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D E C I S I O N
of 14 January 2005

Case Number: T 0330/02 - 3.4.2

Application Number: 91905729.9

Publication Number: 0495930

IPC: G02B 21/00

Language of the proceedings: EN

Title of invention:
Multicolor confocal fluorescence microscopy system

Patentee:
Regents of the University of Minnesota

Opponent:
Leica Microsystems Heidelberg GmbH

Headword:
-

Relevant legal provisions:
EPC Art. 56

Keyword:
"Inventive step (yes)"

Decisions cited:
-

Catchword:
-

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Case Number: T 0330/02 - 3.4.2

D E C I S I O N
of the Technical Board of Appeal 3.4.2
of 14 January 2005

Appellant: (Proprietor of the patent)
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Decision under appeal: **Decision of the Opposition Division of the European Patent Office posted 29 January 2002 revoking European patent No. 0495930 pursuant to Article 102(1) EPC.**

Composition of the Board:

Chairman: A. G. Klein
Members: M. A. Rayner
J. H. P. Willems

Summary of Facts and Submissions

I. The patent proprietor lodged an appeal against the decision of the opposition division revoking European patent 495 930 (application number 91 905 729.9, published application WO92/02839), which concerns a multicolour confocal fluorescence microscopy system. In the decision under appeal, reference was made, amongst others, to the following documents:

E1 K Mossberg et al., J. Microscopy, Vol. 158, Pt. 2, 215-224 (1990), "Detection of doubly stained fluorescent specimens using confocal microscopy."

E2 US-A-5 161 053

E4 R DeBiasio, J. Cell Biol., vol. 105, 1613-1622 (1987)

E5 M R Loken, Cytometry, vol. 1, No. 2, 136-142 (1980), "Simultaneous Quantitation…."

E9 R H Webb et al., Applied Optics., Vol. 19, No. 17, 2991-2997 (1980), "Flying spot TV opthalmoscope."

II. In the decision under appeal, the opposition division focused mainly on prior art document E1, explaining that it saw two systems disclosed, which it denoted as E1.1 and E1.2. The division considered the subject matter of claim 1 as granted to differ from system E1.1 by virtue of use of three rather than two laser excitation lines, and simultaneous rather than consecutive operation. The first difference was only of minor significance, reference being made to table 2 of document E4 and column 2, line 54 of document E2. The provision of fully spectroscopically separated and simultaneously operated excitation and detection channels can be regarded as the teaching of page 216,

penultimate paragraph; page 217, second paragraph; page 217 penultimate paragraph; page 216, last paragraph and the second half of the third paragraph on page 223 of document E1. Thus the subject matter of claim 1 as granted could not be regarded as involving an inventive step. Obviousness was further supported by the fluorescence flow cytometer of document E5. For analogous reasons, the method of claim 12 also does not involve an inventive step. Document E9 was not admitted by the opposition division, since it was not considered more relevant than other document involved in the procedure.

III. The appellant requested that the appeal board set the decision under appeal aside and that the patent be maintained as granted or, in the alternative, on the basis of claims according to auxiliary request 1 to 8 filed with its letter of 14 December 2004. The respondent (=opponent) requested the appeal board to dismiss the appeal. Consequent to requests for oral proceedings on an auxiliary basis from both parties, such proceedings were appointed by the board. In an annex attached to the summons to the oral proceedings, the board observed that application of the problem solution approach to inventive step may be appropriate in the present case.

IV. In support of its position, the appellant submitted that the objective problem is to perform triple staining of a specimen, which stains fluoresce when excited by light of particular frequencies, without undesired system movement. The inventive idea is choosing wavelengths from a single laser for excitation, while preventing overlapping of excitation and emission

in simultaneous operation. With respect to document E1, the microscope system according to E1.1 can be considered to be the closest prior art. Two excitation wavelengths are used after one another, the splitter and filter being exchanged. Mechanical alignment problems associated with the microscope system can be overcome by comparing characteristics of the pictures produced and use of a better quality splitter. With respect to microscope system E1.2, use is made of two detectors that simultaneously can detect two different wavelength intervals, a single line stimulating two fluorescences. In the patent, there are three wavelengths with a separation around 80nm and simultaneous emission. There are three dyes, sufficiently separated and no movement. Microscope system E1.2 meets the movement problem, yet not for three excitations as in the patent in dispute. The problem of cross-talk is recognised for the E1.2 microscope system, but different frequencies are explicitly excluded in strong terms, i.e. "cannot be used". Using different wavelengths as in the E1.1 system cannot be suggested because the way back is closed as it is E1.2 which is designated as the new version, so going backwards would have been track of the teaching of the document. Even this, in the light of document E1, misguided step would not provide the claimed subject matter as only one emission line is used in both of the alternative cases.

In the decision under appeal, no objective problem was formulated with respect to the closest prior art, which can be taken as document E1. Following the problem solution approach to inventive step, there is no reason for the skilled person to see a solution for the

objective technical problem in the teachings of any of the teachings of documents E2, E4, E5 or E9 or to combine these teachings with that of document E1. An Ar-Kr laser is briefly mentioned in document E2, but with no configuration details. Document E4 uses a lamp as light source. Document E5 concerns a flow cytometer where movement of cell populations takes place intentionally. Towards the end, document E9 suggest a Ar-Kr laser source will provide three simultaneous wavelengths in the context of producing a colour display system, not in relation to a plurality of stains. Wavelengths mentioned with respect to the flying spot opthalmoscope with which the document is concerned are 502, 514 and 568 nm. The appellant therefore concluded that the subject matter of independent claims 1 and 12 as granted involved an inventive step.

V. According to the respondent one should first note that, in the present case, the skilled person should be considered to be a higher degree physicist with several years experience of confocal microscopy, who accordingly reads the prior art with specialist knowledge. Document E1 should be read as a single document, in particular the prologue down to and including the penultimate paragraph on page 216 applies to both embodiment E1.1 and E1.2 as defined by the opposition division. Thus document E1 is basically concerned with confocal multicolour microscopy, where a specimen is marked with different fluorescent dyes, four being suggested for possible combination in the introduction. Therefore the possibility of using two or more dyes is disclosed, no reason being apparent as to why colouring should be limited to two dyes (page 215,

second paragraph of the introduction). The penultimate paragraph on page 216 discloses the concrete case of a specimen with two dyes excited by different wavelengths. In the alternative version of PHOIBOS (E1.2), a specimen is illuminated with light of one wavelength, two detectors detecting two different fluorescent emissions. In the second variant only one scan per probe is necessary. A pulse is mentioned, i.e. a laser is suggested. Small movements between scans, entailing complex alignment, is recognised as a problem with the first version E1.1, as is mitigating this problem by choice of beam splitter and aligning resulting images on the basis of there features. At all events, the skilled person realises that technical complexity is involved in meeting the movement problem. A way of overcoming this problem is explained on page 223 as detecting a number of dyes, in this case two, excited by a single wavelength at the same time. A resulting further problem is explained to the skilled person as the emitted light not being as easily separable as in the case of the E1.1 system, i.e. there is cross-talk. One solution is to mitigate cross-talk with software. However, the skilled person knows that a problem can either be solved by mitigation of effects or removal of the source of the problem. Emission behaviour depends not only on the dyes but also interaction with the sample and is thus not so easy to counter by software means. The preceding system E1.1 shows how to remove the problem, i.e. without introducing any disadvantage to use different frequencies. Therefore the problem clearly presented to the skilled person by the teaching of document E1 is, while avoiding problems produced by movement between scans, to excite several, for example three, fluorescent dyes, avoiding cross talk. It is

clear from document E1 for the skilled person, that simultaneous illumination has to be used for avoiding the movement problem and different wavelengths to avoids cross-talk.

The problem would therefore have been to look for a suitable light source in an obvious way, where the knowledge of the skilled person is illustrated for example in Table 2 of document E4. An Ar-Kr laser was known at the priority date of the patent, see column 6, line 54 of document E2 relating to a confocal microscope, and was an obvious choice as source meeting the desiderata of small spectral overlapping with simultaneous illumination. The knowledge of the skilled person is also illustrated by publications such as document E9, relating to an opthalmoscope but disclosing an Ar-Kr laser source which is suggested to provide three simultaneous beams. Thus, as it is obvious to provide the Ar-Kr source to solve the problem of document E1, there can be no inventive step in the subject matter of the independent claims.

VI. The independent claims according to the main request of the appellant are worded as follows.

"1. A multi-color confocal microscopy system for use with an optical microscope in viewing a specimen stained with a plurality of stains, which stains fluoresce when excited by light of particular light frequencies, comprising:
a single laser means (601) for producing multi-line incident laser light having multiple excitation lines from a single laser light source at wavelengths of 488nm, 568nm and 647nm, each of said excitation lines

corresponding to an excitation frequency of only one of the plurality of stains;
means (613, 913) for directing said incident laser light into the microscope and for receiving emitted light from the microscope said emitted light having simultaneous multiple fluorescent emissions each of said fluorescent emissions corresponding to an excitation frequency of one of the stains and each excitation frequency of each stain corresponds to a single excitation line from the single laser;
detector means (615, 915, 921, 923) positioned to receive said emitted light for converting said emitted light into electrical signals; and
control means (620) connected to said detector means for accumulating said electrical signals and for producing a plurality of images of the specimen at a precise focal plane, each of images corresponding to one of said lines of said laser.

12. A method of viewing a specimen stained with a plurality of stains, which stains fluoresce when excited by light of particular frequencies, comprising the steps of:
producing multi-line incident laser light from a single laser having multiple excitation lines from a single laser light source at wavelengths of 488nm, 568nm and 647nm, each of said excitation lines corresponding to single
excitation frequency of one of the stains;
directing said incident laser light into a microscope holding the specimen;
receiving emitted light from the microscope, said emitted light having multiple fluorescent emissions,

each of said fluorescent emissions corresponding to a single excitation frequency of one of the stains: detecting said emitted light and converting said emitted light into electrical signals; and accumulating said electrical signals and producing a plurality of images of the specimen at a precise focal plane, each of said images corresponding to one of said lines of said laser."

The wording of the independent claims according to the auxiliary requests is not given since no decision is taken on these requests (see section 5 of the Reasons below).

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

Reasons for the Decision

1. The appeal complies with the provisions referred to in Rule 65(1) EPC and is therefore admissible.

2. The closest prior art can be taken to be disclosed in document E1. Wording of paragraphs of document E1 included amongst those upon which the proceedings have focused is given in sections 2.1 to 2.6 below. The first and second sentences in section 2.4 concern the confocal microscope which has been referred to in the proceedings as E1.1 and E1.2, respectively.

2.1 (first and second sentence of introduction, page 215) "Double staining is a commonly used technique in fluorescence microscopy. In this paper we will present

the experiences we have acquired from studying doubly stained fluorescent specimens with confocal laser microscopy."

2.2 (last paragraph, page 215) "In some of our projects in co-operation with physicians and biologists, the study of specimens stained with two fluorescent dyes has been of interest. Here, examples are given mainly from three applications: 3-D reconstructions of different types of neurons (Brodin et al., 1988), co-localization of signal substances in axon terminals (Mossberg et al., 1990) and visualization of the distribution of a hormone related to different cytoskeletons in human fibroblasts. Four fluorophores were used: Lucifer Yellow (LY), Texas Red (TR), fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC). They were studied in the combinations FITC— TRITC, FITC—TR and LY—TRITC, Their absorption and emission spectra are discussed. The labelling of the specimens and the chemical properties of the fluorophores are only briefly mentioned since they are not major topics in the present studies."

2.3 (page 216, penultimate paragraph) "The differences in absorption and emission spectra make it possible to distinguish two or more fluorophores in the same preparation. The method of multiple staining has proved useful in biological and medical applications, for example where coexistence of substances or contact surfaces between cells are of interest. If the absorption and/or emission spectra for the fluorophores are sufficiently separated, it is possible to register them separately using different excitation wavelengths and filter settings."

2.4 (paragraph bridging pages 216, 217 and following paragraph) "To separate the stains with PHOIBOS, the specimens are scanned twice, changing filter settings and excitation wavelength in between the scans. There is a new version of PHOIBOS, using two detectors that simultaneously can detect two different wavelength intervals. With this instrument only one scan of each specimen is required, but on the other hand different excitation wavelengths for the fluorophores cannot be used. This paper focuses on the results with the single detector instrument. The excitation wavelength is chosen so that as little as possible of the unwanted fluorescence is excited at the same time as the relevant fluorescence. Since the absorption spectra of the fluorophores always overlap to some extent, it is difficult to suppress completely the fluorescence from one of the stains. However, the emission spectra of the stains also differ and the fluorescent light from the two fluorophores can be separated by the optical filters in front of the PM tube. These filters can either be single high-pass filters or high-pass filters combined with low-pass filters. Total separation is often difficult to obtain and the result also strongly depends on the relative strength of the stains."

2.5 (page 217, penultimate paragraph) "In the applications where the specimens were embedded in a fluid medium, slight movements were observed between the recordings. This is possibly due to the very short working-distance of the objective, which might let the objective touch the cover glass. A solid embedding of the substances would be preferable, but is not always possible. To compensate for such movements, the images recorded for

two different fluorophores sometimes must be shifted in relation to each other until they are aligned. This can be done easily, if there are some characteristic parts of the specimen which can be recognized in both images. Another cause of the translations between two images may be the change of beam-splitter. This effect, which is of the order of 0.5 µm using the 100x objective, can probably be reduced by selecting a beam-splitter of better quality."

2.6 (penultimate paragraph, page 223) "The changes of the laser wavelength and the filters were made manually and two scans of each specimen were performed. An improvement would be to scan every pixel or row of pixels twice, automatically changing excitation wavelength and filters in between. This would make the scanning procedure faster and also eliminate the risk that the specimen moves between the scans. The version of PHOIBOS that simultaneously can detect light in two wavelength bands overcomes the problem with undesired movements. Since the excitation wavelength is the same for the two stains, the signals from the fluorophores cannot be separated as well as in the above mentioned method. However, if the magnitude of the cross-talk is known, the separated images can be reconstructed with software calculations."

3. The subject matter of claim 1 in dispute distinguishes from the disclosure of document E1 at least by virtue of a single laser means for producing multi-line incident laser at wavelengths of 488nm, 568nm and 647nm, each of said excitation lines corresponding to an excitation frequency of one of the plurality of stains, received emitted light having multiple fluorescent

emissions, each excitation frequency of each stain corresponding to a single excitation line.

4. In view of the wavelengths and their separation chosen, the problem solved by the novel subject matter is that of providing a confocal microscopy system with multiple staining without problems with alignment or cross-talk.

4.1 The solution offered is not provided in the other documents in the prior art for the reasons given in section 4.4 below. Thus the board is satisfied that the problem solution approach usually applied in considering inventive step leads to a positive conclusion in the present case.

4.2 In the approach adopted by the respondent, the problem to be solved was simplified down to picking a source for simultaneous illumination at different wavelengths. However, this approach diverges from the problem solution approach by relying on the level of knowledge of the skilled person to mean that it is permissible to pick out features from the document out of context to amplify its teaching to fit the claims in dispute in a hindsight driven way. For example in the teaching of document E1, the microscope system E1.2 is, at first, referred to as "a new version" (see point 2.4, above in the middle). In its argumentation, the respondent focuses on E1.1 and E1.2 as alternatives or variants, which in the view of the board is an incomplete designation not considering this temporal factor. The board does not consider that the skilled person, in the light of this temporal consideration, would glean a hint to take a step backwards and pick part of the teaching relating to microscope system E1.1 to modify

the newer E1.2 version. A very explicit statement in document E1 reinforces the unlikely nature of taking this backward step, namely with reference to microscope system E1.2 that different excitation wavelengths for the fluorophores cannot be used (see section 2.4 above). Moreover, document E1 is essentially concerned with doubly stained fluorescent specimens (see section 2.1 above). While four fluorophores are mentioned and "two or more" and "multiple staining" are mentioned in section 2.3 above, actual use was in combinations of two fluorophores at a time (see the combinations mentioned in section 2.2 above). For the E1.1 system there were two scans and for the E1.2 system two detectors. Thus the general approach of the respondent that because there is no reason given why more than two fluorophores should not be used, either the E1.1 or E1.2 version should be modified towards multiple staining in the sense of the present claims is not persuasive.

4.3 The board reached the view that, while document E1 begins with general discussion passages, once the E1.1 and E1.2 systems are reached, the teaching takes a more specific system related line. Both disclosed systems have problems (movement and cross-talk, respectively) and document E1 suggests how for each system these can be overcome, for E1.1 automatically changing excitation wavelength and filters between pixels or rows, selecting the beam splitter or avoiding objective contact, and for system E1.2 using software. The board considers the skilled person reading document E1 would simply have followed these suggestions. In order to adapt the teaching to its case by rejecting unhelpful teaching relating to E1.2, the respondent argued that

the skilled person knows emission behaviour is not so easy to counter by software means. This approach uses hindsight in the view of the board as no such suggestion is present in document E1. Hindsight is again involved in the next step of the respondent's approach, namely that rather than using software, the source of the problem should be removed by using different excitation wavelengths as in the E1.1 system, because taking this step overlooks it being explicitly ruled out by the "cannot be used" terminology of document E1.

4.4 According to the respondent, reaching the particular laser claimed results from the skilled person looking around for an available and suitable simultaneous multi-wavelength light source to follow the suggestion of and avoid the problems known from document E1. The respondent found candidates in, for example, documents E2, E5 and E9. However, in the view of the board, not only do the disclosures concerned not exactly meet the wording of the claims, but they have also only been selected in an attempt to do this with impermissible hindsight knowledge of the invention as they do not contain teaching relating to the real objective technical problem to be solved in relation to document E1. For example, while document E2 relates to a confocal microscope, an Ar-Kr laser is only one of numerous lasers referred to, not to mention references to incandescent sources such as tungsten filament, halogen, xenon discharge and so on. There is no mention of multiple specimen staining. It cannot therefore be obvious to pick the claimed configuration. Document E5 pertains to an application using a flow cytometer where, on an intentional basis, moving cells are analysed, the

source operating across a band in a multi-wavelength mode with a two colour detection system. Document E9 also concerns a different application, namely an opthalmoscope with an Ar-Kr laser light source most commonly used at 502, 514 and 568 nm. There is a suggestion towards using three beams at appropriate wavelengths simultaneously, but this is in the context of colour display. Another document mentioned in the proceedings was document E4, which is only of background interest in showing a number of fluorophores. Accordingly, none of these documents can be considered to solve the objective technical problem in the light of the teaching of document E1. It is necessary to use hindsight to select features from the documents in an attempt to reach the claimed subject matter. The board was not therefore persuaded by the line taken by the respondent.

4.5 The board accordingly reached the conclusion that the subject mater of claim 1 can be considered to involve an inventive step within the meaning of Article 56 EPC. A similar conclusion applies to corresponding method claim 12.

5. Since the board was satisfied as to patentability of the subject matter of the independent claims of the main request, it was not necessary to consider the auxiliary requests of the appellant and submissions of the parties relating thereto.

Order

For these reasons it is decided that:

1. The decision under appeal is set aside.

2. The patent is maintained unamended.

The Registrar: The Chairman:

P. Martorana A. G. Klein