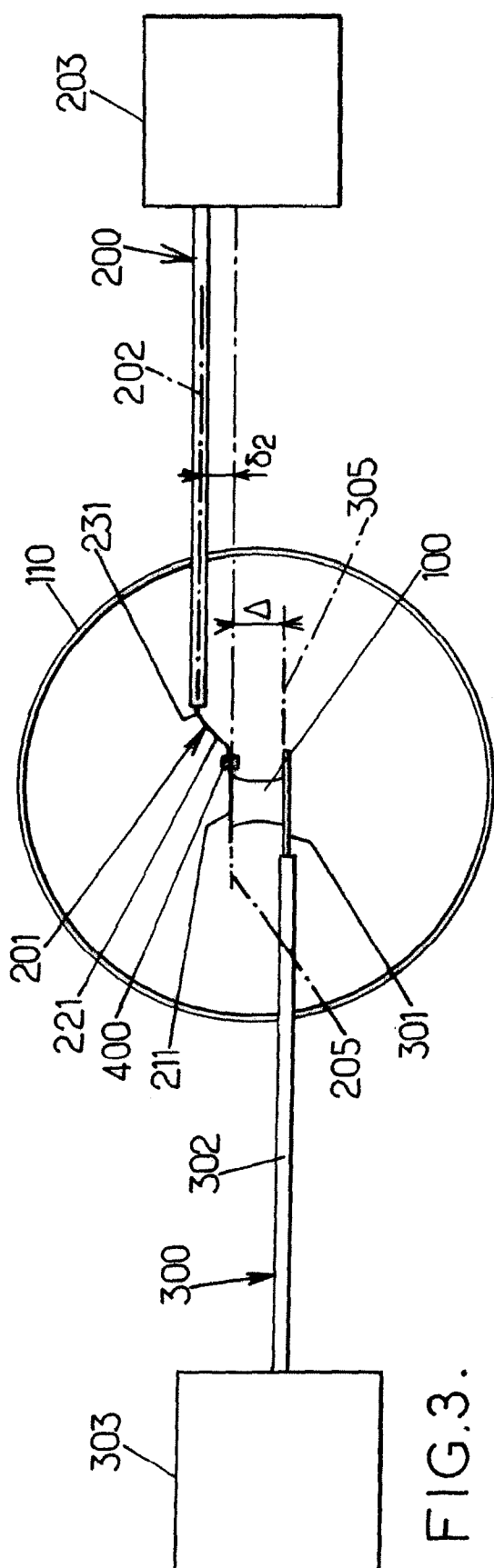


FIG. 2.

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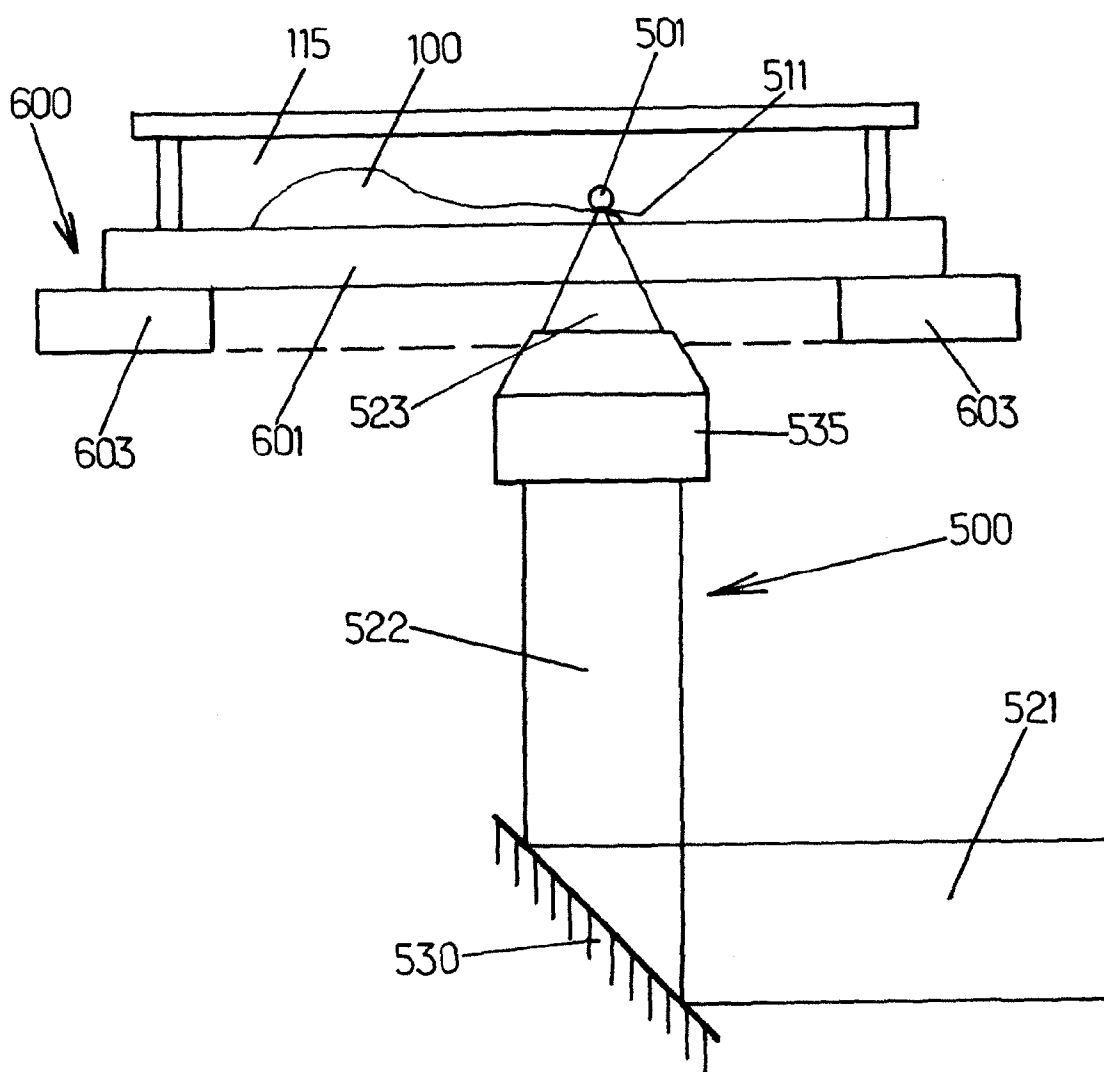


FIG. 5.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/065742

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N3/24

ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N

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

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

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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See patent family annex.

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Date of the actual completion of the international search

14 January 2011

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Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Cantalapiedra, Igor

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2010/065742

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