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**Datasheet for the decision
of 25 February 2011**

Case Number: T 0177/09 - 3.4.02

Application Number: 96930802.2

Publication Number: 0852716

IPC: G01N 21/64

Language of the proceedings: EN

Title of invention:
Multi-photon laser microscopy

Patentee:
CORNELL RESEARCH FOUNDATION, INC.

Opponent:
-

Headword:
-

Relevant legal provisions:
-

Relevant legal provisions (EPC 1973):
EPC Art. 56

Keyword:
"Inventive step (no)"

Decisions cited:
-

Catchword:
-



Case Number: T 0177/09 - 3.4.02

D E C I S I O N
of the Technical Board of Appeal 3.4.02
of 25 February 2011

Appellant: CORNELL RESEARCH FOUNDATION, INC.
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Decision under appeal: Decision of the Opposition Division of the
European Patent Office posted 22 October 2008
revoking European patent No. 0852716 pursuant
to Article 102(1) EPC.

Composition of the Board:

Chairman: A. G. Klein
Members: F. Maaswinkel
B. Müller

Summary of Facts and Submissions

I. The appeal lies from the decision of the opposition division dated 22 October 2008 revoking European patent 0 852 716, since the subject-matter of the independent claims of the Main Request lacked novelty over the disclosure in document D1; the set of claims according to the First Auxiliary Request was not admissible under Art. 123(2) EPC; and the claims according to the Second and Third Auxiliary Requests did not involve an inventive step having regard to documents D4 and D2, or D1 and D4.

D1: EP-A2-0 627 643

D2: EP-A1-0 666 473

D4: US-A-5 034 613.

II. Against this decision the patent proprietor has lodged an appeal and requested that the decision under appeal be set aside and that the patent be maintained. Furthermore the appellant has filed an auxiliary request for oral proceedings.

III. With the letter of 10 February 2009 the opponent requested that the appeal be dismissed. With a further letter of 21 April 2009 the opponent withdrew its opposition.

IV. With a letter of 2 March 2009 the appellant requested that the decision of the opposition division be reversed and that the patent be maintained unamended (Main Request) or on the basis of the sets of claims according to Auxiliary Requests 1 to 8 annexed to this letter. The Auxiliary Request for oral proceedings was

maintained in case the board did not intend to maintain the patent unamended.

- V. In an annex to the summons to oral proceedings pursuant to Rule 115(1) EPC sent on 9 November 2010 the board expressed its opinion that provisionally it concurred with the position of the opposition division concerning the Main Request; furthermore that the compliance of the claims of the Auxiliary Requests with Articles 52(1) and 56 EPC was in doubt.

At the oral proceedings on 25 February 2011 the appellant requested as a Main Request that the decision under appeal be set aside and that the patent be maintained as granted or on the basis of the sets of claims according to Auxiliary Requests 1 to 8 filed with the letter of 2 March 2009. At the end of the oral proceedings the board gave its decision.

- VI. The wording of claim 1 of the Main Request reads as follows:

" A method of microscopy by a three or more photon excitation technique of a target material (14) containing molecules which are excitable by a photon of a characteristic energy, the method comprising the steps of:

illuminating said material (14) with a beam (16) of intense, subpicosecond pulses of laser light comprising incident photons of an energy less than said characteristic energy, and

focusing said illumination to a focal volume within said material (14) to produce an illumination intensity sufficiently high to produce molecular

excitation by simultaneous absorption of n of said incident photons, where n is greater than or equal to three and the combined energy of said n photons is equal to said characteristic energy ".

The wording of claim 7 of the Main Request reads as follows:

" An apparatus (10) for laser scanning fluorescence microscopy, comprising:

stage means (15) and on said stage means a target material (14) including a fluorescent component responsive to excitation by a photon of characteristic energy to produce a fluorescence photon;

at least one source (12) of subpicosecond coherent light pulses comprising incident photons of an energy approximately $1/n$ of said characteristic energy, wherein n is greater than or equal to three;

means (20, 22, 24) for focusing coherent light pulses on said target material (14), thereby causing said target material simultaneously to absorb n incident photons and produce a corresponding said fluorescence photon;

detector means (42, 50, 56) for detecting said fluorescence photon; and

means (34,36,38) for directing said fluorescence photon to said detector means ".

Independent claim 1 of the First Auxiliary Request is identical to claim 1 of the Main Request. Independent claim 7 of this Request differs from claim 7 of the Main Request by the addition "...to a focal volume within..." in the feature: "means (20, 22, 24) for focusing coherent light pulses to a focal volume within

said target material (14)..." [*emphasis added here and in the subsequent passages by the board*].

Independent claim 1 of the Second Auxiliary Request basically reads as claim 1 of the Main Request, but includes the additional feature at the end of the claim "(...said n photons is equal to said characteristic energy); and scanning said beam (16) to scan said focal volume (19) through said material (14), and detecting the fluorescence produced by said material".

Independent claim 6 of this Request reads as claim 7 of the First Auxiliary Request with, at the end of the claim, the additional feature: "(...to said detector means); and scanning means (18) for scanning said light pulses through said material".

Independent claim 1 according to the Third Auxiliary Request reads as follows:

" A method of microscopy by a three or more photon excitation technique of a target material (14) containing molecules which are excitable by a photon of a characteristic energy, wherein said incident photons have an energy approximately $1/n$ of said characteristic energy and wherein said material includes fluorescent molecules which produce fluorescence upon said molecular excitation, the method comprising the steps of:

- (a) illuminating said material (14) with a beam (16) of intense, subpicosecond pulses of laser light comprising incident photons of an energy less than said characteristic energy,

- (b) focusing said illumination to a focal volume within said material (14) to produce an illumination intensity sufficiently high to produce molecular excitation by simultaneous absorption of n of said incident photons, where n is greater than or equal to three and the combined energy of said n photons is equal to said characteristic energy;
- (c) scanning said beam (16) to scan said focal volume (19) through said material (14), and
- (d) detecting the fluorescence produced by said material ".

Independent claim 3 according to this Request is identical with claim 6 according to the Second Auxiliary Request.

Independent claim 1 of the Fourth Auxiliary Request reads as claim 1 of the Third Auxiliary Request with the following modification in step (c): "scanning said beam (16) to scan said focal volume (19) through said material (14) to achieve a 3-dimensional spatial resolution, and". Similarly, independent claim 3 of this Request reads as claim 3 of the Second Auxiliary Request with the additional feature at the end of the claim: " (... , and scanning means (18) for scanning said light pulses through said material) to achieve a 3-dimensional spatial resolution".

Independent claim 1 of the Fifth Auxiliary Request reads as claim 1 of the Main Request, the only difference being in the expression "focusing said illumination to a focal point (19)" instead of "focusing said illumination to a focal volume". Similarly, claim 7 of this Request reads as claim 7 of

the Main Request, only differing in the expression "means (20, 22, 24) for focusing coherent light pulses to a focal point (19) within said target material (14)".

Claim 1 according to the Sixth Auxiliary Request reads as claim 1 of the Fifth Auxiliary Request with the additional feature at the end of the claim "(...is equal to said characteristic energy) ; and scanning said beam (16) to scan said focal point (19) through said material (14), and detecting the fluorescence produced by said material". Claim 6 of this Request is identical to claim 6 of the Second Auxiliary Request with replacement of the expression "to a focal volume within..." by the expression "to a focal point (19) within...".

Claim 1 according to the Seventh Auxiliary Request reads as claim 1 of the Third Auxiliary Request where in steps (b) and (c) the expression "focal volume" has been replaced by "focal point (19)". Claim 3 of this Request is identical to claim 6 of the Sixth Auxiliary Request.

Claim 1 according to the Eighth Auxiliary Request reads as claim 1 of the Fourth Auxiliary Request where in steps (b) and (c) the expression "focal volume" has been replaced by "focal point (19)". Similarly, claim 3 of this Request reads as claim 3 of the Fourth Auxiliary Request, only differing in the expression "means (20, 22, 24) for focusing coherent light pulses to a focal point (19) within said target material (14)".

VII. The arguments of the appellant may be summarised as follows:

With respect to the objection in point 2.1 of the Decision under appeal that the subject-matter of claims 1 and 7 of the Main Request would be anticipated by the disclosure in document D1, it is argued that this document discloses a laser scanning optical system including a Bessel beam which is not a "focused" beam as required by the independent claims. Furthermore, there is no disclosure in D1 of a "simultaneous absorption" of three or more photons. Therefore the subject-matter of claims 1 and 7 is novel by virtue of these features.

In its Communication the board had considered that for the discussion of inventive step the closest prior art was disclosed in document D4. This document discloses a laser scanning microscope which produces molecular excitation in a target material by simultaneous absorption of two photons to provide intrinsic three-dimensional resolution. A drawback to the two-photon laser microscopy technique disclosed in document D4 is that its applications are limited by the available laser technology. In particular, the two-photon technique requires use of a laser at specific wavelengths, depending upon the application, so that the sum of energy levels of the two photons provides the specific energy level needed to generate the desired fluorescent emission. Unfortunately, some laser microscopy applications would require use of a laser having a wavelength which is not technologically feasible at the present time. For example, excitation of chromophores that have very short wavelength

absorption, such as amino acids and nucleic acids, would require a laser having a 540 nm wavelength using the two-photon technique, and such a laser does not exist at the present time.

The subject-matter of the independent claims according to the Main Request differs from the apparatus and method from document D4 in that simultaneous absorption of n photons is produced where n is greater than or equal to three. According to the opposition division, the underlying technical effect is that near-infrared photons may be used to excite fluorophores which normally absorb in the UV region and that, due to the even higher intrinsic confocality of three-photon excitation, photobleaching and photodamage to living cell specimens are further reduced. The opposition division concluded that the technical problem could therefore be seen in modifying the method of D4 in order to enable excitation of fluorophores absorbing in the UV region with light having even longer wavelengths than visible light, in particular near-infrared light, and to further reduce photobleaching and photodamage to living cells. In the opinion of the appellant such a formulation of the objective problem is, however, not correct since by referring to longer wavelengths of the photons to be used this formulation contains hints to the solution of the technical problem. Therefore it appears more correct to define the technical problem as aiming at reducing the costs of the elements of the laser scanning microscope and avoiding damage to the sample.

The skilled person trying to solve this technical problem would not find any hints to the claimed

solution in the cited prior art. In particular document D1 discloses a laser scanning optical system which fundamentally differs from the apparatus disclosed in document D4 in that it teaches to use a Bessel beam for scanning at a higher speed but which does not allow to reach a 3-dimensional spatial resolution. Furthermore this document does not disclose a simultaneous absorption of a plurality of photons. Therefore the person skilled in the art would definitely not combine D4 with D1 since this combination would again result in the disadvantage (extensive scanning time) contrary to the aim of D1 which, therefore, teaches away from a combination with D4. As to document D2, this document describes a method for two-photon excitation of long-lived fluorescent or phosphorescent dyes with long low-power pulses. According to D2, using long excitation pulses and consequently low excitation power is possible due to the long life-time (see column 5, lines 31-34 of D2). In column 6, lines 47-51 of D2, it is described that time-resolved detection of a dye with a half-life of 1 ms allows an excitation pulse of 0.1 ms in order to avoid any significant interference by the duration of the pulse with the time-resolved detection of 2-photon fluorescence. This, however, is completely different compared to the intense sub-picosecond pulses used according to the present invention. Furthermore, a skilled person would even be directed away from the present invention by the teaching of D2. Simultaneous multiphoton absorption as referred to in the present patent is a process similar to the process shown in Fig. 1A of D2 for the case of two-photon absorption, that is, a direct excitation of three or more photons without intermediate energy state. This process, however, requires that the photons are

absorbed simultaneously within approximately 10^{-15} seconds (see column 3, lines 29-33 of D2), i.e., the probability for such a simultaneous absorption when using pulse length in the order to 0.1 ms as described in D2 would be extremely small. This is even more important when more than two, i.e., at least three incident illuminating photons must be absorbed simultaneously as it is the case according to the invention described in the present patent. This fundamental difference is even mentioned in document D2 in the context of two-photon absorption of D4. In numerous places, D2 explicitly refers to document D4 (US-A-5 034 613, see col. 2 lines 18 and 31, col. 3 line 5, col. 4 line 48, and col. 6 lines 25 and 35), and stresses the differences between D4 and D2. In particular, in column 4, line 47 to col. 5 line 2, D2 refers to D4, and correctly states that ultra-short pulses are advantageously used for two-photon excitation. Thus, by stressing the advantages of low excitation powers and, thus, long excitation pulses, D2 teaches not to combine it with D4 and, thus, teaches away from the subject-matter of the contested patent.

Therefore, the subject-matter of the independent claims according to the Main Request is not only novel, but also involves an inventive step in view of the teaching of prior art document D4 in combination with D1 or D2.

Auxiliary Requests 1 to 8

Auxiliary Request 1 corresponds to the Main Request but with a clarification of independent claim 7 in order to bring it into conformity with the wording of claim 1, namely that the coherent light pulses are focused "to a focal volume within the target material". The same

amendment is made in the independent apparatus claims of Auxiliary Requests 2 to 4, which correspond to previous Auxiliary Requests 1 to 3, respectively, apart from the use of the expression "focal volume" (rather than "focal point"). Auxiliary Requests 5 to 8 correspond to Auxiliary Requests 1 to 4 apart from the use of the expression "focal point" (rather than "focal volume").

As regards patentability, it is referred to the arguments provided for the Main Request above with the detailed discussion of the relevance of the prior art documents D1 to D4. The same arguments hold true for the subject-matters of the independent claims of all Auxiliary Requests 1 to 8 which are even more limited compared to independent claims 1 and 7 of the Main Request.

Reasons for the Decision

- 1.1 The appeal is admissible.
- 1.2 In the Decision under appeal the opposition division had found the subject-matter of the independent claims according to the Main Request to be objectionable for lack of novelty and those of the then First Auxiliary Request not admissible under Art. 123(2) EPC. According to the opposition division, the claims of the further Auxiliary Requests did not involve an inventive step. Since the board at the oral proceedings similarly found that the subject-matter of the claims of all Requests does not involve an inventive step, the further

objections in the decision under appeal need not be considered.

2. *Main Request - inventive step*

2.1 The board considers that the closest prior art is disclosed in document D4. It concurs with the appellant that the subject-matter of claims 1 and 7 of the Main Request differs from the laser scanning microscope and the method of microscopy disclosed in this document in that the molecular excitation is produced by simultaneous absorption of n photons, where n is greater or equal to three.

2.2 According to the opposition division, the technical problem addressed by this difference could be defined as modifying the method of D4 for enabling excitation of fluorophores absorbing in the UV with long-wavelength light, for instance infrared light, and to further reduce photobleaching and photodamage to living cells.

2.3 The appellant has argued that the explicit referring to longer-wavelength light in this formulation of the technical problem introduces hints to its solution. Therefore the technical problem was rather related to reducing the costs of the laser scanning microscope and avoiding damage to the sample.

2.4 The board has some reserve whether this definition of the technical problem by the appellant is actually solved in the patent specification: for instance, paragraph [0005] discloses that "In practice, the configuration of three-photon laser scanning microscopy

can be identical to the existing two-photon systems". Therefore, presumably, the costs of the systems are not reduced. Furthermore in paragraph [0011] it is disclosed "Empirical studies have shown that two-photon excitation elicits far less damage than one-photon excitation for comparable fluorescence image acquisition. It is not clear whether further improvement can be made by stepping up to three- or four-photons for excitation".

2.5 Therefore, since the patent specification does not provide a clear teaching that the problems formulated by the appellant are indeed solved, it appears more appropriate to define the objective technical problem over the prior art as to further increase or diversify the possibilities of the prior art two-photon laser microscopy, because this problem is of common interest to the man skilled in the art. To this aim he will as a matter of course consult the available literature in the particular technical field and neighbouring fields.

2.6 Document D1 discloses a fluorescence microscope (see col. 10, l. 40) employing a laser system as a light source having very similar pulse properties as the laser system of document D4 (D1, col. 11, l. 8 - 13: laser having an optical pulse duration of several ten to several hundred fsec and a repetitive frequency of several ten to several hundred MHz; D4, col. 6, l. 37 and 38: pulses less than 100 fsec duration at about 80 MHz repetition rate). Therefore the skilled person reading the disclosure in document D1 immediately understands that the system of D4 can be used for the same applications as disclosed in document D1. In particular in the same passage in col. 11 of D1 it is

further disclosed that the wavelength of the laser may be "two or three times longer than the peak wavelength in an absorption spectrum of a fluorescent dye for labeling the sample...". Furthermore col. 16, l. 2 - 9 discloses that the fluorescent dye "emits ...as a secondary light source proportional to an amount of fluorescent dye similarly as in case of excitation by a peak wavelength in the absorption spectrum corresponding to a half or a third of the oscillation wavelength of the laser beam" (*emphasis by the board*). Thus, similarly as in document D1, which clearly presents multiphoton excitation with n equal two or three as alternatives ("two or three times longer"; "a half or a third") the skilled person will employ these alternatives in the apparatus of D4 according to his particular needs.

2.7 In this respect it is irrelevant that document D1 discloses three-photon absorption together with the use of a Bessel beam for providing the excitation zone: the skilled person understands that the choice of a Bessel beam implies advantages (high illumination intensity in a long focal depth which increases the scan speed) but also inherent disadvantages (additional costs of the axicon-components and, in particular, the loss of three-dimensional resolution of the scanned image). Therefore for three-dimensional microscopy purposes he will readily consider adapting the apparatus of document D4 to provide the proper wavelengths for the three-photon absorption process.

2.8 Similarly document D2, while referring to document D4 in acknowledging the advantages of two-photon excitation (improved background discrimination,

reducing photobleaching of the fluorophores, and minimising the photodamage of living cell structures; see D2, col. 2, l. 2 - 10) also discloses that the use of more than two photons increases the nonlinearity and that this can be exploited to improve resolution (col. 2, l. 37 - 55), in particular lines 37 - 40 disclosing that "The emission caused by two photons is thus an exponential function to the power of two, the emission caused by three photons an exponential function to the power of three, etc".

- 2.9 The board does not concur with the argument of the appellant that, because the gist of the disclosure in document D2 resides in the use of low excitation powers and thus long excitation pulses, its teaching would be irreconcilable with the multiphoton excitation relying on simultaneous excitation by very short pulses disclosed in document D4. Rather, the person skilled in the art, for instance a physicist being familiar with quantum optics and spectroscopy, understands that the phenomenon of multiphoton (in particular, two or three photon) excited fluorescence is not dependent on the the duration of the excitation pulse, and that either simultaneous excitation of the fluorescent dye by very short incident pulses can be accomplished (like in the system with fsec pulses as in D4 or in D1) or by the long pulses proposed in document D2 (albeit only for very particular, rare-earths based dyes). The skilled person, if considering to further increase or diversify the possibilities of two-photon laser microscopy as disclosed in document D4, would learn from document D2 that, also for multiphoton fluorescence microscopy relying on simultaneous absorption as in document D4, it would be attractive to extend the possibilities of

this instrument by including the option to three-photon fluorescence microscopy.

2.10 Therefore, since the use of three-photon excitation processes for the detection of dyes is known from the prior art (documents D1, D2) its implementation in the device of D4 for carrying out three-photon laser scanning microscopy would appear obvious to the skilled person.

2.11 It is concluded that the subject-matter of independent claims 1 and 7 of the Main Request does not involve an inventive step (Article 52(1) EPC and 56 EPC 1973). Therefore this Request is not allowable.

3. *The Auxiliary Requests*

3.1 In the Statement of the Grounds of Appeal of 2 March 2009 the appellant had explained that the wording of the independent claims of the Auxiliary Requests differed from claims 1 and 7 according to the Main Request mainly for bringing the independent claims into conformity (Auxiliary Request 1); using the expression "focal volume" rather than "focal point" (Auxiliary Requests 2 to 4); and, instead, using the formulation "focal point", rather than "focal volume" (Auxiliary Requests 5 to 8).

3.2 The main reason for these amendments appears to reside in a more clear distinction with respect to the disclosure of document D1, which, during the first instance proceedings, had been regarded as anticipating the subject-matter of the independent claims. These amendments should in particular define an unambiguous

distinction between the Bessel beam used in the apparatus of document D1 and the beam focused towards a spot as in the patent under dispute.

3.3 However, since the apparatus for laser scanning fluorescence microscopy disclosed in document D4 employs exactly the same type of focusing as in the patent, namely forming a focal point 26, see Fig. 1A of D4, and thus enabling a three-dimensional scanning, see col. 4, l. 54 and 55, the amendments in the Auxiliary Requests do not define a further restriction over the apparatus disclosed in D4. Therefore, as admitted by the appellant, the same arguments with respect to the patentability of the Main Request hold for the Auxiliary Requests. Since the Main Request is not allowable because its subject-matter does not involve an inventive step, the same must be concluded with respect to the Auxiliary Requests.

4. Hence, since the independent claims of all requests on file are not allowable, none of the requests is allowable.

Order

For these reasons it is decided that:

The appeal is dismissed.

The Registrar:

The Chairman:

M. Kiehl

A. G. Klein