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(54) Title: METHOD FOR SAMPLE PREPARATION FOR CRYOELECTRON MICROSCOPY (CEM), MICROREACTOR AND LOADING PLATFORM

(57) Abstract: A method for sample preparation for cryoelectron microscopy (CEM), wherein the sample is held in a microreactor, wherein the conditions in the microreactor are regulated relative to the environment, wherein the sample in the microreactor is frozen according to a quench freeze process, whereupon the sample, in frozen condition, is placed in the electron microscope. A microreactor for use with cryoelectron microscopy (CEM), comprising a first and second membrane, which membranes, at least in a condition of use, enclose a chamber, while the membranes are configured to last until at least the beginning of a quench freeze process.

Title: Method for sample preparation for cryoelectron microscopy (CEM), microreactor and loading platform.

The invention relates to a method for sample preparation for cryoelectron microscopy (CEM).

The invention also relates to a microreactor for use with CEM.

In addition, the invention relates to a loading platform for use with CEM.

CEM is often used for research on biological samples. Here, a sample is cooled at a low temperature, for instance below approximately 150° K. After cooling, the sample, still in cooled condition, is introduced into an electron microscope, in particular a transmission electron microscope (TEM). Through the cooling, any damage to the sample caused by the electron radiation is limited.

The sample is typically in a liquid and/or contains much liquid, while, in this description, water is used as example, because this liquid is often used with CEM. In order to realize, during cooling, as little modification as possible of the sample through crystallization of the water, the water must very rapidly be formed into amorphous ice. The amorphous form of the ice prevents morphological change of the sample, for instance a cell, through crystallization of the water, for instance through perforation of a cell wall. Furthermore, crystal formation results in the formation of undesired contrasts in the image result of the electron microscope.

For rapid cooling in CEM, a quench-freeze process, also called plunge-freeze process, is used. Here, preferably, the sample is cooled below 150° K, preferably at or below approximately 140° K, while the sample is frozen in a period of time of, for instance, approximately 10^{-5} seconds. After this, the liquid is kept cool, in particular at approximately the 140° K

mentioned, so that the liquid therein, in particular water, retains the amorphous form for as much as possible and does not take a crystalline form.

Due to the amorphous, frozen condition of the sample obtained through the quench freeze process, it is possible to examine the sample in a chemically virtually unchanged condition. Furthermore, by cooling according to the quench freeze process, a sample can be captured at a particular moment and/or in a particular condition, for instance during a biological or chemical reaction or development.

In order to be able to freeze in a short span of time according to the quench freeze process, it is advantageous if the sample is thin. It may for instance be so that before freezing, a sample is thinned or is sliced into thin slices, however, not all samples are suitable thereto.

A sample should also be thin for a sufficient degree of electron transparency. To that end, after the quench freeze process, the sample can for instance be cut into thin slices at approximately 100° K, with the aid of, for instance, a cryo ultra microtome. These slices can have a thickness of, for instance, between 20 and 200 nm. Another method is removing layers of the sample material, for instance of a particular cell structure, while cooling, with the aid of a focussed ion beam ("ion milling") until the desired location to be examined is reached, for instance the core of the cell structure.

In order to place the sample in unmodified condition in the electron microscope, the sample is typically placed in a loading platform where it is kept cool, for instance at or below approximately 140° K. What should also be prevented here is that water from the environment deposits as ice on the surface of the sample. To that end, the temperature and humidity in the loading platform are to be controlled well.

The entire process the sample goes through, placing the sample into the electron microscope included, is indicated in this description as sample preparation. A good sample preparation allows for transfer of the sample in the desired, frozen condition into the electron microscope.

The space in which, with CEM, the sample presentation takes place is typically maintained so as to be moisture free and at the proper temperature. Here, typically, spaces are concerned in which also, people are present and therefore, climate control has to meet high requirements. The control of the entire environment is relatively difficult, and can be considered as a cause due to which, often, only after many repetitions of the CEM process, a suitable image result is obtained.

A method for sample preparation for cryoelectron microscopy (CEM) according to the invention comprises, in a first aspect, holding the sample in a microreactor, wherein the conditions in the microreactor are regulated relative to the environment, while the sample is frozen according to a quench freeze process, whereupon the sample in the microreactor is placed in frozen condition into the electron microscope.

Such a method allows for a better regulation of the conditions of the sample in the microreactor, at least temporarily, for instance until the quench freeze process, than with conventional methods, as the microreactor allows relatively local regulation of the conditions. Regulating conditions such as, for instance, temperature, air humidity, pressure et cetera, locally in the microreactor is simpler than regulating such conditions for a larger space. After the quench freeze process too, the microreactor can prevent the occurrence of certain exchanges, for instance of moisture, between the sample and the environment. Placed into a microscope, the microreactor should then preferably be sufficiently electron transparent for examination of the sample.

In particular embodiments of the method, the conditions are regulated after the quench freeze process, for instance, by sliding the sample in the microreactor in cooled environment in a simple manner into a sample holder. As a result, undesired deformation of the sample and/or crystallization after the quench freeze process are prevented. As, owing to the invention, the process of sample preparation for CEM needs to be repeated less often than

with conventional methods, owing to the invention, the efficiency of the sample preparation is increased.

In a second aspect, the invention comprises a microreactor for use with cryoelectron microscopy (CEM), comprising a first and a second membrane, which membranes, at least in condition of use, enclose a chamber, while the membranes have been configured to last until at least the start of a quench freeze process.

The thin membranes of the microreactor, often with a thickness of, at least locally, only a few nanometres, appear in some embodiments to last sufficiently under the extreme temperature conditions of the CEM process until the desired moment in time.

Furthermore, in particular embodiments, the membranes can be constructed such that they offer transparency for electron beams over relatively large membrane surfaces relative to the membrane thickness.

A larger electron transparent area offers, inter alia, a higher striking probability. This means, for instance, that more sample particles can be observed and/or that sample particles can be spread more within the electron transparent area of the membranes.

In a third aspect, the invention comprises a loading platform for cryoelectron microscopy (CEM), comprising a bottom and at least one upstanding wall, wherein in a wall an opening is provided for introducing a CEM sample holder, while in the bottom of the loading platform, a first recess is provided, preferably with a rectangular shape, so that a microreactor, cartridge and/or sample holder can rest at least partly in said first recess, while the first recess links up with the opening so that, if the sample holder has been introduced partly through the opening into the loading platform, the microreactor and/or cartridge can be slid into the sample holder substantially parallel to the bottom of the first recess.

In clarification of the invention, exemplary embodiments of a method, loading platform and microreactor according to the invention will be further elucidated with reference to the drawing. In the drawing:

Fig. 1 schematically shows a CEM process;

Fig. 2 schematically shows an embodiment of a microreactor in perspective;

Fig. 3 schematically shows an embodiment of a microreactor in top plan view;

Figs. 4A-4D schematically show different alternative embodiments of microreactors in front view;

Fig. 5 schematically shows a loading platform in use;

Fig. 5B schematically shows, in side view, a part of a cross-section of a loading platform in use;

Fig. 5C schematically shows, in top plan view, a part of a loading platform in use,

Figs. 6A-6I show an illustration in steps of embodiments of sliding a sample into a sample holder;

Fig. 7 shows a cartridge for use in a method according to the invention; and

Figs. 8A - 8C schematically show an alternative embodiment of a microreactor.

In this description, identical or corresponding parts have identical or corresponding reference numerals. In the drawing, embodiments of the invention are shown merely by way of example. The elements used therewith are mentioned only by way of illustration and should not be construed to be limitative in any manner. Other parts, embodiments and methods too can be used within the framework of the present invention. The proportions in the drawings too should be understood to be purely schematic and given by way of illustration.

In Fig. 1, four steps 1, 2, 3, 4 of a method according to an embodiment of a sample preparation for CEM (cryoelectron microscopy) according to the invention are shown. A set up with which this method can be carried out according to one embodiment contains, for instance, an optical microscope 32, a quench freezer 7, a loading platform 33 and a transmission electron microscope 11.

In step 1, with the aid of, for instance, water, a sample 6 to be examined is flowed into a microreactor 5. The microreactor 5 comprises a chamber 19, which is enclosed by two membranes 16, 17 within which the sample 6 is present, see, by way of illustration, Fig. 2. The microreactor 5 is explained further in the description. For flowing the sample 6 into the microreactor 5, the microreactor 5 comprises an inlet 14 and an outlet 15 which lead to and from the chamber 19, respectively. This means that water flows through the microreactor 5, while the water carries along a part of a sample 6, or one sample, or several samples 6. Naturally, instead of water, also, a different fluid can be used for flowing into the microreactor 5. The sample 6 can also be laid, for instance with fluid, in an open microreactor 5, whereupon the microreactor 5 is closed.

The sample 6 can, for instance, be a very thin slice of a bulk sample 6. It can also be so that the sample 6 to be examined still has to be formed in the microreactor 5, or that the sample 6 is, for instance, an entire cell. It is noted that the types of sample 6 as discussed in the description serve merely as illustration and should not be construed to be limitative to the invention. For instance, the invention should not be limited to research on biological samples, but it can also be used in other fields.

In the embodiment shown in Fig. 1, the microreactor 5 is placed under a measuring instrument 32, for instance an optical microscope or X-ray diffraction meter 32. The microreactor 5 allows, for instance, that therein, the sample 6 remains relatively stable under relatively controlled conditions, undergoes a particular process or reaction and/or can be selected. The

microreactor 5 is also at least partly sufficiently transparent to light in the visible spectrum, so that the sample 6 can be observed with an optical microscope 32.

In step 2, a quench freezer 7, also called plunge freezer, is arranged. At a selected moment in time, the microreactor 5 with the sample 6 therein is placed the quench freezer 7. Herein are present a bath 31 of liquid ethane 8, arranged in a bath 30 with liquid nitrogen 9. In the quench freezer 7, the sample 6 is frozen according to the quench freeze process in a short period of time at a low temperature, for instance 140° K, in substantially amorphous condition.

In step 3, the microreactor 6 with the sample 6 is placed in a cooled loading platform 33, which, with the aid of liquid nitrogen, is held at low temperature, preferably below 150 ° K, in particular at or below 140° K. A cooling holder 34 cools a sample holder 10. In the present field of technology, the cooling holder 34 and the sample holder 10 are sometimes integrated, while the integrated cooling holder 34 and sample holder 10 are also called "cryo holder" 10, 34. In this description, sample holder 10 is also understood to include such a cryo-holder 10, 34. The cooled sample holder 10 projects into the loading platform 33, so that the microreactor 5 containing the sample 6 can be slid into the sample holder 10 and, in step 4, can be positioned in the electron microscope 11 in preferably virtual amorphous condition.

In step 4, with the aid of the sample holder 10, the sample 6 is placed into the transmission electron microscope (TEM) 11, at least is introduced into an electron beam thereof, so that a preferably accurate image of the sample 6 can be obtained.

In Fig. 2, schematically, an embodiment of said microreactor 5 is shown, comprising walls, designed here as a first chip 12 and a second chip 13. In the first chip 12 are arranged an inlet 14 and an outlet 15 for the passage of liquid and the sample 6. A first membrane 16 and a second membrane 17 are provided, which enclose a chamber 19, in which chamber 19 the liquid and the

sample 6 can flow from the inlet 14 in the direction of the outlet 15. Two windows 18A, 18B are provided in the chips 12, 13 to allow the passage of an electron beam and/or for examining the sample 6 between the membranes 16, 17 for instance visually and/or in a different type of measuring instrument 32 than a TEM 11.

The chips 12, 13 can be made with the aid of chip technology, for instance lithography. With it, the chips 12, 13 can for instance be designed in several layers. Materials that may be utilized are SiN (silicon nitride) SiO2 (silicon oxide) and/or C (carbon). The thin membranes 16, 17 are for instance clamped between the chips 12, 13. Favourable materials for the membranes 16, 17 are, for instance, SiN (silicon nitride) or C (carbon).

In one use, the first chip 12 with membrane 16 and the second chip 13 with membrane 17 are interconnected only after the provision of the sample 6. This can be favourable with large samples 6, if, for instance, a relatively large bulk sample 6 or an entire cell is involved. Here, the cell is placed in an open microreactor 5, after which the microreactor 5 is closed. With this embodiment, the inlet 14 and the outlet 15 may be omitted. In this microreactor 5, for instance particular conditions can be temporarily maintained, such as, for instance, air humidity.

The membranes 16, 17 are sufficiently electron transparent and preferably have a thickness d across the surface of the window that is as electron transparent as possible (see Fig. 2A). The thickness d can be, for instance, 20 nm and, depending on the size of the window surface 20A, 20B, this thickness d can be selected to be, for instance locally, greater or smaller, for instance 5 or 100 nm, or therebetween. Here, window surface 20A, 20B is understood to mean the surface of the membrane 16 or 17 through which at least one electron beam can radiate. This window surface 20A, 20B is shown in schematic top plan view in Fig. 3. Preferably, the membranes 16, 17 have sufficient electron transparency across the window surface 20A, 20B for obtaining an image result that is as good as possible. If the membrane 16 or 17

has a window surface 20A, 20B that is as large as possible, the chance that the desired part of the sample 6 or the samples 6 is struck by the electron beam is greater. For instance, particular sample particles 6 that represent a different condition can be spread over a larger window surface, while, with a smaller window surface 20A, 20B, there would be the chance of these parts influencing each other. Also, the chance that the sought sample part 6 is within the window surface, is greater.

It is preferred that the membrane 16 or 17 lasts at least until the start of the quench freeze process. After the quench freeze process, the sample 6 is, in principle, fixed in the microreactor 5, while the membranes 16, 17 have or have not lasted. The sample 6 can be irradiated with an electron beam with membranes 16, 17 that are broken or that have lasted. Also, the choice can be made to remove the membranes 16, 17 and/or a part of the microreactor before the sample 6 is irradiated with electrons.

A window surface 20A, 20B with a length l and a width b of approximately 100 micron by 100 micron, but also greater, for instance 1 mm by 1 mm, is conceivable. The maximum maintainable size of the window surface 20A, 20B depends on, for instance, the pressure difference to be used inside and outside the chamber 19, but also on the type and/or number of sample (s) 6, the thickness d of the membrane 16 or 17 and/or the material of the membrane 16 or 17.

In one embodiment according to the invention, counter pressure means 21 are provided, in the embodiment of Fig. 2 designed as partitions 21. These are designed for supporting the membrane 16 or 17 inside or outside the chamber 19 and/or for applying a counter pressure. Counter pressure means 21 prevent thin membranes 16, 17 from breaking, in other words, effect a reinforcement and/or effect that the membranes 16, 17 are held, locally or over the entire membrane 16, 17, at a particular distance relative to each other. Generally, the counter pressure means 21 can be realized in different manners, for instance by exerting a fluid pressure 21D on the

membranes 16, 17 inside or outside the chamber 19, as in Fig. 4A, 4B and 4D. Also, mechanical counter pressure means can be provided, for instance in the form of partitions 21, as in Fig. 2 and 3, columns 21A, 21B 21C, as in Fig. 4C, thickenings 21E in the membranes 16, 17 as in Fig. 4D, and combinations thereof. Further explanation of these different embodiments of counter pressure means 21 follows in this description.

In particular embodiments of a microreactor 5, different types of samples 6 can be flowed. For instance, a sample 6 with a relatively large cross section can be used in the microreactor 5 by arranging a larger window surface 20. The proportion of the thickness of the membranes 16, 17 and the window surface 18 is then to be selected to be as favourable as possible. It may be favourable then to provide the earlier mentioned counter pressure means 21, to prevent a membrane with a large surface and a relatively limited membrane thickness from breaking prematurely. It can, for instance, be realized that the membrane thickness d is approximately 20 nm with a membrane surface of 50 by 200 micron. In a different embodiment, with a similar or greater surface, the thickness is approximately 5 nm or 10 nm, or therebetween, but counter pressure means 21 can for instance be provided, in the form of, for instance, micro columns 21A, 21B, 21C between the membranes 16, 17 as can be seen in Fig. 4C.

Counter pressure means 21 can be utilized in several configurations. For instance, a reduced pressure in the chamber 19 can be realized via the inlet and outlet 14, 15, as can be seen in the embodiment represented in Fig. 4C. Here, the inlet and outlet 14, 15 are for instance connected to a pressure regulator. Arrows 21D indicate fictitious counter pressure means 21 at the outside, with which, as a result of the reduced pressure in the chamber 19, the membranes 16, 17 are pressed inwards. In another case, an excess pressure may be utilized in the chamber 19 so that the membranes 16A, 17A, indicated in dotted lines in Fig. 4C, are pressed outwards. In still further cases, a particular pressure is utilized outside the

microreactor 5 to effect pressure difference between the inside and the outside of the chamber 19.

The micro columns 21A – 21C in the chamber 19 hold the membranes 16, 17, at least locally, together and/or at a particular distance from each other, as can be seen in Fig. 4C. In combination with varying the gas pressure inside and/or outside the chamber 19, the columns 21A, 21C also contribute to possible local control of the distance between the membranes 16, 17, 16A, 17A. In particular embodiments, through utilization of particular air pressure differences between the pressure inside and outside the chamber 19, the membranes 16, 17 create an air tight sealing 25 in that the membranes 16, 17 are pressed together locally. This property can contribute to a better control of the conditions in the microreactor 5.

As can be seen in Fig. 4D, in one embodiment, counter pressure means 21 are provided in the form of thickenings 21E in the membrane 16 and/or 17. The thickness d_1 of the membranes 16, 17 across the surface is, for instance, approximately 5 nm, and the thickness d_2 at the thickenings 21E is, locally, thicker, for instance approximately 100 nm. A grid of such thickenings 21E may be provided so that over a relatively large surface, an average relatively thin membrane 16, 17 is obtained which lasts at least until the quench freeze process and preferably also after that. A favourable embodiment of a window 18 with thickenings 21E with said thicknesses d_1, d_2 has, for instance, a window surface 20 of approximately 1 mm by 1 mm.

For some uses, a membrane thickness d of 50 nm can offer sufficient electron transparency. If this electron transparency is still insufficient, with the aid of a focussed ion beam (ion milling), at least one layer of a membrane 16 and/or 17, or optionally, one and/or both membranes could be completely removed after the quench freeze process, in said window or a part thereof. Naturally, the choice of the window surface 18, the choice in thickness(es) d, d_1, d_2 and the choice whether a particular type of counter pressure means 21 is used depends on the application. The above-mentioned

embodiments serve as illustration and can be combined and/or varied per application.

In one embodiment, the distance h (see Fig. 2) between the membranes 16, 17 varies. Particular embodiments for instance are provided with distances between the membranes 16, 17 of, for instance, 10 and 200 nm. A liquid layer of approximately 100 nm with a slice of sample 6 therein may be flowed into the microreactor so that the microreactor 5 with the sample 6 can then be frozen in step 2 according to a quench freeze process. Tests have shown, for that matter, that the membranes 16, 17 can withstand the thermal shock, also if liquid is present in the microreactor 5.

In yet another embodiment, greater distances between the membranes 16, 17 are provided, for instance 5 μm. This embodiment is for instance favourable with larger samples 6, while a sample 6 with a diameter of, for instance, 5 μm, for instance a complete cell 6, is flowed into the microreactor 5 after which it is placed into the quench freezer 7.

The membranes 16, 17 can, for instance, exhibit a curve, as can be seen in Fig. 4A, so that samples with still greater diameters D than 5 μm can be placed in the microreactor. For electron transparency, the sample 6 is then made thinner, for instance layer after layer, until it is electron transparent, with the aid of, for instance, a focussed ion beam or a cryo ultra microtome.

As, upon freezing, the sample 6 becomes fixed in the microreactor 5, it is not necessarily disadvantageous if the microreactor 5 is damaged. The membranes 16, 17 may, in principle, break and/or be removed during/after the quench freeze process.

As stated, controlling the conditions in particular embodiments of the microreactor 5 is done via the inlet 14 and outlet 15. Here, for instance, the air humidity, temperature and pressure are controlled. Through control of the conditions, for instance particular processes in the microreactor 5 can be examined and even driven. The microreactor also prevents particular reactions and/or exchanges between the sample 6 and the environment. With thin slices

of the sample 6, for instance, the control produces advantages, in particular before the quench freeze process, as the relatively large surface loses and/or takes up water very easily, which can be prevented with the microreactor 5. Generally, it is simpler to control the conditions locally in the chamber 19 than in a relatively larger environment, as is the case with the conventional manners of sample preparation. Then, at a selected moment, the microreactor 5 with the sample 6 can be frozen in the quench freezer 7 according to the quench freeze process. This may for instance be at a chosen moment, for instance when a sample part 6 is in a particular condition, which is observed by a measuring instrument 32. After the quench freeze process too, the microreactor 5 can favourably prevent contact and/or exchange between the sample 6 and the environment.

In particular embodiments, additional positioning means are provided in the microreactor 5 with which the sample 6 can be positioned. In one embodiment, the insides of the membranes 16, 17 are, for instance, locally hydrophilized or hydrophobized, so that a layer of water with the sample 6 will fix itself on the hydrophilic portion. A hydrophobic sample 6 may, after flowing into the reactor 5, be present on a likewise hydrophobic part of a membrane 16, 17. Positioning the sample 6 can also be done by locally exerting pressure on the resilient membrane 16, 17 as illustrated in Figs. 4A and 4B.

A different manner of positioning the sample 6 is, for instance, by "playing" with liquid streams through the use of pressure differences via the inlet 14 and the outlet 15.

In one embodiment, the loading platform 33 is designed for receiving a sample holder 10 and a microreactor 5, as schematically shown in Fig. 5a. The sample holder 10 is preferably also specially designed for receiving the microreactor 5 and keeping it cooled, at least the sample 6 in the microreactor.

In one embodiment of the loading platform 33, a bottom 22 and a circular, upstanding wall are provided. In the bottom 22, a first recess 23 is provided for placing the microreactor 5. On the sides of the first recess 23, two

recesses 26 are provided for placing and taking up a microreactor 5 into and from the first recess 23, respectively, with a schematically indicated auxiliary means 27. An opening 24 is provided for sliding at least the end of the sample holder 10 into the loading platform 33. Accordingly, the microreactor 5 can be introduced into the loading platform in a slide-in opening 28 of the sample holder 10. To that end, the first recess 23 links up with the opening 24, so that the end of the sample holder 10 rests in the first recess 23 and the microreactor 5 can be slid in a relatively simple and safe manner via the slide-in opening into the sample holder 10.

Preferably, the first recess 23 encloses the microreactor 5 at least partly at the sides, so that the microreactor 5 is guided when sliding into the sample holder 10, and can be slid into the sample holder 10 with relatively little effort. All this is shown in Figs. 5B and 5C. It is also favourable if a sunken part 23B is provided in the first recess 23, so that the height of the location of the microreactor 5 links up with the height of the slide-in opening 28 in the sample holder 10. In this manner, the microreactor 5 can be slid into the sample holder 10 parallel to the bottom of the first recess 23.

The sample holder 10 is provided with a clamping wedge 29 so that the microreactor 5 is clamped in the sample holder 10. At least one, preferably several wall parts of the microreactor 5 link up with the inside of the sample holder 10, at least the inside near the slide-in opening 28. As a result, a greatest possible thermal contact between the sample holder 10 and the microreactor 5 can be effected so that, via the cooled sample holder, the sample 6 is maintained cooled. Owing to this feature, the cryo conditions of the sample 6 are controlled better. At that location, in the holder 10, a low temperature is preserved for transporting the sample holder 10 with a sample 6 to the electron microscope 11 so that crystals are prevented from, still, being formed in the frozen liquid. In principle, the sample holder 10 can be a known sample holder 10 or cryo holder 10, 34, but, in a favourable embodiment, the end of the sample holder 10, at least adjacent the slide-in

opening 28, is specially arranged for keeping a microreactor cool, for instance through the greatest possible thermal contact between sample holder 10 and microreactor 5. Here, the dimensions of the insides of the sample holder 10 link up with the dimensions of the microreactor 5. This is contrary to conventionally cooled sample holder 10 for CEM, which are, in principle, not arranged for holding microreactors 5 according to the invention.

In Figs. 6A – 6I, the sliding of the sample 6 into the sample holder 10 is illustrated in steps. In Fig. 6A, it can be seen that the sample holder 10 has been introduced through the opening 24 into the loading platform 33 and that the microreactor 5 rests in the first recess 23 of the bottom 22 of the loading platform 33. The microreactor 5 is not yet present in the holder 10. In Fig. 6B, the microreactor 5 is slid, via the slide-in opening 28 of the sample holder 10, into the sample holder 10 and then, in Figs. 6C – 6E, the clamping wedge 29 is positioned so that the microreactor 5 is secured in the sample holder 10 preferably with sufficient thermal contact between microreactor 5 and sample holder 10. It will be clear that instead of a microreactor 5, also, other intermediate means for holding the sample, such as, for instance, a cartridge 36, can be suitable for sliding the sample 6 according to such a principle into the sample holder 10.

In a known embodiment of a sample holder 10, the sample 6 should be at the location of the hole 41. After the sample holder 10 is then introduced into the electron microscope 11, the electron beam will irradiate the sample 6 through the hole 41 through the window surface 20. As can be seen in Fig. 6F, before placement in the electron microscope 11, a sealing cover 35 is slid over the end of the sample holder 10 so that the hole 41 is closed and the sample 6 is somewhat screened off from formation of ice during transport to the electron microscope 11. In the electron microscope 11, the sealing cover 3 is slid back and the hole 41 is opened.

An alternative manner for screening off is offered with an embodiment of a sample holder 10, as can be seen in Figs. 6G – 6I, wherein the

microreactor 5 is slideable within the sample holder 10. Here, the sample 6 can be slid below the hole 41. However, with this embodiment, it is not the hole 41 that is screened off, as can be seen in Fig. 6F, but, as is represented in Fig. 6H and 6I, the sample is slid away from the hole 41, so that the sample 6 can be transported with sufficient thermal exchange with the sample holder 10 outside the loading platform 33 to, for instance, an electron microscope 11. Thus, the sample 6 is still screened off. Even if it still projects somewhat outside the sample holder 10, the sample 6 is still sufficiently cooled, also because the sample holder 10 encloses the microreactor 5 in a thermally conductive manner, while the microreactor 5 is also guided along the insides of the sample holder 10 in a longitudinal direction 1 of the sample holder 10. Here, the sealing cover 35 is not necessary. If the sample holder 10, at least the hole 41, is positioned in the electron microscope 11, the sample 10 will be slid under the hole 41.

If other sample carriers (for instance cartridges 36) than a microreactor 5 are used for holding the sample 6, it is favourable if the contact surface between the sample holder and the other sample carrier is such that the cooling of the sample holder 10 is transmitted. For instance, the sample holder 10 can be arranged for transferring the sample 6 in the other sample holder in a simple and secure manner to an electron microscope 11, while the ice does not lose its amorphous form and the sample 6 will remain virtually unchanged. Sample carriers for holding the sample 6 can comprise, for instance, a cartridge 36 (see Fig. 7) while for the invention, the dimensions of the cartridge 36 are adapted so that it advantageously fits into the sample holder 10 according to the invention. Here, also, it is preferred that a clamping wedge 29 suitable for the cartridge is provided, which clamps the cartridge 36 in the sample holder 10.

An example of a cartridge 36 that is suitable for CEM is represented in Fig. 7. The cartridge 7 is equipped with a swinging mechanism 37 with hinge 37A. The hinge mechanism 37 can be raised, so that the sample 6 can be

placed in the cartridge 36, for instance on a grid or between membranes. Then, the hinge mechanism 37 is closed while a lock 38 is provided for keeping it closed. The sample 6 can now be irradiated through the window 18 with electrons or ions. An advantage of the use of the cartridge 36 can for instance be that layers of the sample 6 can be removed relatively simply with, for instance, the focussed ion beam, as, for a best possible result, the ion beam is to irradiate the sample 6 at a preferably obtuse angle, which will be explained in further detail in the following. A cartridge 36 often presents the possibility thereto as it is typically of flat design. The cartridge 36 can also be slid into the sample holder 10 in manner comparable to that of the microreactor 5. A drawback may be that when a cartridge 36 is utilized, the environmental factors such as for instance the pressure, temperature and humidity just adjacent the sample 6 can be controlled less well than with the microreactor 5.

Figs. 8A-C schematically show an embodiment of a microreactor 5 with which layers of material can be removed relatively simply from the sample 6, in particular with the aid of for instance a focussed ion beam and/or an ultra microtome. This embodiment is also advantageous for use with cryo tomography. With this microreactor 5, environmental conditions of the sample 6, such as humidity and temperature are, still, controlled. To that end, an inlet 14 and an outlet 15 are provided which lead to and from the chamber 19 between the membranes 16, 17. Here too, the microreactor comprises a first and a second chip, between which the membranes 16, 17 can be placed as is represented in Fig. 8B.

When using a microreactor 5 according to Fig. 8A, the sample 6 which, for the purpose of clarity of the drawing, is not represented here, is flowed into the chamber 19 or placed therein in a different manner. With this embodiment, the window 18 is placed in a relatively flat part 39. The construction of the flat part 39 can be somewhat reinforced by the inlet 14 and outlet 15, whereby some reinforcement of the flat part 39 is achieved. It is favourable if the window surface 20 is relatively large relative to the depth d_3

of the window 18, at least the thickness of the flat part 39. The fact is that this enables the sample 6 to obtain a follow up-treatment at a relatively obtuse angle α with, for instance, an ion beam or electron beam 40. Herein, an angle α can be understood to include the angle between the window surface 20 and the beam 40. This is favourable as, upon ion beam bombardment, generally, uniform thinning of the sample 6 is obtained better according as the angle α is more obtuse. The flat part 39 also simplifies cutting the sample 6, for instance with an ultra microtome. It is facilitated to also remove a part of the microreactor 5.

As stated, through the flat part 39 with the relatively great window surface 20, a focussed ion beam 40 can irradiate the sample 6 for removing layers of material, as can be seen in Fig. 8C. Also, an electron beam 40 of an electron microscope 11 can irradiate the sample 6 at a relatively obtuse angle α. This obtuse angle α is achieved by tilting the microreactor 5 with the aid of the sample holder 10, for instance about different axes, relative to the electron beam 40. It will be clear that with such a flat part 39 of the microreactor 5, and by tilting the microreactor 5 about particular axes, the sample 6 can be irradiated at more angles. With this, the present embodiment is favourable to cry-tomography.

In one embodiment of the microreactor 5, the flat part 3 still projects from the sample holder 10 if this is placed in the sample holder 10. Here, the microreactor 5 is still sufficiently cooled, while it is possible to irradiate the sample 6 at a larger multiple of angles, and at relatively more obtuse angles α than when the microreactor 5 is placed completely in the sample holder 10.

The variations described and many comparable variations, as well as combinations thereof, are understood to fall within the framework of the invention as outlined by the claims. Naturally, different aspects of different embodiments and/or combinations thereof can be combined with each other and be exchanged within the framework of the invention and delimitation should not be restricted to only the embodiments mentioned.

Claims

1. A method for sample preparation for cryoelectron microscopy (CEM), wherein the sample is held in a microreactor, wherein the conditions in the microreactor are regulated relative to the environment, wherein the sample in the microreactor is frozen according to a quench freeze process, after which the sample, in frozen condition, is placed in the electron microscope.

2. A method according to claim 1, wherein the controlled conditions comprise at least humidity, temperature and/or pressure.

3. A method according to claim 1 or 2, wherein a part of the sample that is placed in the microreactor is removed in frozen condition, in particular at a temperature below 150° K, more particularly below approximately 140° K, with the aid of means suitable thereto, for instance a focussed ion beam and/or ultra microtome.

4. A method according to any one of the preceding claims, wherein the microreactor holding the sample is placed in a cooled environment, for instance a loading platform, and is slid into a cooled sample holder, at a temperature below 150° K, preferably at approximately 140° K.

5. A method according to claim 4, wherein the cooled sample holder contacts the microreactor placed therein over a relatively large contact surface.

6. A method according to any one of the preceding claims, wherein the microreactor is provided with a relatively flat part with a window, while the microreactor is slid at least partly into a cooled sample holder, such that the window of the microreactor projects at least partly outside the sample holder.

7. A method according to any one of the preceding claims, wherein the sample in the microreactor, before freezing, is observed by means of an optical microscope or an X-ray diffraction microscope.

8. A microreactor for use with cryoelectron microscopy (CEM), comprising a first and second membrane, which membranes enclose a

chamber, at least in a condition of use, while the membranes are configured to last until at least the start of a quench freeze process.

9. A microreactor according to claim 8, wherein at least one inlet and/or outlet is provided for feeding fluid into and/or through the chamber.

10. A microreactor according to claim 8 or 9, wherein the membranes are at least partly transparent to an electron beam of an electron microscope.

11. A microreactor according to claim 10, wherein the membranes are configured to provide transparency to electron beams over a relatively large surface, relative to the thickness of the membranes.

12. A microreactor according to any one of claims 8 – 11, wherein the membranes, at least with the microreactor in closed condition, extend opposite each other at a mutually approximately equal distance from each other, and/or wherein the microreactor is designed for varying the distance between the membranes.

13. A microreactor according to any one of claims 8 – 12, wherein counter pressure means for the membranes are provided.

14. A microreactor according to any one of claims 8 – 13, wherein the counter pressure means comprise at least one column and/or partition at the inside and/or the outside of the chamber.

15. A microreactor according to any one of claims 8 – 14, wherein the counter pressure means comprise a pressure regulation.

16. A microreactor according to any one of claims 8 – 15, wherein the counter pressure means comprise at least one thickening in at least one membrane.

17. A microreactor according to any one of claims 8 – 16, wherein the thickness of the membranes is at least locally smaller than or equal to approximately 50 nm, in particular smaller than or equal to approximately 20 nm, more particularly smaller than or equal to approximately 10 nm.

18. A microreactor according to any one of claims 8 – 17, wherein the membranes are longer and/or wider than approximately 50 μm, in particular longer and/or wider than approximately 200 μm.

19. A microreactor according to any one of claims 8 – 18, wherein the distance between the membranes is smaller than 5 μm, preferably smaller than approximately 200 nm.

20. A microreactor according to any one of claims 8 – 19, for use in a method according to any one of claims 1 – 7.

21. A cryoelectron microscopy (CEM) sample holder for cooling and holding a microreactor according to any one of claims 8 – 20, wherein adjacent the end of the sample holder, insides of the sample holder link up with outsides of the microreactor.

22. A cartridge for holding a sample, wherein the dimensions of the cartridge are designed for placement in a sample holder according to claim 21.

23. A clamping wedge for clamping a microreactor and/or cartridge according to claim 22 in a sample holder according to claim 21.

24. A loading platform for use with cryoelectron microscopy (CEM), comprising a bottom and at least one upstanding wall, wherein in a wall, an opening is provided for introducing a CEM sample holder, while in the bottom of the loading platform, a first recess is provided, preferably with a rectangular form, so that a microreactor, cartridge and/or sample holder can rest at least partly in said first recess, wherein the first recess links up with the opening so that, if the sample holder has been partly introduced through the opening into the loading platform, the microreactor and/or the cartridge can be slid into the sample holder substantially parallel to the bottom of the first recess.

25. A loading platform according to claim 24, wherein the first recess contains a sunken part so that the microreactor and/or cartridge can be slid substantially horizontally into the sample holder.

26. A loading platform according to claim 24 or 25, wherein second recesses are provided at the first recess for creating space for an auxiliary means for placing and/or removing the microreactor.

27. A system which is suitable for use of a method according to any one of claims 1 – 7, comprising a microreactor according to any one of claims 8 – 20 and/or a loading platform according to any one of claims 24 – 27 and an electron microscope.

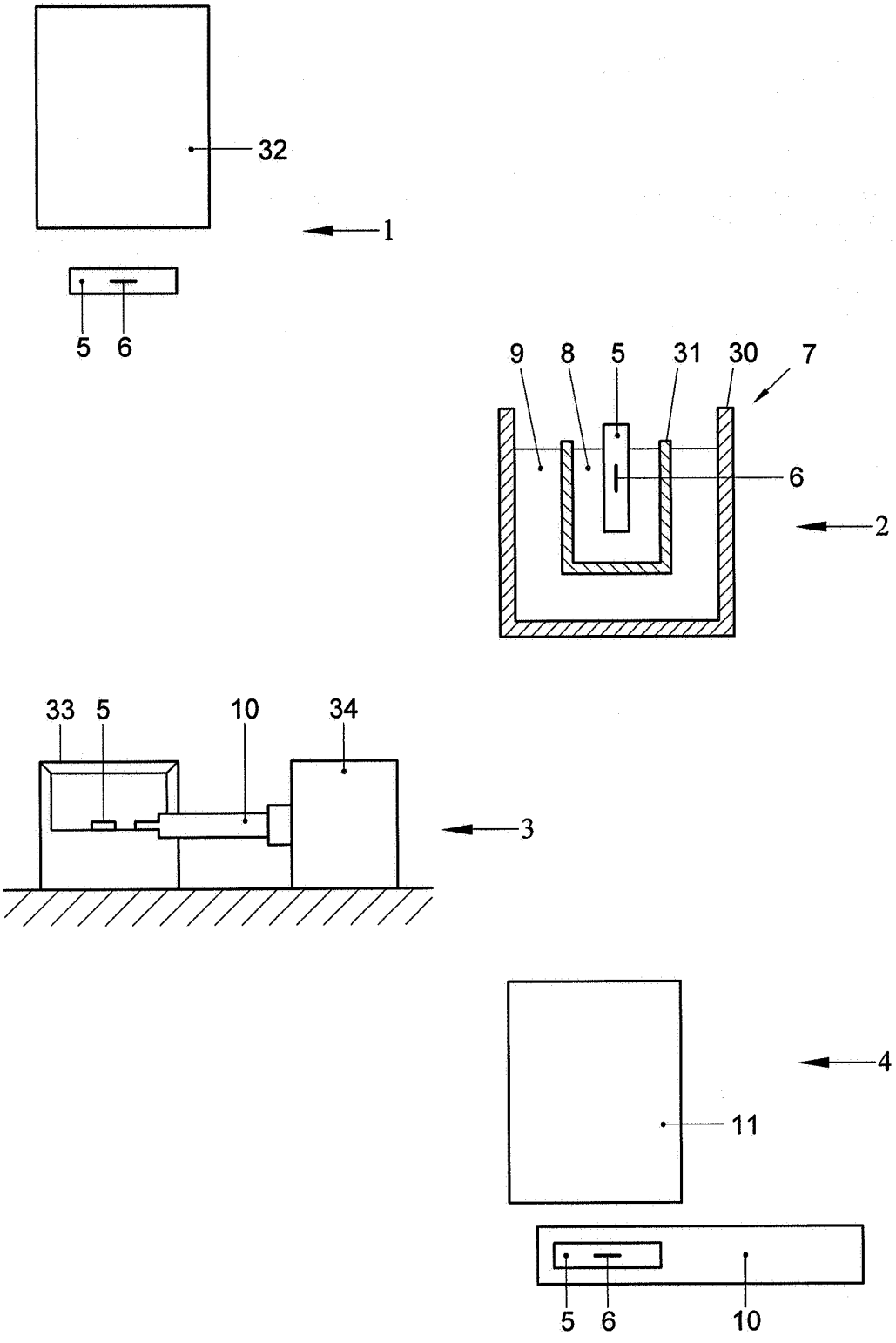

Fig. 1

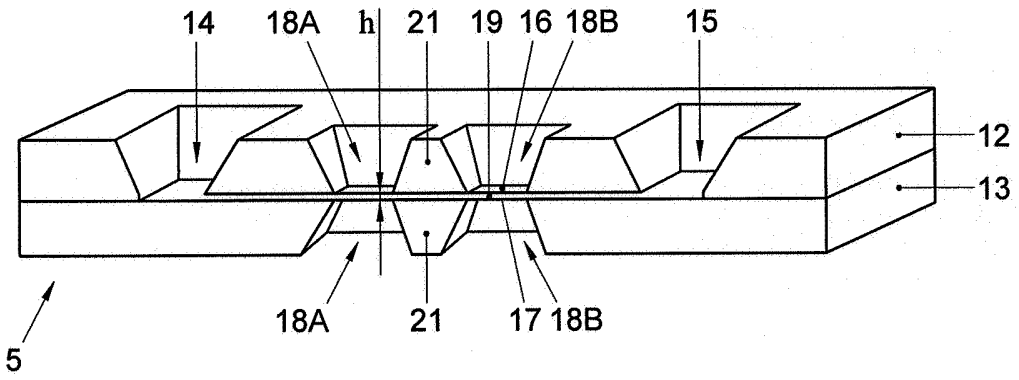

Fig. 2

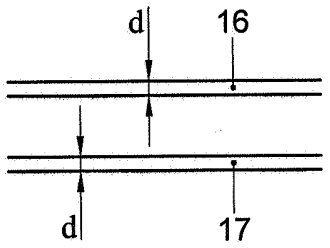

Fig. 2A

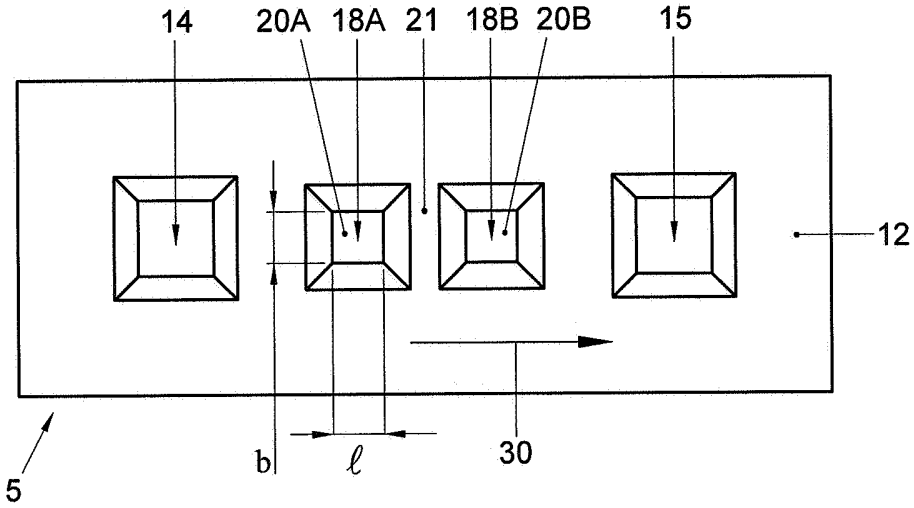

Fig. 3

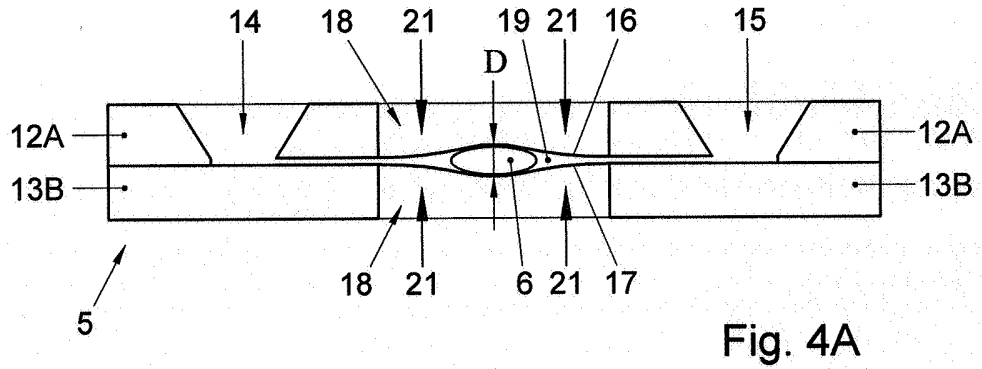

Fig. 4A

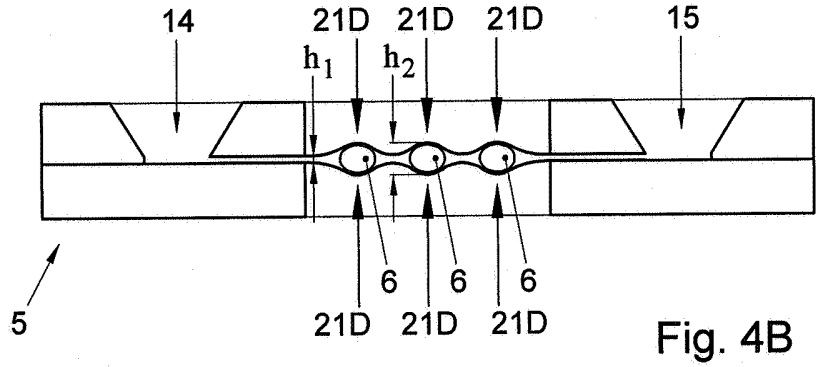

Fig. 4B

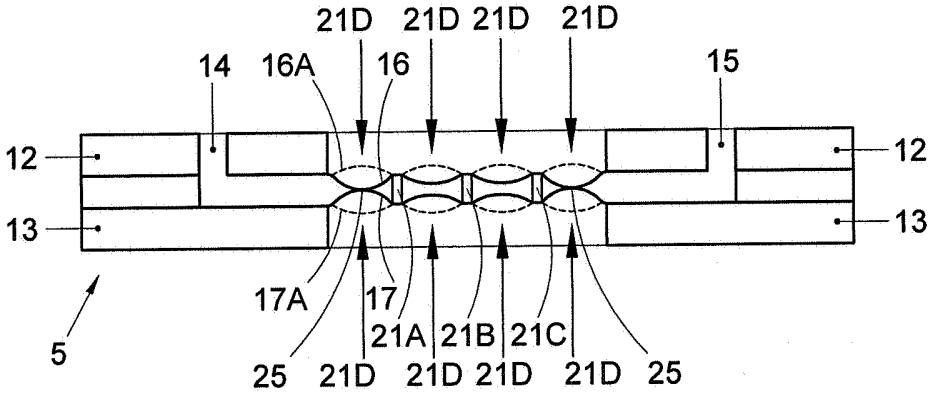

Fig. 4C

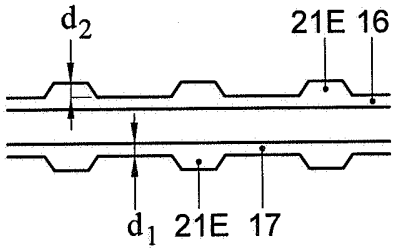

Fig. 4D

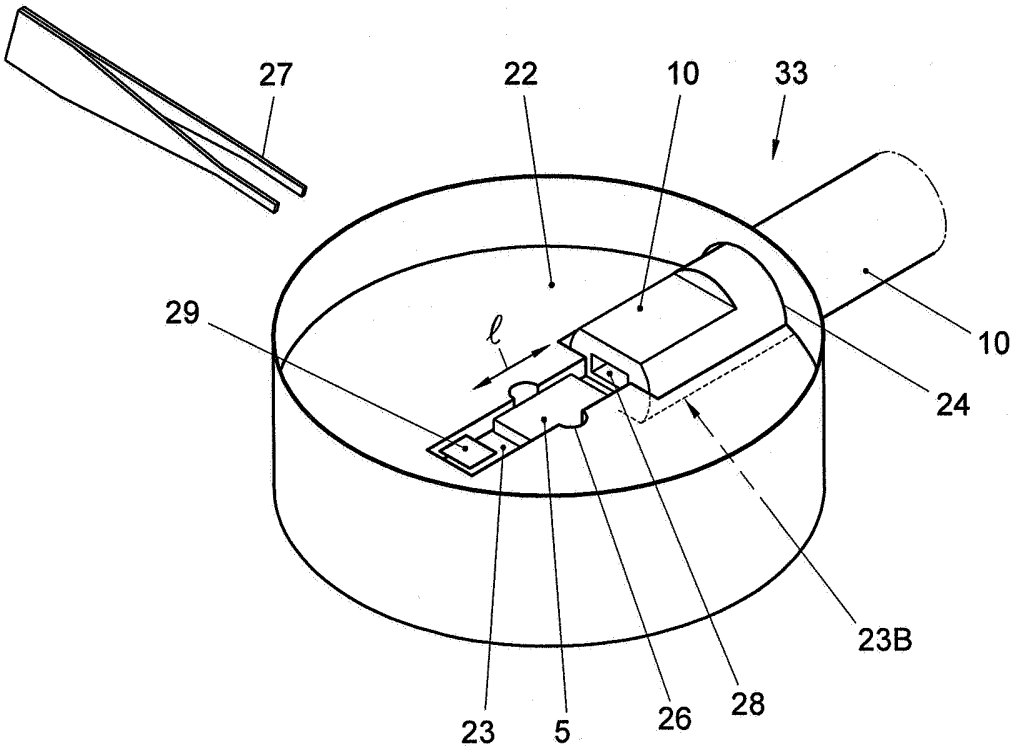

Fig. 5A

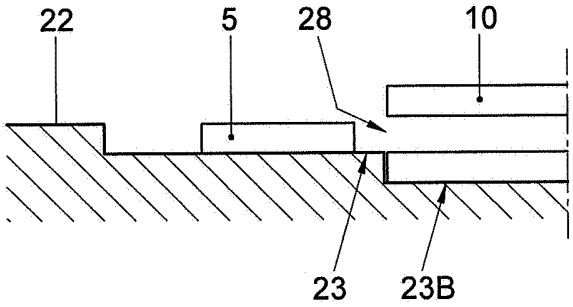

Fig. 5B

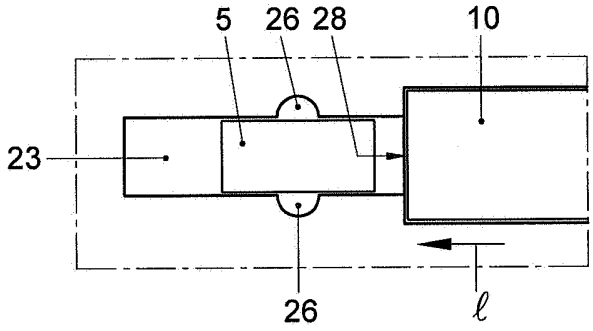

Fig. 5C

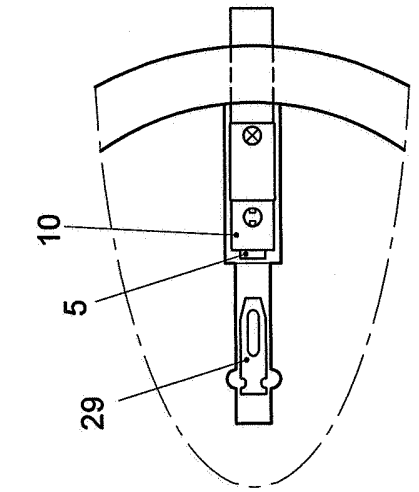

Fig. 6C

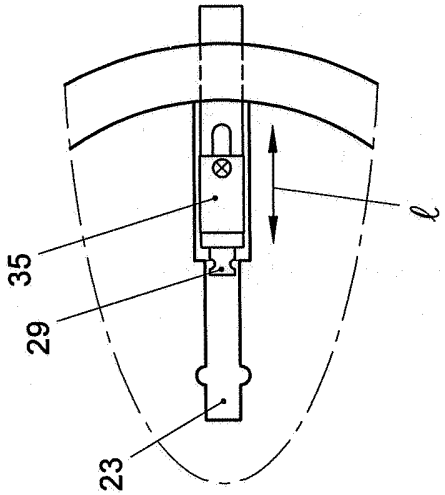

Fig. 6F

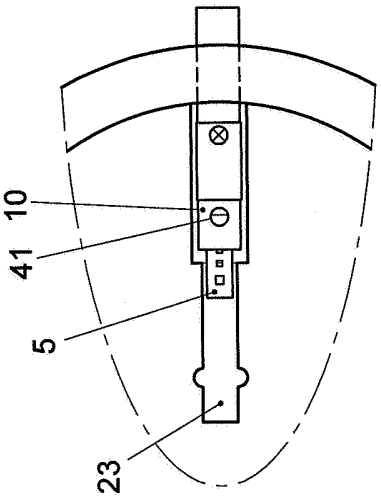

Fig. 6B

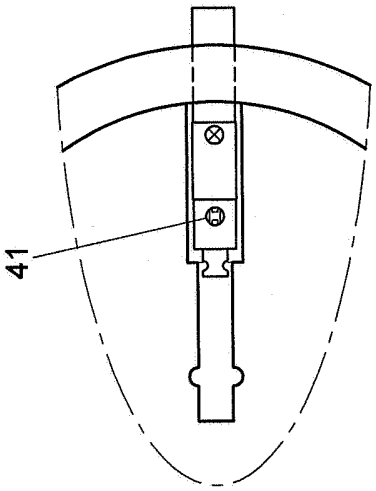

Fig. 6E

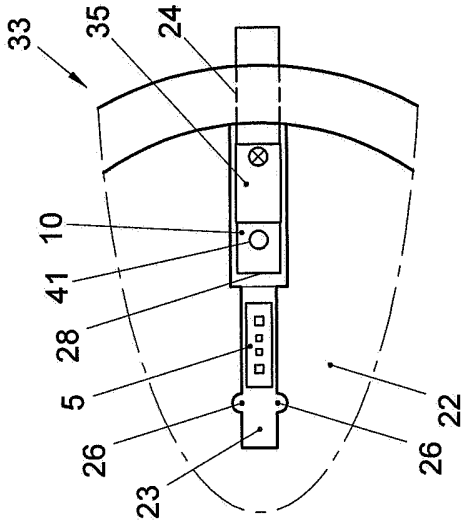

Fig. 6A

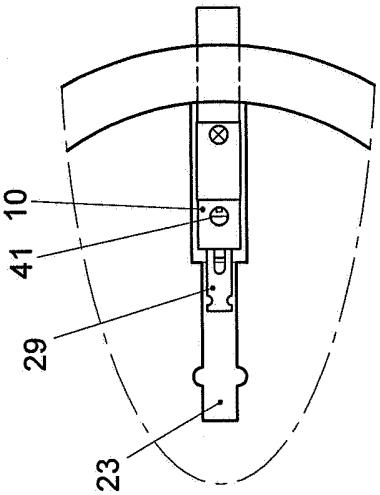

Fig. 6D

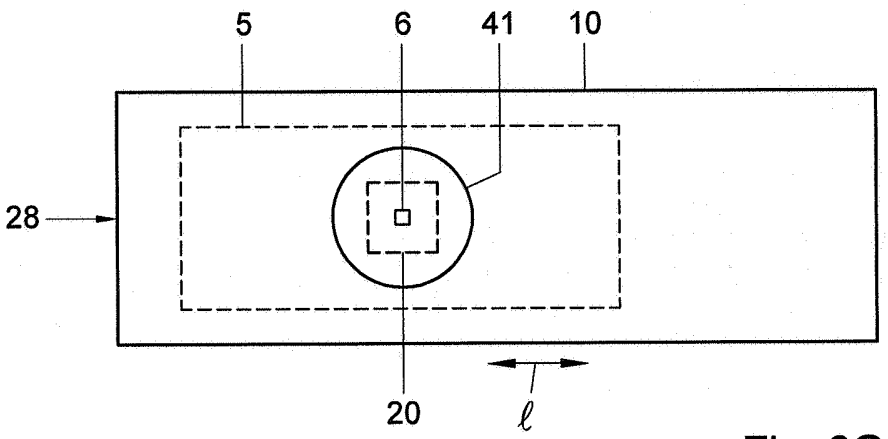

Fig. 6G

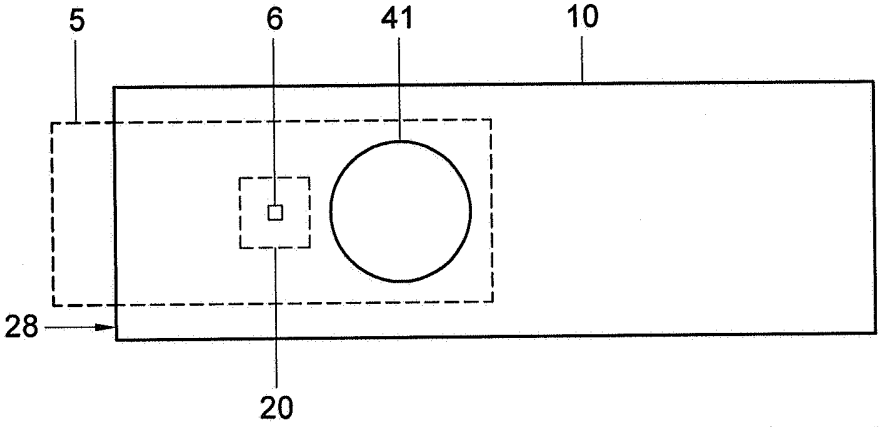

Fig. 6H

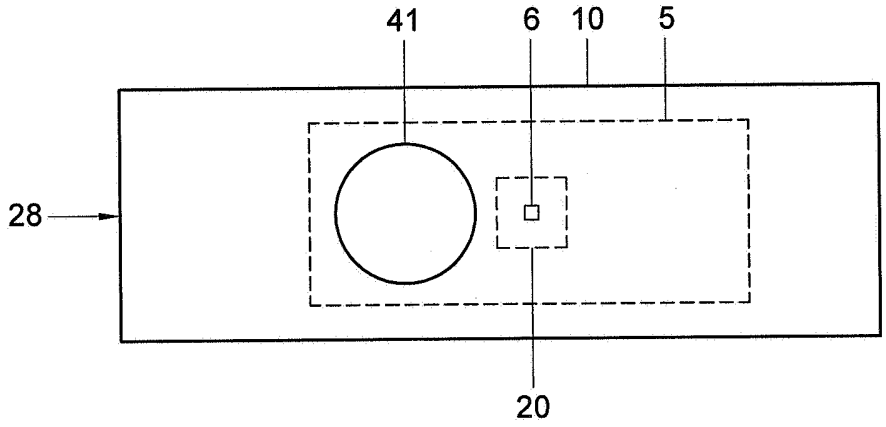

Fig. 6I

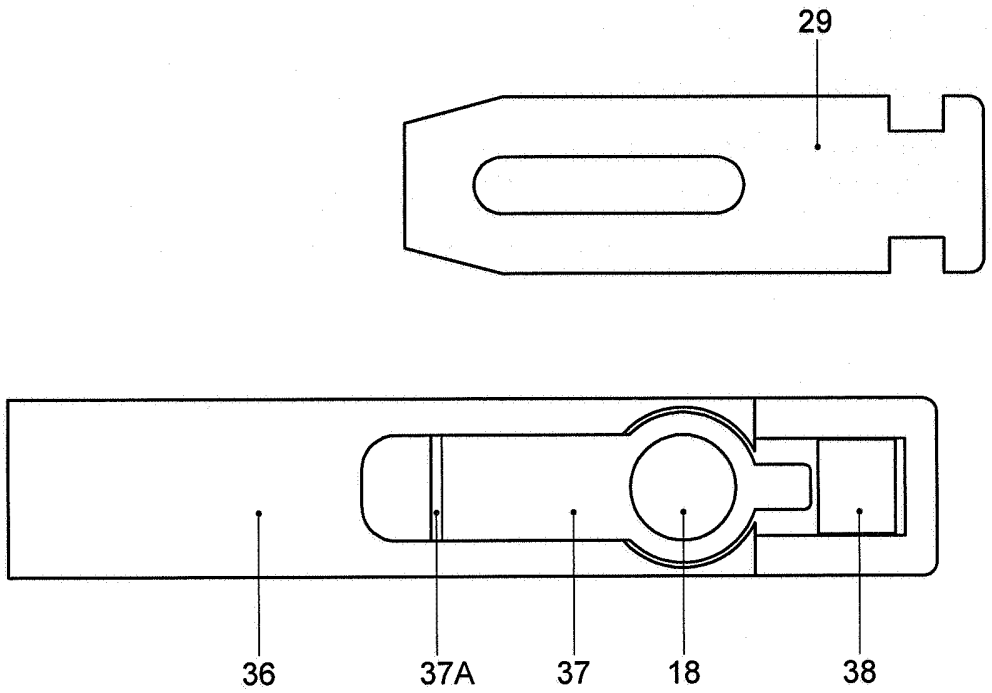

Fig. 7

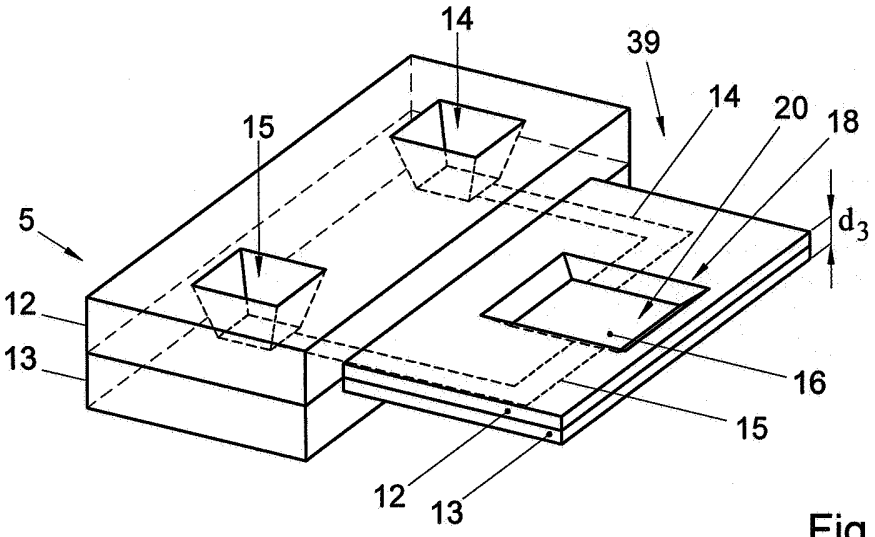

Fig. 8A

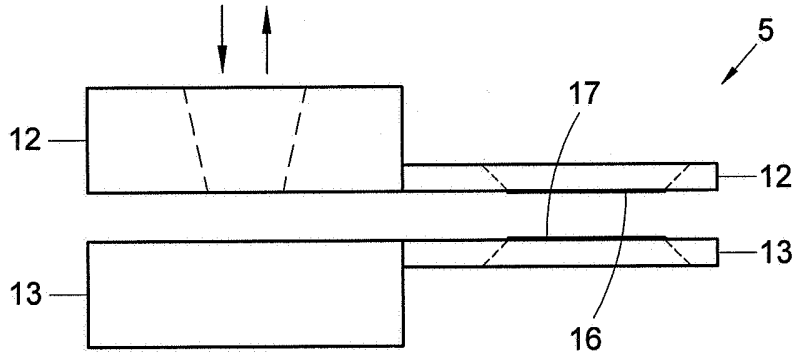

Fig. 8B

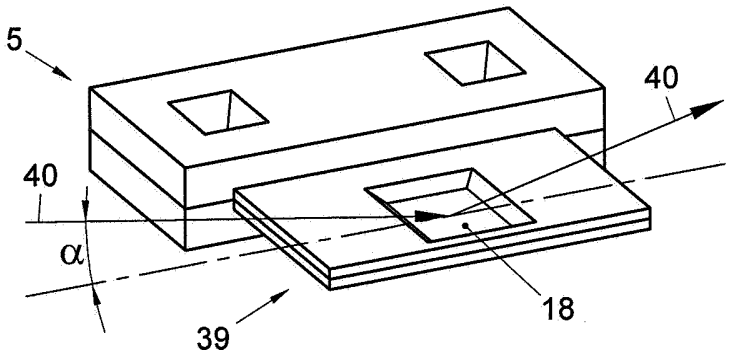

Fig. 8C