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**Datasheet for the decision
of 21 December 2023**

Case Number: T 0302/19 - 3.5.06

Application Number: 11733297.3

Publication Number: 2524338

IPC: G06K9/00

Language of the proceedings: EN

Title of invention:

CELL CHARACTERIZATION USING MULTIPLE FOCUS PLANES

Applicant:

Bio-Rad Laboratories, Inc.

Headword:

Cell characterization/BIO-RAD

Relevant legal provisions:

EPC Art. 56, 111(1), 113(1)

EPC R. 103(1) (a)

RPBA 2020 Art. 11, 13(2)

Keyword:

Inventive step - automation of a known manual practice (no)

Remittal for further prosecution (yes)

Catchword:

For an argument that a claimed method is a straightforward automation of a known manual practice of a laboratory assistant, it should be clear what is the alleged manual practice, it should be convincing that it was indeed an existing practice at the relevant date and that it would have been obvious to consider automating it (see point 20 et seq. of the reasons).



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Case Number: T 0302/19 - 3.5.06

D E C I S I O N
of Technical Board of Appeal 3.5.06
of 21 December 2023

Appellant: Bio-Rad Laboratories, Inc.
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Representative: dompatent von Kreisler Selting Werner -
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Decision under appeal: **Decision of the Examining Division of the
European Patent Office posted on 29 August 2018
refusing European patent application No.
11733297.3 pursuant to Article 97(2) EPC.**

Composition of the Board:

Chairman M. Müller
Members: M. Domingo Vecchioni
B. Müller

Summary of Facts and Submissions

- I. The appeal is against the decision of the examining division to refuse the European patent application, which was issued in written proceedings after a single communication pursuant to Article 94(3) EPC.
- II. The examining division refused the application on the basis that independent claims 1 and 7 of the sole request then on file did not meet the requirements of Article 56 EPC.
- III. The contested decision cites the following documents:
- D1: US 2004/101912 A1,
D2: WO 03/095986 A1,
D3: WO 97/20198 A2,
D4: WO 2011/066837 A1,
D5: H. Harms et al., "Comparison of digital focus criteria for a TV microscope system", *Cytometry*, vol. 5, no. 3, May 1984, pages 236-243, XP055110858,
D6: D.M. Prescott, *Methods in Cell Biology*, vol. XIII, Academic Press, 1976, pages 55-56, XP002722905,
D7: WO 2006/055413 A2,
D8: P. Roux et al., "Focusing light on infection in four dimensions", *Cellular Microscopy*, vol. 6, no. 4, 23 February 2004, pages 333-343, XP055284171,
D9: A. Sacan et al., "CellTrack: an open-source software for cell tracking and motility analysis", *Bioinformatics*, vol. 24, no. 14, 29 May 2008, pages 1647-1649, XP055284324,

but D4 and D7 are not referred to in the reasoning of the examining division.

IV. With the statement of grounds of appeal, the appellant requested that the decision of the examining division be set aside and a patent be granted on the basis of a newly filed main request filed.

V. With the summons to oral proceedings, the board presented its preliminary opinion on the appeal. The claims appeared to be neither clear nor supported by the description, Article 84 EPC, for a number of reasons. As far as inventive step could be discussed under these circumstances, the board noted that it tended to not agree with the assessment by the examining division. In case the objections under Article 84 EPC could be overcome, the board would be minded to remit the case to the first instance for further prosecution as an additional search might be required. The board cited the following further documents:

D10: W. Strober, "Trypan blue exclusion test of cell viability", in: Current Protocols in Immunology, John Wiley & Sons, Inc., 1997, appendix 3B, XP55034746,

D11: K.S. Louis et al., "Cell viability analysis using trypan blue: Manual and automated methods", in: M.J. Stoddart (ed.), Mammalian Cell Viability: Methods and Protocols, Methods in Molecular Biology, vol. 740, 2011, pages 7-12, XP9185447.

The board also noted that the appellant did not appear to have been given, in the first-instance proceedings, an opportunity to comment on an essential element of

the examining division's reasoning. The appellant's right to be heard, Article 113(1) EPC, appeared thus to have been infringed.

- VI. In a letter dated 10 October 2023, the appellant filed an amended set of claims of a new sole request aimed at overcoming the objections under Article 84 EPC. In favour of admittance of the request, the appellant noted that these objections had been raised for the first time in the board's communication. If the case was to be remitted to the examining division for further prosecution, it agreed that the oral proceedings could be cancelled.
- VII. In a communication dated 6 November 2023, the board pointed out remaining issues under Article 84 EPC.
- VIII. On 9 November 2023, the appellant filed an amended set of claims to replace any previous set of claims.
- IX. The oral proceedings were thereupon cancelled.
- X. Independent claim 1 of the sole request on file (filed on 9 November 2023) reads:

"A method of characterizing cells, the method comprising:
tagging cells in a sample with Trypan blue dye such that live cells have a different appearance than dead cells;
forming, using a camera, a series of digital images of the sample containing the cells, each digital image taken at a different focus plane in relation to the sample;
automatically identifying, using a specially programmed computer, a digital image of the series of digital

images taken at a plane of best focus, wherein identifying the digital image taken at a plane of best focus comprises evaluating a contrast metric for each of the digital images to identify the digital image having the highest measured contrast; automatically identifying, using a specially programmed computer, a cell in the image taken at the plane of best focus; and automatically analyzing, using a specially programmed computer, at least the digital image taken at the plane of best focus to characterize the cell either live or dead, by performing the following steps:

- determining whether the cell can be classified as live on the basis of the digital image taken at the plane of best focus; if the cell can be classified as live on the basis of this digital image, characterizing the cell as live;
- if the cell cannot be classified as live on the basis of this digital image, locating the cell in a second digital image of the series of digital images, taken at another focus location, and determining whether the cell can be classified as live on the basis of the second digital image; if the cell can be classified as live on the basis of the second digital image, characterizing the cell as live; otherwise repeating the locating and determining steps on the basis of further digital images of the series of digital images, taken at other focus locations, until either the cell can be classified as live on the basis of any of the further digital images, and then characterizing the cell as dead;

wherein determining whether the cell can be classified as live on the basis of a digital image comprises comparing a ratio of a number of pixels significantly lighter than a background value to a number of pixels significantly darker than the background value to a

predetermined threshold;
and wherein locating the cell in the second or a further digital image comprises generating a respective score for each object found in a region of the second or the further digital image corresponding to a region containing the cell in the previously analyzed digital image, each score indicating a likelihood that the respective object is the cell; and selecting as the cell the object having the highest score."

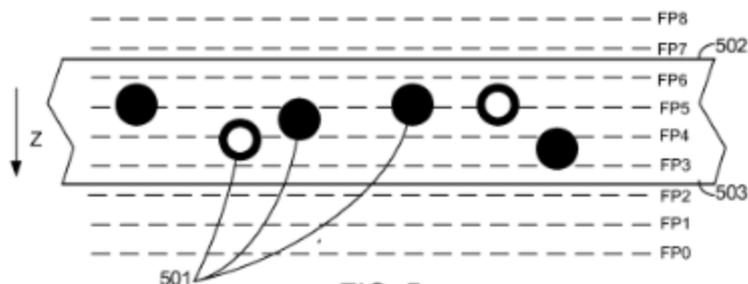
Independent claim 5 is directed to a "system for characterizing cells in a sample" formulated in closely corresponding language.

Reasons for the Decision

The application

1. The application is related to the technical field of cytometry, concerned with counting and characterizing biological cells. More particularly, it relates to an automated image-based method for determining whether particular cells in a sample are live or dead (page 1, lines 12-25, of the published application).
2. The method is based on the trypan blue dye exclusion test. When the sample containing the cells is treated with trypan blue dye, the dye is readily absorbed by dead cells, but live cell membranes tend to prevent absorption of the dye. The live cells should therefore appear to have lighter centers than dead cells. See page 6, lines 23-28, and figure 4.
3. A sample slide carries a sample of fluid in which cells are suspended. The sample is constrained between transparent plates (502, 503) so that the cells (501)

are within a narrow range of vertical positions (page 4, lines 14-17). See figure 5:



4. A series (stack) of digital images of the sample, treated with trypan blue dye, are taken at different focus planes (FP0, FP1, ...), from a focus plane where the sample is out-of-focus to a focus plane beyond the "plane of best focus". See page 6, line 29 to page 7, line 14, and figure 5.
5. For each digital image, a "contrast metric" is computed. The digital image with the highest measured contrast is designated as having been taken at the "plane of best focus". See page 7, lines 15-22.
6. Identifying individual cells in the sample is done using "known digital image processing techniques" (an example is provided) on the basis of the digital image taken at that plane of best focus. According to the description, "the most accurate counting of cells can be achieved using this digital image". A list of identified cells and their locations is stored. See page 7, line 21 to page 8, line 8.
7. For each identified cell, it is then determined whether it can be identified as a live or a dead cell.

On the basis of a given digital image, a cell may, for

instance, be classified as (apparently) live or dead based on whether the ratio of the number of pixels of the cell that are significantly lighter than the background to the number of those that are significantly darker exceeds a predetermined threshold. See page 8, lines 12-15.

8. For any given cell, it is first determined whether the cell may be classified as live on the basis of the digital image taken at the plane of best focus.

If so, the cell is identified as a live cell and the next cell is processed.

Else, a second digital image in the stack is considered. The cell is located in that second digital image and it is determined whether the cell may be classified as live on the basis of that second digital image. If so, the cell is identified as a live cell and the next cell is processed. Else, this process is repeated for the cell on the basis of other digital images in the stack until the cell is classified as live on the basis of one of the digital images or a stopping condition is met (e.g. all digital images or a predetermined maximum number of digital images have been considered), in which case the cell is identified as a dead cell. In both cases, the next cell is then processed. See page 9, lines 7-20.

In an embodiment, only the digital image taken at the plane of best focus and at most the three images taken at the next locations farther from the camera are considered. See page 9, lines 16-18.

9. Figure 7 illustrates this iterative ("sequential") process for the cases of cells 701 and 702, which are

ultimately identified as live resp. dead, even though both appeared to be dead cells on the basis of the digital image at the plane of best focus, FP4 (see page 9, lines 21-30):

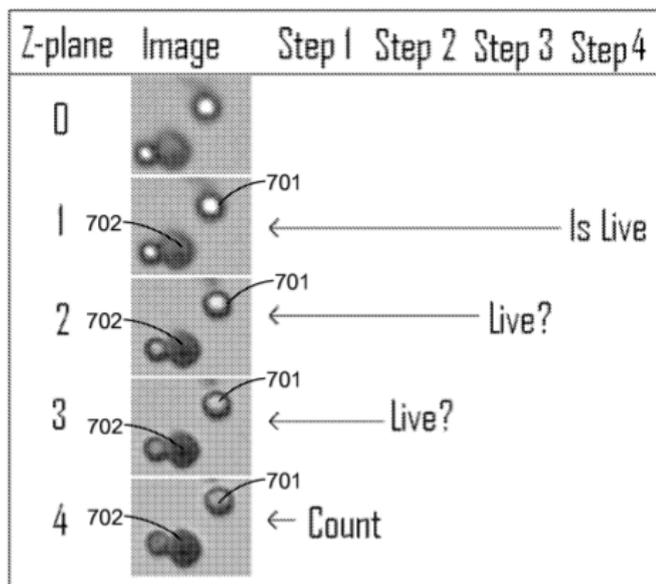


FIG. 7

10. What may be reported after having performed this analysis for all cells are statistics such as the concentration of live cells in the sample. See page 9, line 31 to page 10, line 4.

11. The proposed method is based on the following insights disclosed in the description:
 - the image taken at the "plane of best focus" is the best for counting cells (page 7, lines 21/22; page 8, lines 20-27, and figure 6);

 - it may however not be the best for identifying whether a given cell is live or dead (page 8, lines 15-17);

- while dead cells tend to appear as dead in all images of the stack, live cells may appear live in some images but dead in others (page 8, lines 17-19);

- errors in total cell count and in live cell count on the basis of a single image tend to be undercounting errors (page 8, lines 26/27 and 33/34);

- no single digital image is accurate for both total cell counting and live cell characterization (page 9, lines 1/2);

- no single image allows one to correctly reveal the live or dead status of the cells (page 9, lines 2/3).

12. The proposed method requires in one of its steps *locating* a cell identified in the first image (e.g. the one taken at the plane of best focus) in a second image (e.g. the next one in the stack). In an embodiment, "cell drift" is taken into account in the location step, i.e. that cells may move in the fluid during the taking of the stack of digital images.

A "region" centered around the location of a cell A in the first image is selected, large enough to accommodate for drift of cells within the region but small enough for computational efficiency. Each "object" within that region in the second image is evaluated and assigned a "score" that indicates a likelihood that the object is cell A. The score may be based on size, position and position of other objects in the region (details are provided on pages 10-12, with reference to figures 8A to 8F). The object having the highest score is taken to be cell A. See page 10, lines 21-26, and page 12, lines 25-28.

13. According to the description, the proposed method "improv[es] the accuracy of determining whether particular cells are live or dead" (page 1, lines 24/25, and page 9, lines 4-6). Further advantages are indicated from page 12, line 29 to page 13, line 3:

"Embodiments of the invention as described above exploit the multiple available images in a computationally efficient way to arrive at an accurate total cell count and an accurate characterization of the cells as live or dead. Only as many images as [...] needed are analyzed to characterize a particular cell. The methods also enable the use of low-cost, relatively low resolution imaging optics and sensors to perform accurate cell counting and characterization. Because multiple images [...] taken at different focus settings [are used], the system is also tolerant of tilt of the sample, field curvature, or other effects tending to cause nonuniformity of focus across the sample."

Admittance

14. The sole request on file was filed on 9 November 2023, hence just one day before the planned oral proceedings. However, it overcomes all the objections under Article 84 EPC that were raised for the first time in the board's preliminary opinion, without introducing new ones (see below), so that the oral proceedings could be cancelled. In view of these circumstances and because it is not detrimental to procedural economy, the board exercises its discretion under Article 13(2) RPBA in admitting this request.

Articles 84 and 123(2) EPC

15. In its preliminary opinion (see point 22), the board found that the claims that had been filed with the statement of grounds of appeal were neither clear nor supported by the description, Article 84 EPC.

This was, *inter alia*, because the expression "plane of best focus" was unclear, essential features of the invention regarding the use of Trypan blue dye and the iterative process were missing in the independent claims, and several inconsistencies existed between claims and description.

16. The board considers that the present set of claims overcomes all these objections, while being compliant with Article 123(2) EPC. In particular, the method now
- comprises a step of "tagging cells in a sample with Trypan blue dye such that live cells have different appearance than dead cells" (based on page 6, lines 23-28, of the original description),
 - specifies that "identifying the digital image taken at a plane of best focus comprises evaluating a contrast metric for each of the digital images to identify the digital image having the highest measured contrast" (based on page 7, lines 15-21), and
 - comprises steps defining the iterative process summarised at point 8 above (based on the passages of the description cited in that point).

17. Hence, the present set of claims is considered to comply with the requirements of Articles 84 and 123(2) EPC (as regards Article 84 EPC with the proviso that the description might still have to be adapted to the amended claims).

Inventive step

18. The examining division found that the then pending claim 1 lacked an inventive step, Article 56 EPC. The examining division's reasoning, as far as it is understood by the board, comprised the following elements.
- 18.1 The skilled person was a team comprising a person skilled in cytometry and a person skilled in computer-implemented image analysis.
- 18.2 It was common general knowledge that "finding the best focus between specimen and the microscope objective is essential" in microscope applications and that "many of the specimen's morphological characteristics depend upon contrast and edge transitions, which change with the focus setting", as evidenced by **D5**.
- 18.3 The examining division found that "[i]t was [...] known to stain a cell sample with trypan blue [or nigrosin] and to detect visually (by [a] laboratory assistant) viable cell[s] based on the effect that viable cells exclude the dye and hence have a clear/un-stained cytoplasm while non-viable cells include the dye in the cytoplasm which hence appears stained (blue in the case of trypan blue)" and that, as evidenced by **D6**, it was "furthermore known that non-viable cells and cells which are out-of-focus and viable cannot clearly be distinguished". The examining division called this the "ambiguity problem". All this was considered to be common general knowledge in view of the fact that D6 was a book published 1976 that had been often cited since then.
- 18.4 The examining division considered that "the task to automate a trypan blue or nigrosin cell viability assay

was an obvious task before the date of priority of the present application", as it had been known, as evidenced by **D1**, **D2** and **D3**, to "automate procedures in cytometry that were previously performed by laboratory assistants".

- 18.5 The skilled person would also have known "that the cells may change their position in different focus slices" as they are normally immersed in a liquid when being imaged using a microscope, as evidenced by **D8**. Solutions to the "ambiguity problem" trying "to assemble the slice images into one focused 2D (or 3D) image" were thus not possible or at least difficult.
- 18.6 According to the examining division, it would therefore "have been obvious to a skilled person implementing an automated process to identify a cell that cannot be classified in one slice, due to the ambiguity problem, in a different slice, and try to classify it there". This would "anyway directly correspond to what a laboratory assistance (or scientist) used to do when automatic method[s] were not available". A second image would have to be available for analysis whenever it was determined that a cell is ambiguous.
- 18.7 The claimed method differed from "such processing" merely by the last feature of (then) claim 1 relating to how the cell is located in the second image. This last feature was considered "to cover essentially any kind of tracking (in images)". Tracking of objects in images was common general knowledge. **D9** disclosed an application of tracking in the context of cytometry.
- 18.8 In point 3.4 of the contested decision, in response to an argument submitted by the appellant in its letter of 18 November 2016, the examining division noted that

"what has been claimed as steps are [...] results-to-be-achieved where the desired results relate directly to the manual practise".

The examining division held that, in its view, "a laboratory assistant trying to resolve the above mentioned ambiguity problem for a cell at one focus setting would change the focus setting and try to look at the same cell in a (slightly) different focus setting", "[a]fter changing the focus setting he/she would have to identify the same cell which he or she would do to identify the most similar cell in the close vicinity of the cell from the previous focus setting", so that "the laboratory assistant would in fact maximize a similarity score for cells in the vicinity of the previous location while identifying the matching cell".

Claim 1 of the then pending request was thus obvious to a skilled person in view of common general knowledge. The same applied, *mutatis mutandis*, to claim 7.

19. The appellant argued that the examining division's finding was "no longer applicable in light of the amendments of the independent claims", which "now properly reflect[ed] the gist of the invention", namely that (1) the focus plane that was best for counting cells was not necessarily the best for determining whether a cell is live or dead and that (2) this determination needed not be made at best focus for the individual cell (this being implicit, for example, from figures 6 and 7).

This was contrary to the conventional knowledge in the art and thus non-obvious. D5 and D6, in particular, would at best suggest that best focus was also best for cell viability determination and would thus not have

led the skilled person to the feature of claim 1 according to which the second image, on the basis of which the cell was classified as live, "was not taken at a plane of best focus".

20. The board does not follow the examining division's reasoning.

20.1 This reasoning amounts essentially to consider claim 1 as being a straightforward automation of a known manual practice of a laboratory assistant.

For such an argument to succeed, it should be clear what is the alleged manual practice, it should be convincing that it was indeed an existing practice at the relevant date and that it would have been obvious to consider automating it.

20.2 A clear description of the alleged manual practice - in particular of the concrete steps allegedly performed by a laboratory assistant - has, at best, been provided by the examining division in point 3.4 of the decision (see point 18.8 above).

20.3 It appears to be uncontested that the trypan blue dye exclusion test was the basis of a common manual practice for assessing the viability of cells in a sample at the relevant date.

The board is however not convinced, on the basis of the available evidence, that it was part of that practice, to determine the viability of any given cell by first attempting to determine it based on a first focus plane and, *if the cell appeared to be dead on the basis of*

that first focus plane, to try again based on a second focus plane.

- 20.4 D6 contains only pages 55 and 56 of what is volume XIII of a book series called "Methods in Cell Biology", published in 1976. The passage relied on by the examining division appears in the caption of Figure 16(B). It reads: "*A few damaged cells staining darkly with trypan blue can be seen (arrows). Cells that are out of focus may appear dark in the picture, but they have unstained nuclei and are structurally intact*". Page 55 refers only to Figure 16(A) and does not add anything of relevance to the matter at issue.

The board notes that the quoted sentences of D6 do not specify a *practice* - in terms of steps to be carried out - but an *insight* in relation to the trypan blue dye exclusion test: viable cells that are out of focus may appear as if they were non-viable. D6 does not disclose how this insight is or should be taken into account by a laboratory assistant when applying the trypan blue dye exclusion test to assess the viability of cells in a sample. It does in particular not teach that for any given dark cell the focus should be changed to see whether the cell is actually viable. The available excerpt of D6 does not mention any change of focus. Figures 16(A) and 16(B) differ in that they show the "initial cell suspension" and the "final cell suspension".

The board considers therefore that D6 does not establish the existence before the relevant date of a manual practice as described by the examining division.

21. The board refers to prior art document **D10** for a description of a protocol to manually carry out the trypan blue exclusion test of cell viability, included

in the book "Current Protocols in Immunology" (1997).

In step 4, the human operator is told to place the hemacytometer containing the sample comprising the cells mixed with trypan blue dye on the stage of a microscope and to "focus on the cells".

In step 5, the human operator is told to "count the unstained (viable) and stained (nonviable) cells separately in the hemacytometer".

It appears that "focus on the cells" in step 4 instructs the operator to find a single "best" focus setting in which most if not all cells would be in focus, and not a best focus for each individual cell (doing so manually would also be prohibitively time-expensive). There is in particular no disclosure of determining the viability of a given cell initially found non-viable by changing focus and reassessing viability.

22. Automating the manual practice described in D10 would have been an obvious aim.

It appears that a straightforward solution would have been to implement step 4 by determining the focus plane that is globally best, e.g. by a contrast-based auto-focus procedure. This would have involved taking a series of digital images at various focal planes, computing a contrast metric for each of them and selecting the best focal plane as that for which the contrast metric is highest.

An obvious implementation of step 5 would then have been to identify the cells and to classify them as viable or not viable by an analysis of the digital

image taken at that best focal plane.

The skilled person would thereby not have arrived at the invention.

23. The board considers that even consideration of the teaching of D6 in the course of devising an automated version of the manual practice described in D10 would not have led the skilled person to the invention.

The skilled person could have derived from D6 that the viability of each individual cell (to be determined according to step 5) may best be determined if it is ensured beforehand that the cell is in focus. This could have led the skilled person to consider auto-focusing on each individual cell prior to determining its viability. This would however not have led the skilled person to the iterative process of the invention as the classification of a given cell as live or dead would still only have been based on a single image.

24. The board also agrees with the appellant that no prior art on file suggests that classifying a cell as live or dead does not require the digital image on which the classification is based to have been taken at a focus plane that is "best" for the image as a whole or for the cell in particular.

The proposed iterative process exploits this insight to provide a more accurate yet computationally efficient approach to classifying a cell as live or dead in that it searches beyond the focus plane that is best for the whole image, yet does not require to find the focus plane that is best for the considered cell to be in focus.

This iterative approach may not have been properly reflected in claim 1 that was before the examining division, but now it is in present claim 1.

Remittal for further prosecution

25. It follows from the above that the appeal is allowable within the meaning of Article 111(1), first sentence, EPC, because the decision under appeal cannot stand. The board is however not in a position to remit the case to the first instance with an order to grant a patent on the basis of the present application documents.

This is due to the fact that the examining division did not cite any prior art *automated* method for determining cell viability based on the trypan blue dye exclusion test, even though it appears that prior art on such automated methods does exist, as the board noted when studying the background of the case.

Document **D11**, itself published only after the earliest claimed priority date, refers to several devices which are said to have implemented such automated methods and provides references to their manuals, all published before the earliest priority date. See paragraph bridging pages 7 and 8 and the corresponding bibliographical references.

Performing an additional search might thus be required before arriving at a conclusive assessment of inventive step.

26. The board considers this to justify remitting the case to the department of first instance for further

prosecution, Article 111(1), second sentence, EPC and Article 11 RPBA.

Reimbursement of the appeal fee

27. The board notes that a clear description of what the examining division considered to be the known manual practice to be automated appears to have been communicated to the appellant only with the decision under appeal (under point 3.4). The board considers that this description represents an essential element of the examining division's line of reasoning. By not having given the appellant an opportunity to comment on this essential element in the first-instance proceedings, the appellant's right to be heard, Article 113(1) EPC, has been infringed. This constitutes a substantial procedural violation, which makes full reimbursement of the appeal fee equitable.

The appeal being allowable, the appeal fee is to be reimbursed in full, Rule 103(1)(a) EPC.

Order

For these reasons it is decided that:

1. The appealed decision is set aside.
2. The case is remitted to the first instance for further prosecution.
3. The appeal fee is to be reimbursed.

The Registrar:

The Chairman:



L. Stridde

Martin Müller

Decision electronically authenticated