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D E C I S I O N
of 21 November 2002

Case Number: T 0558/01 - 3.4.2

Application Number: 90917225.6

Publication Number: 0500717

IPC: G02B 21/00, G01N 21/64, G11B 7/00

Language of the proceedings: EN

Title of invention:
Two-photon laser scanning microscopy

Patentee:
CORNELL RESEARCH FOUNDATION, INC.

Opponent:
Carl Zeiss Jena GmbH
Leica Microsystems AG Corporate Patent+Trademarks Department

Headword:
-

Relevant legal provisions:
EPC Art. 123, 54, 56
EPC R. 89

Keyword:
"Novelty and inventive step (yes)"

Decisions cited:
T 0095/90

Catchword:
-



Case Number: T 0558/01 - 3.4.2

D E C I S I O N
of the Technical Board of Appeal 3.4.2
of 21 November 2002

Appellant:
(Proprietor of the patent)

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Decision under appeal:

Decision of the Opposition Division of the
European Patent Office posted 6 March 2001
revoking European patent No. 0 500 717 pursuant
to Article 102(1) EPC.

Composition of the Board:

Chairman: E. Turrini
Members: A. G. Klein
G. E. Weiss

Summary of Facts and Submissions

- I. The appellant (patentee) filed an appeal against the decision of the opposition division revoking European patent No. 0 500 717 (application No. 90 917 225.6) on the ground that its subject-matter did not involve an inventive step.
- II. Oral proceedings were held on 21 November 2002, at which the appellant requested that the decision under appeal be set aside and that the patent be maintained with the claims and drawings as granted and a description comprising amended pages 4 and 8 as filed at the oral proceedings (main request).

Claims 1 and 6, the only independent claims of the set of claims in accordance with the appellant's main request, read as follows:

"1. Apparatus (10) for laser scanning fluorescence microscopy of a target material (20) said apparatus comprising:

stage means (22) bearing target material (20) including a fluorescent component responsive to excitation by a photon of a characteristic energy to produce a fluorescence photon;

focusing means (40,12) positioned to direct light toward said stage and having an object plane (18) in said target material (20) at said stage means (22);

a repetitive source (16) of subpicosecond monochromatic coherent light pulses comprised of photons of energy less than said characteristic energy;

detector means for detecting said fluorescence photons (54);

means directing said coherent light pulses along

an optical path including said focusing means (40,12), said focusing means (40,12) focusing said light pulses at a focal volume (26) on said object plane (18) to impinge on said target material (20) at said stage means (22) so that two-photon excitation induces said fluorescence photons in said fluorescent component, and means for directing said fluorescence photons from said focal volume (26) to said detector means (54).

6. A method of laser scanning fluorescence microscopy by a two-photon excitation technique of a target material (20) containing a fluorescent component which is excitable by a photon of a characteristic energy to produce a fluorescence photon; by the steps of repetitively illuminating said material (20) with a beam of intense, subpicosecond pulses of monochromatic coherent laser light comprising photons of an energy less than the said characteristic energy; and focusing said illumination to a small focal volume (26) within said material (20) to produce an illumination intensity sufficiently high only at said focal volume to produce molecular excitation by simultaneous absorption of two of said incident illuminating photons to induce fluorescence in said fluorescent component, and detecting the fluorescence induced."

The appellant further requested that the patent be maintained on the basis of one of the alternative sets of amended claims filed as auxiliary requests 1 to 6.

The respondents (opponents) requested that the appeal be dismissed.

The following documents were discussed during the oral proceedings:

- R7: C.J.R. Sheppard et al., "Resonant scanning optical microscope", Applied Optics, Vol. 17, No. 18, 15 September 1978, pages 2879 to 2882;
- R9: Denk et al., "Two-photon Laser Scanning Fluorescence Microscopy", Science, Vol. 248 (4951), 6 April 1990, pages 73 to 76;
- R14: C.J.R. Sheppard, "Scanning optical microscope", Electronics and Power, pages 166 to 172, February 1980, reprinted in SPIE Milestone Series, Volume MULTIMODE SEMICONDUCTOR LASER DEVICES 131, pages 113 to 119;
- R16: A. Parthenopoulos et al., "Three-Dimensional Optical Storage Memory", Science, Vol. 245, 25 August 1989, pages 843 to 845;
- R20: Demptroder, "Laser Spectroscopy", Springer-Verlag Berlin, Heidelberg, New York, 1981, pages 422 to 441 and 672 to 675;
- R21: J.A. Valdmanis et al., "Design Considerations for a Femtosecond Pulse Laser...", IEEE Journal of Quantum Electronics, Vol. QE 22, No. 1, January 1986, pages 112 to 118;
- R24: GB-A-1 236 108;
- R27: M. Friedrich, "Two-Photon Molecular Spectroscopy", J. Chem. Educ., Vol: 59(6), 1982, pages 472 to 481;

- R32: Z. Bor, "Distortion of femtosecond laser pulses in lenses and lens systems", J. Modern Optics, Vol. 35(12), 1988, pages 1907 to 1918;
- R33: W. Berns, "A possible Two-Photon Effect *in vitro* Using A Focused Laser Beam", Biophys. J., Vol. 16, 1976, pages 973 to 977;
- R34: R. Shack et al., "Ultrafast Laser Scanner Microscope ", J. Histochem. and Cytochem., Vol. 27(1) 1979, pages 153 to 159;
- R35: F. Docchio et al., "Experimental investigation of optical breakdown thresholds in ocular media under single pulse irradiation with different pulse durations", Lasers in Ophthalmology, Vol. 1(2), 1986, pages 83 to 93;
- R38: D. Stern et al., "Corneal Ablation by Nanosecond, Picosecond and Femtosecond Lasers at 532 and 625 nm", Arch. Ophthalmol., Vol. 107, 1989 pages 587 to 592;
- R65: C. J. R. Sheppard, "Scanning Optical Microscopy" in Advances in Optical and Electron Microscopy, Vol. 10, Academic Press, 1987, pages 1 to 98; and
- R70: V.S. Letokhov, "Recent Results in Laser Biomedicine and Some Prospects of the Future", Berichte der Bunsen-Gesellschaft für Physikalische Chemie, Vol. 93, No. 3, March 1989, pages 233 to 422.

The board gave its decision at the end of the oral proceedings.

The order of the decision as announced at the oral proceedings was corrected pursuant to Rule 89 EPC by decision of 28 January 2003.

- III. In support of its requests the appellant stressed that the claimed two-photon laser scanning fluorescence microscope was a further development of the normal fluorescence microscope, both being based on the detection of fluorescent signals uniformly emitted by the observed material in all directions.

In contrast, document R14 which the opposition division considered to disclose the closest prior art and the articles R7 and R65 by the same author essentially addressed the obtaining of images from second harmonic generation of signals propagating predominantly in the same direction as the excitation.

Prior to the patentee's invention, scanning fluorescence microscopy was limited in many respects. The fluorophores used to stain specimens generally required energy corresponding to the ultraviolet or shorter visible wavelengths of light to excite fluorescence. Such light, however, often damaged living cells, making scanning confocal fluorescence microscopy unsuitable for use with biological specimens. The intensity of illumination necessary to produce satisfactory images also often photobleached the fluorophores. Because the illumination irradiates the full depth of the specimen, it also contributed to emission of out-of-focus fluorescence, which reduced image contrast. In addition, using UV or shorter visible wavelengths of light required microscope optics that were chromatically corrected and transparent to the excitation and emission wavelengths. Such optics at the priority date of the present invention were not readily available.

The inventors discovered that two-photon excitation using subpicosecond laser pulses with a scanning fluorescence microscope could eliminate or minimize the effects of photobleaching, out-of-focus fluorescence, and photodamage, thereby making it possible to record images of impressive clarity revealing details never before accessible to optical microscopes without the need for complex optics.

The patentee also submitted a number of documents and declarations by Dr Amos and Dr Walmsley to show that the person skilled in the art of non-linear optics and confocal microscopy would not have been motivated to use subpicosecond pulses in conjunction with two-photon fluorescence microscopy before the date of the patent.

Dr Walmsley summarised these submissions during the oral proceedings.

IV. The opponents' submissions can be summarised as follows.

As concerns novelty, document R14 is a review article which beyond the disclosure of laser scanning microscopy by optical harmonics generation clearly teaches both the suitability of other non-linear optical effects, such as two-photon fluorescence, for the production of micrographic images and the desirability of operating the laser source in a pulsed mode. At the date of the invention, subpicosecond lasers were well known, as is shown for instance by document R21 (see the titles of the references 16 to 18 at the end of this document). Document R14 - when considered in its entirety as is recommended for instance in paragraph 4.2 of the reasons of decision T 95/90 (not published in the OJ EPO) according to which different passages of one document may be

combined provided that there are no reasons which would prevent a skilled person from such a combination and the technical teaching of examples may be combined with that disclosed elsewhere in the same document - therefore clearly provides an enabling disclosure of a two-photon pulsed laser scanning fluorescence microscope in accordance with claim 1 as granted .

The claimed microscope is not novel either in view of the contents of document R16. This citation discloses a three-dimensional optical memory system based on two-photon laser fluorescence writing, reading and erasing of information. Such an apparatus is encompassed by the scope of claim 1 as granted, as is expressly set out for instance in the second paragraph of page 8 of the patent specification.

Concerning inventive step, the only feature of claim 1 which is not explicitly described in document R14 is the subpicosecond length of the coherent light pulses. The interest of using such short light pulses and the relationship between pulse length and probability for a two-photon transition were well known to the skilled person at the filing date of the patent in suit, as is evidenced for instance by documents R20, R24, R27 or R70. Adequate laser sources, such as femtosecond lasers, were commercially available at that time, and using them for the purpose of putting to practice the teaching of document R14 cannot be considered to involve an inventive step, accordingly.

The respondents in this respect refuted the appellant's submissions, based in particular on documents R32, R33, R34, R35, R38 and R70, that the expectation of tissue damage, light scattering and reduction of the signal-to-noise ratio would have deterred the skilled person from using such short pulses for the imaging of

biological material. These arguments were backed up by two written statements by Professor Sheppard who presented his view also at the oral proceedings.

The respondents at the oral proceedings also explained the absence in the prior art documents of any representation of a micrography obtained by two-photon fluorescence laser scanning microscopy in that the required femtosecond laser sources were simply too expensive before the date of the patent for having been readily available to the research institutions which worked in this field. The great number of publications referring to the citation R9 published by the inventors of the patent in suit shortly after the filing of their patent can be explained by the particularly large diffusion of the publication "Science" in which the article was published and by the special advertising efforts made by BioRad, the company which commercialises the patented microscope.

Reasons for the Decision

1. The appeal is admissible.
2. *Amendments*

The claims and drawings in accordance with the appellant's main request are those of the patent as granted.

Only the description was amended by deletion of the two paragraphs which on page 4, lines 29 to 31 and page 8, lines 3 to 8 referred to the possibility of using the two-photon fluorescence excitation technique for the writing or reading of data information in a three-dimensional optical memory device. This deletion does

not add any subject-matter, neither does it extend the protection conferred by the claims, in conformity with the requirements of Article 123(2) and (3) EPC.

This was not disputed by the respondents.

3. *Novelty*

- 3.1 Document R14 was held by the opposition division to disclose a two-photon luminescence laser scanning microscope operating in a pulsed mode.

Document R14, a review article by Professor Sheppard, is dedicated to scanning optical microscopes (see the title). From page 113 to the end of the first paragraph in the middle column of page 118 and in conjunction in particular with the schematic representation of Figure 1 on page 140 it presents the principles and mode of operation of conventional and confocal microscopes. In the passage from the second paragraph of the middle column on page 118 to the third paragraph of the right hand column on the same page, the article then describes the production of images from the generation of optical harmonics within a specimen, with details of the laser source used, the power density of the beam and the signal detection device. Figure 9 on page 119 shows an example of a harmonic micrography from a crystal and reference is made in the passage also to further macrographs published elsewhere by the same author (see lines 9 to 11 of the third paragraph of the right hand column on page 118).

Two-photon fluorescence is referred to only in the following (penultimate) paragraph of the right hand column on page 118, which reads as follows:

"Other nonlinear interactions could also be used to get interesting new ways of studying matter in microscopic detail hitherto not available. Nonlinear interactions include the generation of sum-frequencies, Raman scattering, two-photon fluorescence, and others. We feel that the method will be of particular interest in studying biological materials, some of which, such as d.n.a and proteins, have large second-harmonic generation coefficients; and the wide range of these coefficients should give very strong contrast in the images formed. Furthermore, frequency mixing should give information concerning the chemical structure of the object" (emphasis added).

Thus, two-photon fluorescence for the production of images from a scanning laser microscope is evoked in document R14 in a single sentence, as only one of several non-verified possibilities. The document does not set out any constructional detail of a two-photon fluorescence microscope, neither does it show any micrography obtained by two-photon fluorescence. The merely speculative character of the reference in this document to two-photon fluorescence in conjunction with scanning microscopes is confirmed by citation R65, published seven years later by the same author, in which two-photon fluorescence is still only evoked as a possibility which could be envisaged for the future; see page 84, the first sentence of paragraph C: "If the energy density in the focused spot of a scanning optical microscope is sufficiently large, non-linear effects such as harmonic generation, generation of the sum frequencies, coherent Raman scattering, parametric oscillations and two-photon fluorescence may result" (emphasis added).

The fact, not disputed by the parties, that before the filing date of the patent in suit there has been disclosed no micrography obtained by two-photon fluorescence laser scanning microscopy in the board's view is a strong indicator of the actual non-availability of such a microscope in the prior art. The respondent's explanation in this respect that two-photon fluorescence macrographs had not been produced earlier simply because of the high costs of femtosecond pulse lasers is not convincing. Documents R14 and R65 do not indeed call for the use of femtosecond lasers and document US-a-4 777 732, a patent filed on behalf of one of the respondents after the filing date of patent in suit, shows that two-photon fluorescence can be achieved also with laser pulses of relatively long duration such as above a picosecond, or even with continuous light (see reference 55 as filed by the appellant with its statement of grounds of appeal, the title and column 1, lines 42 to 45).

The respondents referred to decision T 95/90 *supra* to support the argument that teachings from different portions of a prior art document can be combined to define its actual disclosure. This decision relates to the question of whether a claimed chemical composition can be anticipated by the disclosure in a prior art document of a specific example of a composition comprising certain components of the claimed composition, together with a further teaching in the document that the remaining components of the claimed composition could be advantageously included in the exemplified composition as well. The components in this case were all precisely defined. This decision is accordingly hardly relevant to the question at issue

here, namely whether a speculative proposal, not supported by any specific description of how it should be put into practice, to modify an actually disclosed device shall be held to make a so modified device available to the public within the meaning of Article 54(2) EPC.

Accordingly, neither document R14, nor documents R7 and R65 which originate from the same author and in which two-photon fluorescence microscopy is evoked in substantially the same terms as in document R14, in the board's view make any two-photon fluorescence laser scanning microscope available to the public.

- 3.2 Document R16 discloses an apparatus and a technique based on two-photon fluorescence for the writing, reading and erasing of information in a photochromic material embedded in a polymer matrix.

The respondents submitted that although the subject-matter of claim 1 was designated as an apparatus for laser scanning fluorescence microscopy of a target material, the claim also encompassed an apparatus for the three-dimensional optical storage of data in a memory device, as was clear from the references to such an application in several passages of the specification.

However, independent claims 1 and 6 are explicitly directed to the achieving of laser scanning fluorescence microscopy, which the apparatus of document R16 does not, and passages of the specification mentioned by the respondents have now been deleted, so that the patent documents in

accordance with the appellant's main request do not offer any support for an interpretation of the claims so as to extend their scope to the writing, reading and erasing of information in a three-dimensional structure.

3.3 The other documents in the file do not come closer to the claimed subject-matter.

3.4 For these reasons, the subject-matter of independent claims 1 and 6 in accordance with the appellant's main request is novel within the meaning of Article 54 EPC.

The same conclusion applies to the subject-matter of the remaining claims 2 to 5 and 7, by virtue of their appendance to claims 1 and 6.

4. *Inventive step*

4.1 Although document R14 does not for the reasons set out above in connection with the question of novelty make available to the public a two-photon fluorescence laser scanning microscope, the board can concur with the respondents' submission that the claimed apparatus can be considered as a further development of the scanning-harmonic microscope actually described in this document. Like the claimed microscope, the scanning-harmonic microscope operates on the basis of a non-linear interaction produced by a strongly focused laser beam such as to achieve inherent depth discrimination comparable to that which can be achieved with a conventional confocal microscope. This scanning-harmonic microscope can therefore be reasonably considered to constitute the closest prior art.

4.2 Accordingly, the technical problem underlying the subject-matter of claim 1 of the appellant's main request can be seen in providing an alternative to the known scanning-harmonic microscope of document R14, which moreover does not suffer from the disadvantages of conventional fluorescence microscopy such as excessive photobleaching of fluorophores in the target material, damage of the target material by ultraviolet light and the necessity of using chromatically corrected lenses which are transparent for ultraviolet radiation (see page 3 of the specification of the patent in suit, lines 34 to 45).

4.3 The claimed solution to this technical problem, involving two-photon excitation by a repetitive source of subpicosecond monochromatic coherent light pulses comprised of photons of energy less than the characteristic energy producing a fluorescence photon in conventional fluorescence microscopy, does not in the board's view follow in an obvious manner from the prior art citations in the file.

It is true that document R14 cites two-photon fluorescence as a non-linear interaction which "could also be used to get interesting new ways of studying matter in microscopic detail hitherto not available" (see page 118, right hand column, the fourth paragraph). Two-photon fluorescence is however evoked only as a non-verified possibility amongst others, like the generation of sum-frequencies and Raman scattering and without any indication of how to realise it in practice. Citations R7 and R65, two articles by the same author but published respectively two years earlier and seven years later than document R14, show that the use of two-photon fluorescence in a scanning microscope had remained a mere speculation without any practical application for a long period before the

filing date of the patent. Apart from the above series of articles by Professor Sheppard there are indeed no other prior art citations in the file to refer to two-photon fluorescence in conjunction with the obtaining of images by a microscope.

The appellant also invoked the intense research and development activities which took place over such a long period before the filing date of the patent, the evident scientific and commercial interests at stake, in particular in connection with the studying of biological materials such as DNA and proteins as evoked already in document R14, and the unanimous recognition by the scientific community of the inventors of the patent in suit as the founders of two-photon fluorescence laser scanning microscopy, as is evidenced by more than 800 scientific publications which refer to the article R9 in Science, which the inventors published shortly after the filing date of the patent in suit, as being the first published report of optical image formation by two-photon excited fluorescence and by their awarding of the Rank Price, a prestigious award in the field of optoelectronics.

In the board's view, the number, the consistency and the relevance of these various pieces of circumstantial evidence are such that they can hardly not be brought to the appellant's credit.

In particular, the respondent's submission that the great resonance of citation R9 in the scientific community can be explained simply by the large diffusion of the journal Science in which it was published and by the publicity organised around it by the company which commercialises the claimed apparatus could not convince the board. Even the respondents themselves, ten years after the priority of the patent

in suit still expressly referred to document R9 in the product information brochure "Microscopy from Carl Zeiss, Two-photon excitation with the LSM 510 NLO", August 1999, stating that the non-linear excitation of fluorescence dyes disclosed there "was developed as a new technique for fluorescence microscopy around ten years ago" (see the citation R56 filed with the appellant's statement of the ground of appeal, the first sentence).

For these reasons, taking into due consideration both the objective content of the prior art citations and the strength of the circumstantial evidence submitted by the appellant, the board is satisfied that the subject matter of claim 1 in accordance with the appellant's main request involves an inventive step within the meaning of Article 56 EPC.

The same conclusion applies to the subject-matter of independent claim 6, which recites substantially the same limitations as claim 1 in terms of a method of laser scanning fluorescence microscopy, and to the remaining claims by virtue of their appendancy to either independent claim 1 or independent claim 6.

- 4.4 The question of whether the subpicosecond pulse length set out in claim 1 provides an inventive contribution *per se* was amply debated both in the written and during the oral proceedings. This line of argument developed from the assumption that document R14 already disclosed two-photon fluorescence pulsed laser scanning microscopy and, consequently, that the subpicosecond pulse length was the only feature to distinguish the claimed subject matter from this prior art, as was held by the opposition division in the appealed decision.

This assumption however is incorrect, since for the above reasons already the combination of the other features of the claim could not be derived in an obvious manner from the state of the art. The question of the contribution to inventive step of the subpicosecond pulse length alone did not therefore need be examined further.

5. For the above reasons, the patent as amended in accordance with the appellant's main request and the invention to which it relates meet the requirements of the convention, so that the patent can be maintained as amended (Article 102(3) EPC).

The appellant's auxiliary requests 1 to 6 need not be examined, accordingly.

Order

For these reasons it is decided that:

1. The decision under appeal is set aside.
2. The case is remitted to the department of first instance with the order to maintain the patent as amended in the following version:

Description: pages 3 and 5 to 7 of the patent specification, pages 4 and 8 filed at the oral proceedings of 21 November 2002.

Claims: 1 to 7 of the patent specification.

Drawings: 1, 1A and 2 to 6 of the patent specification.

The Registrar:

The Chairman

P. Martorana

E. Turrini



Case Number: T 0558/01 - 3.4.2

D E C I S I O N
of 28 January 2003
correcting an error in the decision
of the Technical Board of Appeal 3.4.2
of 21 November 2002

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Decision under appeal: Decision of the Opposition Division of the
European Patent Office posted 6 March 2001
revoking European patent No. 0 500 717 pursuant
to Article 102(1) EPC.

Composition of the Board:

Chairman: E. Turrini
Members: A. G. Klein
G. E. Weiss

- I. At the end of the oral proceedings held on 21 November 2002 the board announced its decision to set aside the decision under appeal and to remit the case to the department of first instance with the order to maintain the patent as amended in the version set out in the minutes sent to the parties on 29 November 2002.

- II. In a letter dated 11 December 2002 the appellants (patentees) representative requested that page 3, line 3 of the minutes of the oral proceedings of 21 November 2002 be corrected to reflect the fact that the amended version of the description for maintenance of the patent comprises pages 3, 5 to 7 of the patent specification rather than pages 3, 5 and 7.

- III. The correct version of the description in which the patent is to be maintained in accordance with the appellants main request indeed also comprises page 6 of the patent specification, which was neither deleted nor amended by the appellant.

The mistake mentioned by the appellant appears in the portion of the minutes which - in the boards view faithfully - reproduces the decision announced by its chairman at the end of the oral proceedings.

- IV. This decision is hereby corrected as follows, in accordance with the provisions of Rule 89 EPC:

In the order of the decision of 21 November 2002 the mention in the paragraph "Description" of point 2, the mention of "pages 3, 5 and 7 of the patent specification" is changed to "pages 3 and 5 to 7 of the patent specification".

The Registrar:

The Chairman

P. Martorana

E. Turrini

