BESCHWERDEKAMMERN DES EUROPÄISCHEN PATENTAMTS

BOARDS OF APPEAL OF THE EUROPEAN PATENT OFFICE

CHAMBRES DE RECOURS DE L'OFFICE EUROPEEN DES BREVETS

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File Number: T 822/91 - 3.4.2

Application No.: 84 305 391.9

Publication No.: 0 136 014

Title of invention: Method and apparatus for cryopreparing biological tissue for ultrastructural analysis.

Classification: G01N 1/28

D E C I S I O N
of 19 January 1993

Applicant: Board of Regents the University of Texas System

Headword:

EPC Articles 84 and 56

Keyword: "Clarity (yes)"
"Inventive step (yes)"

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Beschwerdekammern | Boards of Appeal | Chambres de recours

Case Number : T 822/91 - 3.4.2

D E C I S I O N
of the Technical Board of Appeal 3.4.2
of 19 January 1993

Appellant : Board of Regents the University of Texas System
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Decision under appeal : **Decision of the Examining Division 061 of the European Patent Office dated 30 April 1991, written decision sent by post on 28 May 1991 refusing European patent application No. 84 305 391.9 pursuant to Article 97(1) EPC.**

Composition of the Board :

Chairman : E. Turrini
Members : C. Black
L.C. Mancini

Summary of Facts and Submissions

I. European patent application No. 84 305 391.9 (publication No. 0 136 014) was refused by decision of the Examining Division.

II. The reason of the refusal was that the subject-matter of the independent Claims 1 and 16 on file did not meet the requirements of Article 84 EPC and did not involve an inventive step (Article 56 EPC), having regard to the following prior art document:

(D1) The Journal of Histochemistry and Cytochemistry, Vol. 29, No. 9, 1981, pages 1021 to 1028, L. Terracio et al., "Freezing and Drying of Biological Tissues for Electron Microscopy".

III. The Applicant (Appellant) lodged an appeal against this decision in its entirety.

In the Statement of Grounds he cited the following further prior art document:

(D2) The Anatomical Record, Vol. 187, 1977, pages 477 to 493, H.D. Coulter et al., "Preparation of Biological Tissues for Electron Microscopy by Freeze-drying".

IV. In a communication of the Board pursuant to Article 110(2) EPC, amended application documents were proposed.

V. The Appellant stated his approval of the text proposed by the Board and requested that the decision under appeal be set aside and that a patent be granted on the basis of said text.

He further requested oral proceedings, auxiliarily.

VI. Independent method **Claim 1** according to the Appellant's request reads as follows:

"A method for cryopreparing a biological tissue sample for ultrastructural analysis characterized by:

vitrifying the biological tissue sample by rapidly reducing the temperature of the sample to minus 140°C or below at a rate such that vitrification of the water in the tissue takes place without the formation of resolvable ice crystals;

depressurizing the atmosphere surrounding the sample to less than 4.10^{-7} Pa (3.10^{-9} Torr) by drawing a vacuum in less than 300 minutes whereby a vacuum is created such that vitrified water can be removed from the biological sample;

bringing the vitrified tissue sample to equilibrium at a temperature of less than minus 140°C;

dehydrating by sublimation the vitrified tissue sample while the sample is maintained in a state of equilibrium at a temperature of less than minus 140°C;

infiltrating the dehydrated tissue sample with a degassed resin;

polymerizing the resin in the infiltrated tissue sample to form an embedded tissue sample."

Independent apparatus **Claim 16** according to the Appellant's request reads as follows:

"An apparatus for cryopreparing a biological tissue sample for ultrastructural analysis, according to the method of Claim 1, characterized by:

means for vitrifying the biological tissue sample by rapidly reducing the temperature of the sample to minus 140°C or below at a rate such that vitrification of the

water in the tissue takes place without the formation of resolvable ice crystals;

means (10,11) for depressurizing the atmosphere surrounding the sample to less than 4.10^{-7} Pa (3.10^{-9} Torr) by drawing a vacuum in less than 300 minutes whereby a vacuum is created such that vitrified water can be removed from the biological sample;

means for bringing the vitrified sample to equilibrium at a temperature of less than minus 140°C;

means for dehydrating by sublimation the vitrified tissue sample while said sample is maintained in a state of equilibrium at a temperature of less than minus 140°C;

means for infiltrating the dehydrated tissue sample with a degassed resin; and

means for polymerizing the resin in the infiltrated tissue sample to form an embedded tissue sample."

Claims 2 to 15 depend on Claim 1, and Claims 17 to 24 on Claim 16.

VII. In support of the allowability of his request, the Appellant submitted essentially the following arguments.

As to the raised objection of lack of clarity with regard to the state of equilibrium during dehydration, it is clear to the skilled person, from the wording of Claim 1, that the dehydration takes place at a given temperature below minus 140°C and that no changes occur which may disrupt the macroscopic thermodynamic status of the sample to a significant degree. The question of what degree of temperature change is permissible over what period of time (see end of page 13 of the description) is a detail which has to be determined depending on the particular sample.

As to the question of inventive step, the reference D1 teaches that "despite repeated attempts, complete

vitrification of cells or aqueous solutions by cooling has never been demonstrated" (see page 1022, right-hand column, lines 1 to 3); this article not only holds that vitrification has never been accomplished, but it seems to imply that it is intrinsically impossible. This is in direct contrast to the teachings of the present invention and the inventor has, therefore, overcome a technical prejudice. Moreover, D1 fails to appreciate or mention important parameters of the invention, in particular the use of extreme temperatures and pressures involved in the claimed method. The "high" vacuum pressure and the temperature used in the method described at the end of the left-hand column of page 1026 of D1 are not mentioned. The importance of maintaining the sample in a state of equilibrium at a temperature below minus 140°C is likewise not discussed or appreciated. On the contrary, D1 teaches on said page 1026 that one should heat the sample during a period of 1 to 2 days from an undefined freeze-drier temperature to room temperature, so as to allow the sublimation of the water to occur. This dynamic heating procedure is opposed to the quasi-static thermodynamic equilibrium during the dehydration envisaged by the invention.

Reasons for the Decision

1. The appeal is admissible.

2. Allowability of the amendments

2.1 Method Claim 1 is essentially based on the originally filed Claim 6 with the following amendments, for which a basis can be found in the originally filed application:

- the tissue sample is vitrified at a rate such that vitrification of the water in the tissue takes place without the formation of resolvable ice crystals (see the description as filed, page 12, lines 6 to 11),
- a vacuum is created in less than 300 minutes, such that vitrified water can be removed from the biological sample (see the description as filed, page 13, lines 8 to 19),
- the equilibrium temperature is less than minus 140°C (see original Claim 1, feature (c)),
- the original step (e) of adding a contrast enhancing material to the sample has been deleted, whereby the broadening of the claim by this excision of a step is supported by the description as filed, page 15, line 32 to page 16, line 1.

2.2 Apparatus Claim 16 is essentially based on the originally filed Claim 8 and has been amended so as to correspond to the valid Claim 1. In particular, the fact that the apparatus also includes means for bringing (and maintaining) the vitrified sample to (in) equilibrium at a temperature of less than minus 140°C can be inferred from the original description, page 13, line 17 to page 14, line 6.

2.3 Dependent Claims 2 to 15 and 17 to 24 contain features which are disclosed in the description as filed. In particular:

- for Claim 2, see page 12, lines 6 to 11,
- for Claim 3, see page 13, lines 12 to 14,
- for Claim 4, see page 13, line 34 to page 14, line 4,
- for Claim 5, see page 13, lines 32 to 34,
- for Claim 6, see page 13, lines 23 to 28,
- for Claim 7, see page 14, lines 15 to 19,
- for Claim 8, see page 15, lines 2 to 5,
- for Claim 9, see page 14, lines 15 to 21,
- for Claim 10, see page 14, lines 22 to 24,
- for Claim 11, see page 14, lines 21 and 22,
- for Claim 12, see page 15, lines 15 to 30,
- for Claims 13 and 14, see page 15, lines 32 to 34
- for Claim 15, see page 8, lines 6 to 22,
- for Claim 17, see page 12, lines 1 to 14,
- for Claim 18, see Figure 2,
- for Claim 19, see page 19, lines 21 to 33 in conjunction with page 13, lines 11 and 12,
- for Claim 20, see page 20, lines 8 to 10,
- for Claims 21 and 22, see page 22, lines 13 to 15 and page 22, line 33 to page 23, line 14,
- for Claim 23, see page 22, lines 13 to 26,
- for Claim 24, see page 20, line 35 to page 21, line 7.

2.4 Therefore, the amended version of the claims, on the basis of which the Appellant requests that a patent be granted, does not contravene Article 123(2) EPC, since it does not include subject-matter extending beyond the content of the application as originally filed.

3. Article 84 EPC

3.1 Claim 1 pertains to a method for cryopreparing a biological tissue sample, which method essentially comprises the following steps: vitrifying the sample, depressurizing, bringing the sample to equilibrium,

dehydrating the sample, infiltrating the sample with a resin and polymerizing the resin.

The steps of vitrifying, depressurizing and dehydrating, as such, are typical of the freeze-drying technique, or sublimation of water in vacuo, already known in the prior art (see D1, sections "Freezing Techniques" and "Drying of Frozen Tissue"), so that their meaning is clear to the skilled person.

3.1.1 As regards the step of vitrifying the sample, it is characterized in Claim 1 by the temperature range to be achieved (below minus 140°C) and the cooling rate, whereby the cooling rate is defined by means of a functional feature.

According to the established case law of the Boards of Appeal of the EPO, technical features may be defined functionally in appropriate cases. In the decision T 68/85 (OJ EPO 1987, 228), it has been held that functional features defining a technical result are permissible in a claim, if from an objective viewpoint, such features cannot otherwise be defined more precisely **without restricting the scope of the invention**, and if these features provide instructions which are sufficiently clear for the expert to reduce them to practice **without undue burden**, if necessary with reasonable experiments. This approach was confirmed by the decisions T 299/86 (unpublished) and T 204/90 (unpublished).

In the present case, the prerequisites above-mentioned are fulfilled. As to the first prerequisite, it is clear (see the description as filed, page 5, lines 12 to 17) that the cooling rate is related to the rate at which heat is removed from the sample. The heat transfer depends on the freezing system, in particular its temperature, as well as

the size and thermal characteristics of the sample. Different cooling rates are, therefore, possible, even though the temperature of the freezing system is the same, depending on the sample. The need for a fair protection without undue limitations thus justifies the broadening deriving from the choice of the functional feature for the cooling rate, this feature together with the temperature to be achieved satisfactorily characterizing the vitrifying step. Indeed, the temperature is presented in the description as filed, page 12, lines 1 to 14, as the essential feature of the vitrification for the reason given on page 12, lines 35, 36 and page 13, lines 1 to 6, whereas, on the other hand, the cooling rate has an influence on the number and size of ice crystals formed, as it is already known in the prior art (see D1, page 1022, right-hand column, lines 23 to 27 as well as the description as filed, page 4, line 28 to page 5, line 4). Also the second prerequisite above-mentioned is thus fulfilled, since the skilled person can, without undue burden, on the basis of his experience and, if necessary, of reasonable experiments, find out the best cooling rate for a given sample, so as to minimize ultrastructural damage due to ice crystal formation.

3.1.2 As regards the depressurizing step, the essential features, i.e. the pressure to be achieved and the time, are clearly mentioned. The fact that a vacuum is created such that vitrified water can be removed from the biological sample, is considered as a superfluous mention of the result achieved in relation to the following step of dehydrating rather than a genuine functional feature.

3.1.3 As to the step of bringing the sample to equilibrium, it seems to be evident that the wording of a claim has to be understood in connection with the description. This principle is expressed in Article 69 EPC which states that

the extent of the protection conferred by a European patent or a European patent application shall be determined by the terms of the claims, but the description and drawings shall be used to interpret the claims. From page 13, lines 17 to 19 of the description as filed it can be inferred that the equilibrium is achieved by maintaining, i.e. keeping constant, temperature and pressure for a given period of time, this being confirmed by the originally filed Claims 3 and 9 at least with regard to temperature. Claim 1, furthermore, mentions the essential feature that the equilibrium takes place at a temperature of less than minus 140°C, at which critical temperature cubic ice crystallization begins. The mention of a further state of equilibrium on page 13, lines 23 to 34, in particular lines 32 to 34, does not engender any confusion, because it refers to a following step of the method of Claim 1, namely the dehydrating step. Indeed, after equilibration is obtained, dehydration by sublimation takes place, as minimal amounts of energy are incrementally added; after each addition, a state of equilibrium should be achieved, the temperature of the sample remaining constant in the optimum condition (see page 15, lines 2 to 6). The length of time needed for achieving this equilibrium depends on the particular tissue sample and, therefore, this feature does not have to be mentioned in Claim 1.

3.1.4 The last two steps of the method according to Claim 1 are perfectly clear to the skilled person.

3.2 The wording of apparatus Claim 16 corresponds, mutatis mutandis, to that of method Claim 1, so that the remarks of section 3.1 remain valid.

3.3 Dependent claims do not cause any problem of lack of clarity.

3.4 For the reasons set out above, all the features which are essential to the performance of the invention are included in the main claims and the functional feature relating to the vitrification of the sample is admissible. Therefore, the claims are clear in the sense of Article 84 EPC.

4. Novelty

4.1 D1 is a review article on freezing and drying of biological tissues for electron microscopy. It discloses on page 1024, right-hand column, lines 56, 57 and page 1026, left-hand column, line 1, that freeze-drying, or the sublimation of water in vacuo, is the ideal method for removing water without disturbing other cell constituents. As stated on page 1026, left-hand column, lines 46 to 57 and right-hand column, lines 1 to 10, improvements in freeze-drier design have resulted in a method that should remove water with limited effects from devitrification and recrystallization. This method comprises, inter alia, the following steps (see also the abstract of D2 cited in D1 on page 1026 under the number (4), as an example of document disclosing such a method):

- a biological tissue sample is vitrified by inserting a copper block, in which the sample is placed, into a glass freeze-drier bathed in liquid nitrogen (at a temperature of minus 196°C),
- the atmosphere surrounding the sample is depressurized by connecting the drier to a molecular sieve pump producing a high vacuum,
- the copper block is gradually warmed to room temperature over a period of 1 to 2 days, heat from the block being thereby transferred to the tissue surface and creating a temperature gradient within the tissue, so that water is sublimed from the surface of the

tissue, resulting in an insulating dry shell of tissue, and
- the dry tissue is fixed with OsO_4 vapour and vacuum embedded in a degassed resin.

From the fact that liquid nitrogen is, for instance, used for freezing the sample (temperature of less than minus 140°C) and that the effect of the cooling rate on the production of amorphous structures was already known in the prior art (see D1, page 1022, right-hand column, lines 7 to 35), can be inferred that the vitrification takes place substantially without, or with minimal, formation of ice crystals.

The method of Claim 1 is essentially distinguished from said method known from D1 (or D2) by the following features and steps:

- a pressure of less than 4.10^{-7} Pa is achieved in less than 300 minutes,
- the vitrified tissue sample is brought to equilibrium at a temperature of less than minus 140°C,
- the vitrified tissue sample is dehydrated while the sample is maintained in a state of equilibrium at the temperature of less than minus 140°C.

4.2 The apparatus of Claim 16 is essentially distinguished from the freeze-drier known from D1 (or D2) by the following features:

- the means for depressurizing provides a pressure of less than 4.10^{-7} Pa that is achieved in less than 300 minutes,
- means is provided, for bringing and keeping the vitrified tissue sample to equilibrium at a temperature of less than minus 140°C.

4.3 The further documents cited in the Search Report are less relevant than D1 (or D2).

4.4 Therefore, the subject-matter of independent Claims 1 and 16 is novel in the sense of Article 54 EPC.

5. Inventive step

5.1 Claim 1

5.1.1 It is known from D1 (see page 1021, right-hand column, section "Ice Crystal Formation") that freezing of biological tissues may damage cell ultrastructure, primarily because of the mechanical forces produced by expanding ice crystals. A further damage, which is secondary to this physical effect, consists in that ice crystal growth causes chemical changes (eutectic formation). These disadvantages are also mentioned in the application as filed on page 4, lines 1 to 15. To limit the growth of ice crystals was, therefore, an object of several different freezing procedures of the prior art. This search effort has led to the known freeze-drying method mentioned in section 4.1 above, which offers the advantage of removing water with limited effects from devitrification and recrystallization (see D1, page 1026, left-hand column, lines 47 to 50).

Starting from this known method, which is considered to be closest to the subject-matter of Claim 1, the objective problem underlying the patent application in suit can be seen in the achievement of a more effective dehydration of the biological samples without causing damages to the tissue ultrastructure, so as to obtain samples which can be used with modern high-powered magnification apparatus

(see the description on file, page 8, line 24 to page 9, line 9). As this object corresponds to the many efforts of the prior art to freeze-dry tissues for electron microscopy whilst preventing ice crystals and eutectic formation, it appears that no inventive step can be perceived in the fact alone of stating this problem.

5.1.2 The problem is solved by the steps of the method Claim 1, in particular by the provision of a particularly efficient depressurizing step resulting in a pressure of less than 4.10^{-7} Pa in less than 300 minutes, bringing the vitrified tissue sample to equilibrium at a temperature of less than minus 140°C and dehydrating the vitrified tissue sample whilst the sample is maintained in a state of equilibrium.

D1 presents the method referred to in section 4.1 above (see also D2) as a result of the study of the theoretical aspects of freeze-drying, or sublimation of water in vacuo, by several investigators. Although freeze-drying is regarded as the ideal method for removing water without disturbing other cell constituents, it is complicated by the necessity to warm the tissue so as to allow sublimation to proceed in a reasonable period of time. This warming, however, in addition to promoting water sublimation, produces other physical events leading to glass transition, devitrification and recrystallization, depending on the temperature, these being phenomena that cause ultrastructural damage of the tissue adding to that occurring during freezing (see D1, page 1026, left-hand column, lines 11 to 30). Therefore, freeze-drying is a difficult procedure, which has to be carefully designed so as to hold the drying time within practical limits, whilst avoiding formation of ice crystals artifacts. The intrinsic difficulty of this step is an important aspect to be considered when assessing inventive step.

Although it was known in the prior art that temperatures above minus 140°C are critical, because glass transition, devitrification and recrystallization occur, the improved method of the prior art referred to in section 4.1 above does not give up the idea of gradually warming the tissue sample to room temperature. With regard to this known method, the tissue is initially frozen by means of liquid nitrogen, whereby one may assume that the freezing procedure is such that vitrification of the tissue takes place without any substantial, or with low, formation of resolvable ice crystals, as it is the case for the method of Claim 1. A "high" vacuum is then produced, whereby in D2 (cited in D1 on page 1026, left-hand column, line 48) the value of 1.10^{-7} Torr is mentioned (see D2, page 479, right-hand column, lines 10 to 14). It is clear that this value is much higher than the claimed one; moreover, it is important to notice that the copper block, in which the tissue is placed, achieves a steady state temperature of about minus 130°C, at which glass transition already occurs (see D1, page 1026, left-hand column, lines 17 to 19). The following warming step by raising the temperature of the copper block 10°C per hour until room temperature, will lead to negative effects due to devitrification and recrystallization, because it may be assumed that water sublimation is not yet completed, when these phenomena occur.

It is thus clear that the method of Claim 1 is distinguished from that known by an important aspect. It, namely, gives up the idea that an essential warming is necessary for the sublimation of water and introduces the principle of sublimation in thermodynamic equilibrium at a temperature lower than minus 140°C. By remaining below this critical temperature (see D1 and also the description as filed, page 12, line 35 to page 13, line 6), the

negative phenomena above-mentioned do not occur at all or their effect is strongly minimized. Moreover, the sublimation of water takes place efficiently, although the tissue sample is dehydrated in a state of equilibrium without substantial temperature increase, as the results achieved, in particular as regards the corneal tissue (see the description as filed, page 8, lines 6 to 22), show. At this point, it appears appropriate to interject that the content of this passage is not in accordance with Claim 1, because it relates to the transplanting of corneal tissue and, therefore, the last two process steps of Claim 1 would not be applicable. Moreover, a claim directed to the subject-matter of this passage would, in all likelihood, not be allowable under Article 52(4) EPC. Nevertheless, the passage does demonstrate the efficacy of the freeze-drying steps required by Claim 1 and for this reason it may remain.

The choice of optimal conditions for the dehydrating step of a method for cryopreparing a biological tissue sample is, as the prior art teaches, particularly difficult. The method known from D1 (or D2) results in removing water with limited effects from devitrification and recrystallization. These documents, however, fail to give any hint at the possibility of obtaining even better results by letting water sublimate in a state of equilibrium at an unusually high vacuum and a temperature below the critical value of minus 140°C. On the contrary, the known method teaches away from the invention in the sense that it considers a substantial increase of the temperature as essential for the sublimation. This fact and the complexity of the subject-matter in suit, in particular as regards the achievement of the desired results, lead to the conclusion that the skilled person, starting from said method of D1 (or D2), does not have any reason to modify it according to Claim 1, so as to solve the stated problem.

5.1.3 Therefore, in view of the foregoing the subject-matter of Claim 1 involves an inventive step in the sense of Article 56 EPC. Claim 1 is thus allowable (Article 52(1) EPC).

5.2 Claim 16

5.2.1 The same conclusion regarding inventive step must be drawn with reference to the independent Claim 16, which pertains to an apparatus for cryopreparing a biological tissue sample for ultrastructural analysis according to the method of Claim 1.

Indeed, the arguments mentioned above with reference to Claim 1 also apply, mutatis mutandis, to Claim 16.

5.3 Dependent Claims 2 to 15 and 17 to 24 refer to particular embodiments of the invention as defined in Claims 1 and 16. They are, therefore, likewise allowable.

Order

For these reasons, it is decided that:

1. The decision under appeal is set aside.

2. The case is remitted to the first instance with the order to grant a patent on the basis of the text proposed with the communication of 6 November 1992.

The Registrar: The Chairman:

P. Martorana E. Turrini