UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

FLUIDIGM CORP.,
Petitioner,

v.

THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIV.,

Patent Owner.

Case IPR2017-00013
Patent 7,563,584

PETITION FOR *INTER PARTES* REVIEW OF U.S. PATENT NO. 7,563,584 UNDER TO 35 U.S.C. §§ 311 *ET SEQ.* AND 37 C.F.R. § 42.100 *ET SEQ.*

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I. Introduction

The Real Party in Interest, Fluidigm Corp. (hereinafter "Petitioner"), hereby respectfully requests *Inter Partes* Review pursuant to 35 U.S.C. §§ 311 *et seq.* and 37 C.F.R. §§ 42.100 *et seq*., of claims 1-27 of U.S. Patent No. 7,563,584 ("the '584 patent") filed July 10, 2002 to Perez *et al. See* Exhibit 1001.

On its face, the '584 patent acknowledges that each limitation recited in claims 1-27 utilizes well-known techniques and commercially available reagents and detection devices. This includes activation state-specific antibodies specific for selected proteins (Ex. 1001 at 28:8-62), distinguishable labels (*id*. at 31:10-32:59), antibody conjugation procedures and kits (*id*. at 25:35-61 and 35:9-12), and devices to detect distinguishably labeled state-specific antibodies (*id*. at 35:67-36:9 and 37:16-53). That all of these techniques and technologies were well known prior to the time of the invention is further confirmed by the declaration of Dr. Tom Huxford. Ex. 1002 at ¶¶14-35 (describing the state of technology at the time of the invention).

Thus, the '584 patent does not purport to have invented any new antibody, protein, antibody-protein conjugation technique, antibody labeling technique, or detection apparatus. Rather, at best, the '584 patent purports to be the first to use these well-known techniques and commercially available reagents and devices to

provide "an approach for the simultaneous determination of the activation states of a plurality of proteins in single cells." Ex. 1001 at 4:27-29.[1]

However, the Darzynkiewicz and Fleisher references relied on in this Petition disclose determination of the activation state of at least one protein in a cell using labeled antibodies detected via flow cytometry prior to the '584 patent. *See e.g.*, Ex 1004 and 1005. In addition, each of these references provides explicit teachings to apply their methodology to the detection of the activation state of a plurality of proteins using different labeled antibodies. Ex. 1005 at 12:50-59 and Ex. 1004 at 429.

By the time of the '584 patent, one of skill in the art would have been knowledgeable about the numerous commercially available activation state-specific antibodies (as admitted to be known in the '548 patent) that could be used to detect a plurality of activatable proteins of interest using the methods of Darzynkiewicz or Fleisher. Ex. 1002 at ¶¶22-24; Ex. 1001 at 28:8-62.

Therefore, the prior art renders obvious the claims of the '584 patent as one of ordinary skill in the art would be motivated to apply the teachings of Fleisher and Darzynkiewicz to detect the activation state of a plurality of proteins. Accordingly, Petitioner respectfully requests that claims 1-27 of the '584 patent be

[1] The claims do not recite any requirement for a "simultaneous" determination.

canceled based on the grounds of unpatentability set forth below. 35 U.S.C. § 314(a).

II. MANDATORY NOTICES UNDER 37 C.F.R. § 42.8(a)(1)

Petitioner satisfies each requirement for *Inter Partes* Review of the '584 patent pursuant to 37 C.F.R. § 42.8(a)(1).

A. Real Party In Interest Under 37 C.F.R. § 42.8(b)(1)

The Real Party in Interest is Fluidigm Corp., 7000 Shoreline Court, Suite 100, South San Francisco, CA 94080.

B. Related Matters Under 37 C.F.R. § 42.8(b)(2)

Petitioner is not aware of any judicial proceeding involving the '584 patent. Based on the USPTO's Public PAIR website, pending administrative proceedings related to the '584 patent include Application Nos. 14/619,997 and 14/802,366.

C. Lead and Back-Up Counsel Under 37 C.F.R. § 42.8(b)(3)

Petitioner is represented by the following counsel:

Lead Counsel	Backup Counsel
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Pursuant to 37 C.F.R. § 42.10(b), a Power of Attorney has been filed with this Petition.

D. Service Information Under 37 C.F.R. § 42.8(b)(4)

Service information for lead and back-up counsel is as follows:

James P. Murphy
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Petitioner also consents to service by e-mail at the above e-mail addresses provided for lead and back-up counsel.

III. GROUNDS FOR STANDING UNDER 37 C.F.R. § 42.104(a)

Petitioner certifies that the '584 patent is available for *inter partes* review. Petitioner is not barred or estopped from requesting an *inter partes* review of the '584 patent claims on the grounds identified in this Petition. 37 C.F.R. § 42.104(a).

IV. IDENTIFICATION OF GROUNDS FOR WHICH REVIEW IS REQUESTED UNDER 37 C.F.R. § 42.104(b)(1)

Petitioner asserts that claims 1-27 of the '584 patent are unpatentable based on the following grounds:

Ground 1: Claims 1, 2, 4-6, 10-21, and 23-27 are rendered obvious under 35 U.S.C. § 103 by Fleisher;

Ground 2: Claims 3, 7-9 and 22 are rendered obvious under 35 U.S.C. § 103 by Fleisher in view of Belloc;

Ground 3: Claims 1, 2, 4-6, 10-21, and 23-27 are rendered obvious under 35 U.S.C. § 103 by Darzynkiewicz in view of Yen;

Ground 4: Claims 3, 7-9 and 22 are rendered obvious under 35 U.S.C. § 103 by Darzynkiewicz in view of Yen and Belloc.

While some of the above grounds cover the same claims the technical teachings between the different grounds are not cumulative.

Fleisher discloses detection of activated STAT-1 using an activation state specific antibody along with teaching to use the disclosed methodology to detect the activation state of other intracellular proteins including Jak, NFκB, or MAPK. Darzynkiewicz in view of Yen teach a flow cytometric method to concurrently detect the activation state of proteins that regulate the cell cycle such as RB, ERK 1/2 and MEK 1/2.

Thus, the different grounds all render obvious the claims, but they do so based on different and non-cumulative teachings.

V. HOW THE CHALLENGED CLAIMS ARE TO BE CONSTRUED UNDER 37 C.F.R. § 42.104(b)(3)

In an IPR claim terms are to be given their broadest reasonable construction consistent with the specification. 37 C.F.R. § 42.100(b). Here, the specification of the '584 Patent defines certain terms used in the claims as follows:

A. Protein

The '584 patent states "[a]s used herein, the terms 'polypeptide' and 'protein' may be used interchangeably and mean at least two covalently attached amino acids, which includes proteins, polypeptides, oligopeptides and peptides. The protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures." Ex. 1001 at 26:8-13.

Therefore, the broadest reasonable construction of "protein" in light of the '584 patent should be "at least two covalently attached amino acids, which includes proteins, polypeptides, oligopeptides and peptides." *id*.; *see also* Ex. 1002 at ¶¶45-50.

B. Activatable Protein

The '584 patent states:

> As used herein, an "activatable protein" or "substrate" or "substrate protein" or "protein substrate" grammatical equivalents thereof, refers a protein that has at least one isoform (and in some cases two or more isoforms) that corresponds to a specific form of the protein having a particular biological, biochemical, or physical property, e.g., an enzymatic activity, a modification (e.g., post-translational modification), or a conformation.

Ex. 1001 at 26:24-44. In addition, claims 19 and 20 provides specific examples of "activatable protein[s]" that are within the scope of claim 1. Ex. 1001 at 152:35-48.

Therefore, the broadest reasonable construction of "activatable protein" in light of the '584 patent is "a protein that has at least one isoform that corresponds to a specific form of the protein having a particular biological, biochemical, or physical property." *id*. at 26:24-44; *see also* Ex. 1002 at ¶¶51-53.

C. Activation state-specific antibody

The '584 patent states "[a]s used herein, the term 'activation state-specific antibody' or 'activation state antibody' or grammatical equivalents thereof, refer to an antibody that specifically binds to a corresponding and specific antigen." Ex. 1001 at 25: 21-24. Therefore, the broadest reasonable construction of "activation state-specific antibody" in light of the '584 Patent is "an antibody that specifically binds to a corresponding and specific antigen." *See* Ex. 1002 at ¶¶54-56. In addition, claim 21 provides specific examples of "activation state-specific antibod[ies]" that are within the scope of claim 1. Ex. 1001 at 152:49-153:3.

Petitioner notes that the definition provided in the '584 patent for "activation state-specific antibody" would encompass virtually any antibody, as all antibodies bind to a specific antigen. Thus, if the Board determines the above construction is too broad despite what is expressly stated in the specification, then the broadest reasonable construction of "activation state-specific antibody" in light of the '584 patent is "an antibody that specifically binds to a corresponding and specific isoform of an activatable protein." Ex. 1002 at ¶¶54-56.

VI. OVERVIEW OF THE '584 PATENT

A. Summary of the disclosure of the claimed subject matter

The '584 patent states that a problem in prior art intracellular assays is a limited ability to correlate functional subsets of cells based on the activity of signaling agents. Ex. 1001 at 24:49-54. The '584 patent alleges to have solved this problem by providing methods "for simultaneously detecting the activation state of a plurality of activatable proteins in single cells using flow cytometry" despite admitting prior art investigations "have traditionally focused on one *or a few* proteins at a time." *Id*. at 3:43-45(emphasis added)[2] and 24:55-58; Ex. 1002 at ¶37. Moreover, simultaneous detection is not claimed and is not a necessary requirement of the invention as the disclosed method "can be used optionally to *sequentially* detect the activation state of a plurality of activatable proteins in single cells." Ex. 1001 at 24:65-67.

The invention can allegedly be used to "detect any particular protein isoform in a sample that is antigenically detectable and antigenically distinguishable from other isoforms of the protein which are present in the sample." *Id*. at 25:5-9. An "activation state-specific antibody" binds to a corresponding and specific antigen, which is preferably a specific isoform of an activatable protein. *Id*. at 25:21-26. The '584 patent admits the binding of an activation state-specific antibody to a

[2] All emphasis herein added by Petitioner unless otherwise noted.

target protein was well known in the art at the time of the invention. *Id*. at 25:33-49.

The '584 patent describes kinases as an example of proteins that are capable of existing in an activated isoform with an antigenically distinguishable non-activated isoform. *Id*. at 28:65-67. The phosphorylated form of protein kinases are typically the active isoform and the un-phosphorylated form of protein kinases are typically the inactive isoform. *Id*. at 28:2-7. The '584 patent admits that activation state-specific antibodies to detect kinases were known in the art and commercially available. *Id*. at 28:8-15; Ex. 1002 at ¶40. Prior to the '584 patent, these antibodies have been used "for the study of signal transducing proteins." Ex. 1001 at 28:15-17.

To provide detectability, an antibody may be distinguishably labeled, such as with different fluorescent, enzymes and radioisotopes labels. *Id*. at 30:64-31:8. As acknowledged by the '584 patent, the use of distinguishable labels was well known in the art and numerous distinguishable labels that were commercially available before the '584 patent can be used in the present invention. *Id*. at 31:9-32:59; 32:49-59 and 33:67-34:2; Ex. 1002 at ¶41.

In a preferred embodiment a flow cytometer is used to detect fluorescence. Ex. 1001 at 36:28-30. The "detecting, sorting or isolating steps of the methods of the invention can entail fluorescence-activated cell sorting (FACS) techniques…[a]

variety of FACS systems are known in the art and can be used in the methods of the invention." *Id*. at 37:10-20. In a preferred embodiment, a commercially available Becton Dickinson "FACSVantage^{TM}" cell sorter is used. *Id*.

In summary, the claimed invention purports to use well-known state-specific antibodies that bind to well-known activatable proteins which can be detected using well-known labels by using commercially available detection devices. Ex. 1002 at ¶44; *see also id*. at ¶¶14-35 (describing the state of technology at the time of the invention).

B. Reason for Allowance

During the prosecution of the application that became the '584 patent, the Examiner rejected pending claims 1, 2, 4-6, 24-25, 27, 28, 31 and 32[3] as anticipated by USPN 6,821,740 to Darzynkiewicz ("Darzynkiewicz '740") while pending dependent claims 3, 7-9, 29, 30, 33-36[4] were rejected as obvious over Darzynkiewicz '740 in view of other references, including Belloc. Applicant overcame the rejections by amending the independent claim and arguing that Darkzynkiewicz '740 discloses determining the activation state of a single protein in a cell because Darkzynkiewicz '740 only determines the "level of expression (or

[3] These claims later issued as claims 1, 2, 4-6, 10-13 and 16-17 in the '584 patent.

[4] These claims later issued as claims 3, 7-9, 14-15 and 18-21 in the '584 patent.

presence)" of a second protein rather than the activation state of the second protein. Ex. 1015 at 42.

C. Petition is not cumulative to the arguments and evidence relied on in the prosecution history

While this Petition relies on a Darzynkiewicz reference similar to that used by the Examiner[5], Petitioner asserts Darzynkiewicz renders the claims obvious in view of Yen whereas the Examiner only asserted Darzynkiewicz as anticipating the claims. In addition, the Petition also relies on the teachings of Fleisher and the declaration of Dr. Huxford, neither of which were considered when examining the patentability of claims during the prosecution of the application that led to the '584 patent. Therefore, the Board should not exercise its discretion under 35 U.S.C. § 325(d) to deny any ground in the Petition.

VII. OVERVIEW OF THE PRIOR ART

A. Summary of Fleisher

Fleisher teaches a method of using an activation state-specific antibody for STAT-1 to detect and analyze the activation state of STAT-1 protein in peripheral blood mononuclear cells. Ex. 1004 at 425. Like the '584 patent, Fleisher also

[5] Petitioner relies on a PCT publication due to its availability as prior art under pre-AIA 35 U.S.C. § 102(b) while the Examiner relied on a U.S. Patent. The disclosure of each appears substantially the same.

teaches the benefits of using flow cytometry to analyze the active isoforms of other intracellular signaling proteins to obtain an understanding of a fuller range of cellular signaling pathways:

> This approach should allow for the evaluation of different intracellular signaling pathways using a combination of monoclonal reagents that are specific for native and activation modified proteins. ... In addition, this technique should permit dissection of a full range of cellular signaling pathways at the protein level.

Ex. 1004 at 425. Indeed, Fleisher identifies his teachings apply generally to the evaluation of other activatable proteins so long as there is an activation state-specific antibody for that protein. Ex. 1004 at 425 and 429. In fact, Fleisher expressly teaches some other activatable proteins of interest for further study, including MAP kinases. Ex. 1004 at 429.

In sum, Fleisher teaches a method of using flow cytometry to analyze activated intracellular proteins using an activation state specific antibody. The only technical restriction to using his technique is the existence of an antibody that discriminates between the native and active state of the protein of interest. Ex. 1002 at ¶69. At the time of the invention, one of ordinary skill in the art was aware of activation state specific antibodies for many proteins, including STAT-1 and various MAP kinases, which could be assayed together using the teachings of Fleisher. *Id.* at ¶74.

B. Summary of Darzynkiewicz

Darzynkiewicz discloses "methods and reagents that permit the concurrent and discriminable detection of discrete functional conformations of proteins in a single cell." Ex. 1005 at 7:2-6. In addition, Darzynkiewicz uses fluorescently labeled state-specific antibodies to detect the phosphorylation state of proteins in a single cell with the use of a flow cytometer. *Id*. at 8:21-29; 27:22-34. Thus Darzynkiewicz is trying to solve the exact same problem of the '584 patent, namely using state-specific antibodies to permit the concurrent detection of discrete protein isoforms via flow cytometry.

To perform this method, Darzynkiewicz first teaches contacting a cell with "first" and "second" antibodies where the two antibodies are specific for different conformations of the same protein. *Id*. at 9:12-19. In addition, a "third" antibody can be used which is "specific for a second protein and distinguishable from each of said first and second antibodies, and then detecting the concurrent binding of each of said antibodies to said cell." *Id*. at 10:8-16. As his primary example, Darzynkiewicz discloses the first protein (pRB) being activated retinoblastoma and the second protein is chosen from a cyclin, cyclin dependent kinase or a cyclin dependent kinase inhibitor. *Id*. at 9:20-20 and 10:13-16.

Antibodies are labeled to enable multiparametric detection using a flow cytometer, such as using distinguishable fluorescent labels. *Id*. at 22:13-17 and

22:17-23:21. As noted by Darzynkiewicz, "multiparametric flow cytometric techniques permit more than two antibodies to be discriminable detected in a single assay." *Id*. at 27:22-24. Moreover, "further antibodies may, for example, be used to report, concurrently with the phosphorylation status of pRB, the concurrent levels of other proteins that participate in the regulation of the cell cycle." *Id*. at 22:28-34 and 28:13-15.

C. Summary of Yen

Yen is directed to studying the effects proteins in the MAPK pathway, such as activated ERK 2, on cell differentiation and cell cycle arrest associated with hypophosphorylation of the retinoblastoma (RB) tumor suppressor protein. Ex. 1005 at 3163 ("G_0 growth arrest and RB tumor suppressor protein phosphorylation…, *also depended on ERK2 activation by MEK*."); *Id*. at 3165 ("The antibody used to detect activated ERK1 and ERK2 (V6671 rabbit polyclonal antibody; Promega, Inc., Madison, WI)").

Therefore, Yen teaches a method of using activation state-specific antibodies to detect the activation state for ERK 1/2 and other proteins in the MAPK signaling pathway in correlation with the detection of retinoblastoma protein. *Id*. at 3163.

VIII. HOW THE CONSTRUED CLAIMS ARE UNPATENTABLE UNDER 37 C.F.R. § 42.104(b)(4)

A. Level of Skill in the Art

As acknowledged by the Examiner during prosecution, the level of skill in this art field is high. Ex. 1015 at 15. In view of the subject matter of the '584 patent, a person of ordinary skill in the art as of July 10, 2001 was typically a person who had a Ph.D. in the areas of chemistry, biochemistry, cell biology or molecular biology including five or more years of experience in dealing with antibodies, protein labeling, protein interaction, and protein detection. Ex. 1002 at ¶¶12-13.

Dr. Huxford is well versed in the technology of the '584 patent and was personally working on analysis of protein activation at the time of the invention. Ex. 1002 at ¶¶2-7; Ex. 1003. Thus, Dr. Huxford is qualified to provide his opinion on the level of skill and the knowledge of a person of skill in the art at the time of the invention.

B. Claims 1, 2, 4-6, 10-17, and 23-27 are rendered obvious under 35 U.S.C. § 103 by Fleisher

Although Fleisher does not expressly use a state-specific antibody for a second protein, this is because the specific examples provided in Fleisher were only attempting to determine the ratio of the activated state of STAT-1 to the native state of STAT-1. Ex 1002 at ¶¶64 and 67. Fleisher identifies his teachings

apply generally to the evaluation of other activatable intracellular signaling proteins where activation state-specific antibodies exists. Ex. 1004 at 425; Ex. 1002 at ¶64. In addition, Fleisher teaches certain other activatable proteins of interest for further study:

> This technique should find applications in the study of multiple phosphorylation-dependent pathways such as those involving other Jak–STAT combinations, IkB, and MAP kinases.

Ex. 1004 at 429; Ex. 1002 at ¶64.

Therefore, it would have been obvious at the time of the invention to use Fleisher's method to analyze multiple proteins where antibodies specific to the activated state of those proteins were known at the time of the invention. Ex. 1002 at ¶¶15-17, 69-71 and 96; *see also* Ex. 1001 at 28:8-30.

Claim 1

a. (Preamble) 1. A method of detecting the activation state of at least a first and a second activatable protein in single cells, said method comprising the steps of:

Fleisher teaches one of skill in the art a method of detecting the activation state of multiple different activatable proteins in a single cell. Ex. 1004 at 425 and 429; Ex. 1002 at ¶¶70-71. In particular, Fleisher provides the example of detecting the activation state of STAT-1 and expressly teaches one of skill in the art to detect

the activation state of "other Jak–STAT combinations, IκB, and MAP kinases." *Id*.; Ex. 1002 at ¶64.

b. a) providing a population of cells comprising said first and said second activatable proteins, wherein said first and second activatable proteins are distinct proteins that each have at least an activated isoform, and a non-activated isoform;

Fleisher teaches his method can be applied to populations of human monocyte cells, including peripheral blood mononuclear cells (PBMC). Ex. 1004 at 425; Ex. 1002 at ¶¶65-66. As taught by Fleisher and understood by one of ordinary skill in the art, these cells contain activatable proteins such as MAP kinases that can be detected using the method of Fleisher including ERK1/2 and MEK1/2.[6] Ex. 1002 at ¶¶70 and 73.

c. b) permeabilizing said population of cells;

Fleisher teaches that "[f]ollowing incubation, the cells were either treated with specific antibodies *or subjected to fixation and permeabilization before antibody addition."* Ex. 1004 at 425; Ex. 1002 at ¶¶65-66.

[6] The claims of the '584 patent recite "ERK 1/2" and MEK 1/2" are each an activatable protein, which is consistent with the understanding of those of skill in the art. Ex. 1001 at 152:35-48 (claims 19 and 20); Ex. 1002 at ¶24.

d. c) contacting said permeabilized population of cells with at least two distinguishably labeled activation state-specific antibodies,

A specific example taught in Fleisher focuses on contacting the cells with a labeled antibody to detect the active state of the STAT-1 protein (Ex. 1004 at 425); however, Fleisher identifies his teachings apply generally to the evaluation of other activatable proteins. Ex. 1004 at 425 and 429.

In light of Fleisher's teachings that his "technique should find application in the study of multiple phosphorylation-dependent pathways" and his express teaching to apply his technique to "MAP kinases," it would have been obvious to one of skill in the art to apply teachings of Fleisher to use antibodies to detect activated STAT-1 as well as detect ERK1/2 and MEK1/2, which are MAP kinases. Ex. 1002 at ¶¶70 and 73. The only requirement to do so is the existence of an antibody specific to the activated form of the proteins of interest. Ex. 1004 at 429; Ex. 1002 at ¶69. So long as an antibody was available that was specific to an activated protein of interest, it would only require routine optimization to apply the teachings of Fleisher to detect the activation state of a protein using that antibody. Ex. 1002 at ¶69.

Fleisher itself teaches an antibody specific to STAT-1. Ex. 1004 at 425. One of skill in the art at the time of the invention would have been aware that antibodies specific to the phosphorylated form of ERK1/2 and MEK 1/2 were

commercially available, such as anti-phospho-p44/42 MAP kinase (Thr202/ Tyr204) and anti-phospho-MEK1/2 (Ser217/221). Ex. 1002 at ¶¶22-24, 71 and 74; *see also* Ex. 1001 at 28:8-30, Ex. 1008 at 5107-5108, and Ex. 1010 at 39436.[7] One of skill in the art would have used these antibodies in Fleisher to gain a better understanding of the protein phosphorylation cascade for the MAPK signaling pathway. Ex. 1002 at ¶76.

One of skill in the art would understand these primary antibodies could be distinguishably labeled using well-known fluorescent labels, such as FITC (fluorescein isothiocyanate) and PE (phycoerythrin). Ex. 1002 at ¶¶74-75; Ex. 1004 at 426. In addition, multi-parametric flow cytometers were well known at the time of the invention that could simultaneously distinguish between these fluorescent labels. Ex. 1002 at ¶¶32-34, 72 and 74; *see also* Ex. 1005 at 27:22-24 and Ex. 1011 at 79 and 83.

[7] Cited references not named in a ground of rejection are cited for the purpose of showing the background knowledge of a person of ordinary skill in the art at the time of the invention and to corroborate the testimony of Dr. Huxford. *See Randall Mfg. v. Rea*, 733 F.3d 1355, 1362 (Fed. Cir. 2013); *see also*, *Edmund Optics Inc. v. Semrock Inc*., IPR2014-00599, Paper No. 72 at 37 (Sept. 16, 2015) (background knowledge "may be evidenced through secondary references").

Therefore, depending on the proteins chosen for analysis, one of ordinary skill in the art would select the appropriate activation state specific antibodies with distinguishable labels.

e. wherein a first of said at least two distinguishably labeled activation state-specific antibodies is specific for said activated isoform of said first activatable protein;

As discussed above, Fleisher expressly teaches a first distinguishable labeled antibody can be "rabbit anti-human phosphorylated STAT-1" which is specific for the activated isoform of STAT-1 and could have been labeled with FITC. Ex. 1004 at 426; Ex. 1002 at ¶¶66, 68 and 75. Alternatively, it would have been obvious in light of Fleisher's teachings to use his technique on MAP kinases to use FITC labeled anti-phospho-p44/42 MAPK (Thr202/Tyr204) as the first antibody, which is specific for activated ERK1/2. Ex. 1002 at ¶¶76-77; Ex. 1004 at 429.

f. and a second of said at least two distinguishably labeled activation state-specific antibodies is specific for said activated isoform of said second activatable protein; and

While Fleisher does not expressly teach a second activation-state specific antibody, it would have been obvious to one of skill in the art to use a second activation-state specific antibody in light of Fleisher statement to use his technique to "permit dissection of a full range of cellular signaling pathways" which would require investigation into the sequential activation of multiple proteins in a

signaling path and not just the investigation of a single activated protein. Ex. 1002 at ¶70; Ex. 1004 at 425 and 429.

In addition, Fleisher notes his technique is "applicable in any setting where immunoblotting has already been useful to dissect intracellular signaling pathways." Ex. 1004 at 425. One of skill in the art at the time of the invention was aware of numerous immunoblot assays that detected multiple different intracellular signaling proteins at the same time. Ex. 1002 at ¶71; see also Ex. 1006 at 3165, Ex. 1008 at 5107-5108, Ex. 1009 at 1823-1824 and Ex. 1010 at 39436.

There, it would have been obvious in light of Fleisher's teachings to use his technique with MAP kinases. For example, one of skill in the art could select anti-phospho-MEK1/2 (Ser217/221) that has been labeled with PE as the second antibody, which is specific for activated MEK1/2. Ex. 1002 at ¶¶76-77; Ex. 1004 at 429. One of skill in the art understands FITC is distinguishable from PE from since they emit light at distinguishable wavelengths when excited. Ex. 1002 at ¶¶32-33; *see also* Ex. 1010 at 79 and 83.

g. d) using flow cytometry to detect binding of said first and said second distinguishably labeled activation state-specific antibodies to their corresponding activated isoform of said first and second activatable proteins in single cells of said population of cells,

Fleisher expressly teaches using flow cytometry to detect the binding of antibodies with activated proteins of interest in single cells. *See e.g.*, Ex. 1004 at

429 ("Flow cytometry provides a sensitive and rapid method for the evaluation of activation-specific changes in intracellular proteins") and 425 ("enables rapid and quantitative assessment of STAT-1 phosphorylation on a discrete cell basis.") The specific instrument used in Fleisher was a Becton-Dickinson "FACScan" that could excite FITC labels conjugated on an antibody and sort the cells based on such. *Id*. at 426; Ex. 1002 at ¶68.

In addition, multi-parametric flow cytometers that could distinguish between different types of fluorescent labels concurrently, including detecting both FITC and PE using a single laser, were well known at the time of the invention. Ex. 1002 at ¶¶32-34, 72 and 74; *see also* Ex. 1005 at 27:22-24 and Ex. 1011 at 79 and 83.

Therefore, in light of the teachings of Fleisher, it would have been obvious to one of skill in the art at the time of the invention to use flow cytometry to detect distinguishable labels, such as FITC and PE, conjugated to activation state-specific antibodies for STAT-1, ERK1/2, or MEK1/2 that were bound to the activated form of their respective proteins. Ex. 1002 at ¶77. For example, one could use anti-phospho-p44/42 MAPK (Thr202/Tyr204) that has been labeled with FITC to detect activated ERK1/2 and use anti-phospho-MEK1/2 (Ser217/221) that has been labeled with PE to detect activated MEK1/2 via flow cytometry. *Id*. at ¶77.

h. wherein said binding of said first distinguishably labeled activation state-specific antibody is indicative of the activation state of said first activatable protein, and said binding of said second distinguishably labeled activation

state-specific antibody is indicative of the activation state of said second activatable protein.

One of skill in the art understands the binding of a labeled activation state specific antibody is indicative of the activated state of the associated protein. Ex. 1002 at ¶¶25-26. In particular, the binding of labeled "rabbit anti-human phosphorylated STAT-1" is indicative of the activated state of STAT-1. Ex. 1004 at 426; Ex. 1002 at ¶66. The binding of labeled anti-phospho-p44/42 MAPK (Thr202/Tyr204) is indicative of the activated state of ERK1/2. Ex. 1002 at ¶¶73, 74 and 77. The binding of labeled anti-phospho-p44/42 MAPK (Thr202/Tyr204) is indicative of the activated state of MEK1/2. *Id*.

Claim 2

2. The method according to claim 1, wherein said first activatable protein is a kinase.

Claim 1 is rendered obvious as discussed above. Fleisher teaches the first activatable protein can be a protein in the MAPK pathway. Fleisher at 429. As noted above, it would have been obvious for the first activatable protein to be ERK1/2, which is a kinase. Ex. 1002 at ¶78.

Claim 4

4. The method according to claim 1, wherein said first activatable protein is a first kinase and said second activatable protein is a second kinase.

Claim 1 is rendered obvious as discussed above. Fleisher teaches the first and second activatable proteins can be proteins in the MAPK pathway. Fleisher at

429. As noted above, it would have been obvious for the first activatable protein to be ERK1/2 and the second activatable protein to be MEK1/2, both of which are kinases. Ex. 1002 at ¶¶78-79.

Claim 5

5. The method according to claim 4, wherein said activated isoform of said first kinase is a phosphorylated kinase, and said activated isoform of said second kinase is a second phosphorylated kinase.

Claim 4 is obvious as discussed above. One of skill in the art understands that both ERK1/2 and MEK1/2 are kinases activated by phosphorylation which means their activated forms would be phosphorylated kinases. Ex. 1002 at ¶80.

Claim 6

The method according to claim 5, wherein said first distinguishably labeled activation state-specific antibody is specific for said first phosphorylated kinase, and said second distinguishably labeled activation state-specific antibody is specific for said second phosphorylated kinase.

Claim 5 is obvious as discussed above. One of skill in the art would understand to use FITC labeled anti-phospho-p44/42 MAPK (Thr202/Tyr204) that is specific for phosphorylated ERK1/2 and use PE labeled anti-phospho-MEK1/2 (Ser217/221) that is specific for phosphorylated MEK1/2. Ex 1002 at ¶81.

Claim 10

10. The method according to any one of claims 1 to 9, wherein step a) further comprises contacting said population of cells with an agent that induces the activation of at least said first activatable protein.

Claims 1, 2, and 4-6 are obvious as discussed above. Fleisher teaches activation of the Jak-STAT pathway using interferon-ɣ, which induces activation of STAT-1 protein. Ex. 1004 at 425; Ex. 1002 at ¶82. If the proteins to be studied are MAPK kinases, including ERK1/2 and MEK1/2, it would have been obvious to one of skill in the art to induce activation of those proteins by contacting the cells with retinoic acid. Ex. 1002 at ¶82.

Claim 11

11. The method according to any one of claims 1 to 9, wherein step a) further comprises contacting said population of cells with an agent that induces the activation of said first and said second activatable proteins.

Claims 1, 2, and 4-6 are obvious as discussed above. Claims 1, 2, and 4-6 are obvious as discussed above. Fleisher teaches activation of the Jak-STAT pathway using interferon-ɣ, which induces activation of STAT-1 protein. Ex. 1004 at 425; Ex. 1002 at 82. If the proteins to be studied are MAPK kinases, including ERK1/2 and MEK1/2, it would have been obvious to one of skill in the art to induce activation of those proteins by contacting the cells with retinoic acid. Ex. 1002 at ¶82.

Claim 12

The method according to any one of claims 1 to 9, wherein said method further comprises sorting said single cells based on said activation state of said first activatable protein, and said activation state of said second activatable protein.

Claims 1, 2, and 4-6 are obvious as discussed above. Fleisher teaches using a fluorescent label, such as FITC, and a Becton Dickinson FACS device to detect and sort the cells containing the activated proteins of interest. Ex. 1004 at 426; Ex. 1002 at ¶84. In addition, one of skill in the art understood that multi-parametric flow cytometers were well known at the time of the invention that could distinguish between different types of fluorescent labels concurrently, including detecting both FITC and PE using a single laser. Ex. 1002 at ¶84; *see also* Ex. 1005 at 27:22-24 and Ex. 1011 at 79 and 83.

Therefore, it would have been obvious to one of skill in the art at the time of the invention to use FITC labeled phospho-p44/42 MAPK (Thr202/Tyr204) to detect activated ERK 1/2 and PE labeled anti-phospho-MEK1/2 (Ser217/221) to detect activated MEK1/2 via flow cytometry and sort cells based on the detection of those signals. Ex. 1002 at ¶¶82 and 86.

Claim 13

13. The method according to claim 12, wherein said first distinguishably labeled activation state-specific antibody comprises a first fluorescent label, wherein said second distinguishably labeled activation state-

specific antibody comprises a second fluorescent label, and wherein said sorting is by fluorescent activated cell sorting (FACS).

Claim 12 is obvious as discussed above. Fleisher teaches using a fluorescent label, such as FITC, and a Becton Dickinson FACS device to detect and sort the cells containing the activated proteins of interest. Ex. 1004 at 426; Ex. 1002 at ¶84. At the time of the invention, multicolor FACS devices were well known to concurrently analyze and sort based on the detection of multiple fluorescent signals. Ex. 1002 at ¶82; *see also* Ex. 1001 at 37:16-24 ("A variety of FACS systems are known in the art and can be used in the methods of the invention").

It would have been obvious to one of skill in the art to use a second fluorescent label with the method of Fleisher to detect a second protein. A well-known and readily available laser at the time of the invention was an argon laser that could simultaneously excite FITC and PE. Ex. 1002 at ¶84. Simultaneous detection and sorting based on detecting excited FITC and PE only required routine filters known to those of skill in the art. *Id*.

Therefore, it would have been obvious to one of skill in the art at the time of the invention to use a FACS device to detect and sort phospho-p44/42 MAPK (Thr202/Tyr204) that has been fluorescently labeled with FITC and anti-phospho-MEK1/2 (Ser217/221) that has been fluorescently labeled with PE. Ex. 1002 at ¶¶82 and 86.

Claim 14

14. The method according to claim 12, wherein said first distinguishably labeled activation state-specific antibody comprises a first FRET label, wherein said second distinguishably labeled activation state-specific antibody comprises a second FRET label, and wherein said sorting is by fluorescent activated cell sorting (FACS).

Claim 12 is obvious as discussed above. Fleisher teaches using fluorescent labels to sort by FACS, but does not expressly teach FRET labels. However, FRET labels were well-known to those of ordinary skill in the art at the time of the invention and it would have been obvious to use a FRET label for Fleisher's fluorescent labels. Ex 1002 at ¶¶27-30. In particular, one of skill in the art would have looked to tandem labels as a type of FRET label that would provide an added benefit in the method of Fleisher. *Id*. at ¶85. Examples of tandem labels include Cy5PE and Cy7PE. Ex. 1002 at ¶85.

One of skill in the art would use a tandem label since it would broaden the selection of compatible dyes for flow cytometry and enable up to four different colors to be detected using only a single argon laser. Ex. 1002 at ¶85; *see also* Ex. 1011 at 83 and Ex. 1012 at 168-69. In addition, tandem labels could be used to shift the emission wavelength further above the wavelength cellular autofluorescence to reduce background noise. Ex. 1002 at ¶85.

Therefore, based on the teachings of Fleisher, it would have been obvious to one of skill in the art at the time of the invention to use a flow cytometer to simultaneously detect signals from anti-phospho-p44/42 MAPK labeled with FRET label Cy5PE and anti-phospho-MEK1/2 labeled with FRET label Cy7PE and sort cells based on the detection of those signals.

Claim 15

15. The method according to claim 12, wherein said first distinguishably labeled activation state-specific antibody comprises a FRET label, wherein said second distinguishably labeled activation state-specific antibody comprises a fluorescent label, and wherein said sorting is by fluorescent activated cell sorting (FACS).

Claim 12 is obvious as discussed above. As discussed above, both fluorescent and FRET labels are obvious to use in the method of Fleisher. Based on the teachings of Fleisher it would have been obvious to one of skill in the art at the time of the invention to use a flow cytometer to simultaneously detect signals from anti-phospho-p44/42 MAPK labeled with either a fluorescent label (FITC) or a FRET label (Cy5PE) and anti-phospho-MEK1/2 labeled with a either fluorescent label (PE) or a FRET label (Cy7PE) and sort cells based on the detection of those signals. Ex. 1002 at ¶86. Detecting any combination of the above labels (FITC, PE, Cy5PE, and Cy7PE) could be accomplished using a single argon laser. Ex. 1002 at ¶84.

Claim 16

16. The method according to any one of claims 1 to 9, wherein step a) further comprises fixing said cells.

Claims 1, 2, and 4-6 are obvious as discussed above. Fleisher teaches that "[f]ollowing incubation, the cells were either treated with specific antibodies *or subjected to fixation and permeabilization before antibody addition."* Ex. 1004 at 425; Ex. 1002 at ¶87.

Claim 17

17. The method according to any one of claims 1 to 9, wherein said cells are mammalian cells.

Claims 1, 2, and 4-6 are obvious as discussed above. Fleisher teaches the cells can be peripheral blood mononuclear cells (PBMC), which are found at least in humans as well as other mammals. Ex. 1004 at 425; *see also* Ex. 1002 at ¶87.

Claim 18

18. The method according to claim 1, wherein said first activatable protein is a protein within the MAPK, AKT, STAT, NFkB, PKC or WNT signaling pathways and said second activatable protein is a protein within the MAPK, AKT, STAT, NFkB, PKC or WNT signaling pathways.

Claim 1 is obvious as discussed above. Fleisher teaches analyzing the activated form of the STAT-1 protein, which is part of the STAT signaling pathway. Ex. 1004 at 425. In addition, it would have been obvious to one of skill in the art to use Fleisher's method to also analyze ERK1/2 and MEK1/2, which are

both proteins within the MAPK signaling pathway. Ex. 1002 at ¶¶88-89. Any two of STAT-1, ERK 1/2, or MEK 1/2 can be the first and second activatable proteins.

Claim 19

19. The method according to claim 18, wherein said first activatable protein and second activatable protein are independently selected from the group consisting of … p44/42 MAP kinase, …MEK1/2, …ERK1/2, …STAT1, ….

Claim 18 is obvious as discussed above. Fleisher teaches analyzing the activated form of the STAT-1 protein. Ex. 1004 at 425. In addition, it would have been obvious to one of skill in the art to use Fleisher's method to also analyze the activated form of ERK1/2 and MEK1/2. Ex. 1002 at ¶¶88-89. Thus, Fleisher teaches the first and second activatable protein can be independently selected from the group STAT1, ERK 1/2, or MEK 1/2.

Claim 20

20. The method according to claim 19, wherein said first activatable protein and second activatable protein are independently selected from the group consisting of … ERK1/2, MEK1/2, …STAT1, ….

Claim 19 is obvious as discussed above. Fleisher teaches analyzing the activated form of the STAT-1 protein. Ex. 1004 at 425. In addition, it would have been obvious to one of skill in the art to use Fleisher's method to also analyze the activated form of ERK1/2 and MEK1/2. Ex. 1002 at ¶¶88-89. Thus, Fleisher

teaches the first and second activatable protein can be independently selected from the group STAT1, ERK 1/2, or MEK 1/2.

Claim 21

21. The method according to claim 1, 4, 5 or 6, wherein said first distinguishably labeled activation state-state specific antibody is selected from the group consisting of: … anti-phospho-p44/42 MAP kinase - (Thr202/Tyr204), … anti-phosphoMEK1/2 (Ser217/221), …anti-STAT1, ….

Claims 1 and 4 are obvious as discussed above. Fleisher teaches analyzing both the non-specific and activated form of the STAT-1 protein using labeled antibodies for STAT-1 and activated STAT-1. Ex. 1004 at 426; Ex. 1002 at ¶65. In addition, it would have been obvious to one of skill in the art to use anti-phospho-p44/42 MAPK (Thr202/Tyr204) that has been distinguishably labeled to detect activated ERK1/2 and anti-phospho-MEK1/2 (Ser217/221) that has been distinguishably labeled with PE to detect activated MEK1/2. Ex. 1002 at ¶¶88-89.

Claim 23

23. The method according to claim 1, wherein said activated isoform of either the first or second activatable protein is created through a covalent addition, structural change, or conformational change.

Claim 1 is obvious as discussed above. Fleisher teaches that the activation of proteins in the STAT-Jak, NF-κB, and MAPK pathways is generally due to phosphorylation and one of skill in the art understands in particular that STAT-1, ERK 1/2, MEK 1/2 all activate due to phosphorylation. Ex. 1004 at 425; Ex. 1002

at ¶90. One of skill in the art understands that activation by phosphorylation requires the covalent addition of a phosphate group to a protein and causes a conformational change in the protein due to the addition of the phosphate group. Ex. 1002 at ¶90.

Claim 24

24. The method according to claim 23 wherein said conformational change causes said activated isoform of either the first or second activatable protein to present at least one epitope that is not present in a non-activated isoform of either the first or second activatable protein.

Claim 23 is obvious as discussed above. The conformational change of any of STAT-1, ERK 1/2, or MEK 1/2 due to activation via phosphorylation results in at least one epitope being present that is not present prior to the activation. Ex. 1002 at ¶91. For example, anti-phospho-p44/42 MAPK (Thr202/Tyr204) specifically targets the phospho-Thr202/phospho-Tyr204 epitope in ERK 1/2 that is not present in un-phosphorylated ERK 1/2. *Id*. Similarly, anti-phospho-MEK1/2 (Ser217/221) specifically targets the phospho-Ser217/phospho-Ser221 epitope in MEK 1/2 that is not present in un-phosphorylated MEK 1/2. *Id*.

Claim 25

25. The method according to claim 1 wherein the activated isoform of either the first or second activatable protein is a phosphorylated or a cleaved product of either the first or second activatable protein.

Claim 1 is obvious as discussed above. Fleisher teaches that the activation of proteins in the STAT-Jak, NF-κB, and MAPK pathways is generally due to phosphorylation and one of skill in the art understands in particular that STAT-1, ERK 1/2, and MEK 1/2 all activate due to phosphorylation. Ex. 1004 at 425; Ex. 1002 at ¶90. Therefore, the activated isoforms of these proteins are the phosphorylated products of their respective proteins.

Claim 26

26. The method according to claim 1 wherein the activated isoform of either the first or second activatable protein is created through binding to a third protein or protein complex.

Claim 1 is obvious as discussed above. As discussed with respect to claim 1, the first or second activatable protein can be MEK 1/2. It was well-known at the time of the invention, that the active form of MEK 1/2 is created by binding un-activated MEK 1/2 with Raf or MEK Kinase proteins. Ex. 1002 at ¶92.

Claim 27

27. The method according to claim 1 wherein the activated isoform of either the first or second activatable protein is created through binding to a soluble small molecule entity.

Claim 1 is obvious as discussed above. One of skill in the art would recognize GTP as a soluble small molecule which activates Ras. Ex. 1002 at ¶93. Ras is a protein that is within the MAPK signaling pathway like MEK 1/2 and ERK 1/2. *Id.*; *see also* Ex. 1006 at 3163. In addition, antibodies specific to the

activated form of Ras were known at the time of the invention. Ex. 1002 at ¶94; *see also* Ex. 1013 at 119-120.

Therefore, one of skill in the art would have considered it obvious to apply the method of Fleisher to analyze the activation state of Ras since an activation state specific antibody existed and Ras is part of the MAPK signaling pathway. Ex. 1004 at 429; Ex. 1002 at ¶95.

C. Claims 3, 7-9 and 22 are rendered obvious under 35 U.S.C. § 103 by Fleisher in view of Belloc

As noted above, Fleisher teaches a method of using flow cytometry to concurrently detect isoforms of multiple activated intracellular proteins, including STAT-1 and MAP kinases. Belloc adds the teaching that those of skill in the art were also interested in analyzing the active isoforms of caspases, which are also intracellular proteins, to better understand cell cycle regulation.

In particular, Belloc teaches that "[c]aspase activation is now well known as a key event in the apoptotic process." Ex. 1007 at 152. Belloc further teaches that "we found that this caspase 3 activation can be detected by the anti-cleaved caspase 3 antibodies" and "[t]his labeling can be used in multivariate analysis." Ex. 1007 at 152-53. Fluorescently labeled antibodies conjugated to multiple proteins of interest, including activated caspase 3, were concurrently detected using a flow cytometer. *See e.g.*, *Id*. at 153 and 159.

Belloc also recognizes that other caspases regulate cellular activity, including caspase-1. Ex. 1007 at 152. In addition, researchers were actively investigating immunoassays that correlate the activation states of caspase-1 and -3 using activation state-specific antibodies for each. Ex. 1002 at ¶97; *see also* Ex. 1014 at 13902 and Ex. 1001 at 28:56-62.

Therefore, at the time of the invention one of skill in the art would have been motivated to use Fleisher's and Belloc's teachings to concurrently detect activated caspases-1 and -3 using activation state specific antibodies to gain further understanding of the mechanics of how a cell regulates itself, and in particular how caspases regulate aptoptosis within a cell. Ex. 1002 at ¶¶99 and 100.

Claim 3

3. The method according to claim 1, wherein said first activatable protein is a caspase.

Claim 1 is obvious as discussed above. At the time of the invention, one of skill in the art would understand caspase-3 is an activatable caspase. Ex. 1007 at 152; Ex. 1002 at ¶¶15 and 97.

Therefore, in light of Fleisher's teaching that his method should be used to study multiple pathways and applies to "any activation pathway for which antibody reagents exist that discriminate between a native and an activation modified protein," it would have been obvious to analyze activated caspase-3 using

multivariate flow cytometry as taught by Belloc with the methods taught by Fleisher to analyze apoptosis in the cell. *id*. at ¶¶99 and 100.

Claim 7

7. The method according to claim 1, wherein said first activatable protein is a first caspase and said second activatable protein is a second caspase.

Claim 1 is obvious as discussed above. At the time of the invention, one of skill in the art would understand caspase-3 is an activatable caspase. Ex. 1007 at 152; Ex. 1002 at ¶¶15 97, and 130. One of skill in the art at the time of the invention knew researchers were actively investigating assays that correlate the activity of other different caspases as well, such as caspases-1 and -3. Ex. 1002 at ¶97; *see also* Ex. 1014 at 13902 and Ex. 1001 at 28:56-62.

Therefore, it would have been obvious to use caspase-3 for the claimed first activatable protein and another caspase, such as caspase-1, as the claimed second activatable protein to study the regulation of apoptosis. Ex. 1002 at ¶¶98-99.

Claim 8

8. The method according to claim 7, wherein said activated isoform of said first caspase is a cleaved product of a first pro-caspase, and said activated isoform of said second caspase is a cleaved product of a second pro-caspase.

Claim 7 is obvious as discussed above. One of ordinary skill in the art understands the active forms of caspases-1 and -3 are the cleaved products of their respective pro-caspases. Ex. 1002 at ¶98; Ex. 1007 at 152 ("Procaspase 3 is a constitutive proenzyme that is activated by cleavage during apoptosis.").

Claim 9

9. The method according to claim 8, wherein said at least two distinguishably labeled activation state-specific antibodies comprise a first distinguishably labeled activation state-specific antibody that is specific for said activated isoform of said first caspase, and a second distinguishably labeled activation state-specific antibody that is specific for said activated isoform of said second caspase.

Claim 8 is obvious as discussed above. One of skill in the art at the time of the invention understood that activation state-specific antibodies for caspases-1 and -3 were available at the time of the invention. Ex. 1002 at ¶97; *see also* Ex. 1014 at 13902; Ex. 1001 at 28:56-62. Therefore, it would have been obvious to distinguishably label those antibodies to analyze activated caspase-1 and -3 using multivariate flow cytometry. Ex. 1007 at 152 ("[t]his labeling can be used in multivariate analysis." and Ex. 1002 at ¶99.

D. Claims 1, 2, 4-6, 10-17, and 23-27 are rendered obvious under 35 U.S.C. § 103 by Darzynkiewicz in view of Yen

During prosecution, the only limitation found not to be anticipated by the disclosure of Darzynkiewicz is the use of a second state specific antibody to identify a second activated protein. However, this limitation and all other limitations of claims 1, 2, 4-6, 10-17 and 23-27 would have been obvious to one of skill in the art in light of Darzynkiewicz's teachings of using an activation state-specific antibody to detect activated retinoblastoma protein concurrently with other proteins and Yen's teaching of concurrently detecting proteins in the MAPK pathway, including activated ERK 1/2 and retinoblastoma protein.

1. Reason to Combine the Teachings of Darzynkiewicz in view of Yen

Darzynkiewicz states the "third" antibody is "specific for a second protein" but is silent if it is specific for an activated isoform of a second protein. Ex 1005 at 10:8-16. However, the method of Darzynkiewicz is not limited to measuring the correlation of activated pRB with the presence of another protein. Rather, the disclosed method is generally applicable to measuring the concurrent correlation of two different proteins. Ex. 1005 at 10:8-16. One of skill in the art reading Darzynkiewicz would understand the teaching of using distinguishable antibodies generally would include the use of activation state-specific antibodies. Ex. 1002 at ¶¶16-17. There is no change in the approach needed to use and detect a state-

specific antibody compared to using and detecting a non-state-specific antibody. Ex. 1002 at ¶17.

Consistent with Darzynkiewicz's statement to analyze other proteins that participate in the regulation of the cell cycle, Yen teaches an assay that concurrently detects retinoblastoma protein (RB) in conjunction with activated ERK 1/2 to analyze the effect of these proteins on cell cycle regulation. Ex. 1006 at 3163. Yen also discussed the effect other MAPK proteins have on cell cycle regulation including MEK, RAS, and RAF. *See e.g.,* Ex. 1005 at 3163. Yen teaches using activation state-specific antibodies that target the phosphorylated Thr^{183} and Tyr^{185} sites on active ERK proteins used in the cell cycle regulation of the HL-60 cell line, which is the same cell line used in Darzynkiewicz. Ex. 1005 at 11:12-18 and 24:47-65; Ex. 1006 at 3163; Ex. 1002 at ¶104. Both Darzynkiewicz and Yen induce the HL-60 cells with retinoic acid, which further indicates to one of skill in the art the complementary nature of the teachings of Darzynkiewicz and Yen. Ex. 1005 at 11:12-18; *see also* Ex. 1006 at 3163 and 3165; Ex. 1002 at ¶104.

Finally, Darzynkiewicz teaches that multiparametric flow cytometric techniques to concurrently detect two or more antibodies, including state-specific antibodies, was known in the art and provided a reason to apply his technique to proteins other than retinoblastoma:

> Present multiparametric flow cytometric techniques permit more than two antibodies to be discriminably detected in a single assay…In the methods of the present invention, these further antibodies may, *for example, be used to report, concurrently with the phosphorylation status of pRB, the concurrent levels of other proteins that participate in the regulation of the cell cycle*.

Ex. 1005 at 27:22-24; *see also id.* at 23:23-34; Ex. 1002 at ¶102.

Therefore, in light of the teachings of Darzynkiewicz and Yen, one of skill in the art would have been motivated to analyze the effects of cell cycle regulation by concurrently analyzing the activated state of retinoblastoma protein, ERK 1/2, MEK 1/2, or RAS using the multiparametric flow cytometric method of Darzynkiewicz. Ex. 1002 at ¶¶103 and 129.

Claim 1

a. (Preamble) 1. A method of detecting the activation state of at least a first and a second activatable protein in single cells, said method comprising the steps of:

Darzynkiewicz teaches a method of detecting two different proteins in a single cell. Ex. 1005 at 10:13-16; Ex. 1002 at ¶101. While Darzynkiewicz uses activated pRB (retinoblastoma protein) as its primary example to explain his method of detecting an activatable protein, the method of Darzynkiewicz can be used to detect the activation state of other activatable proteins. Ex. 1002 at ¶102; Ex. 1005 at 23:23-34 and 27:22-24.

In the same HL-60 cell line cited in Darzynkiewicz, Yen teaches detection of RB (abbreviate pRB in Darzynkiewicz) in addition to the detection of activated ERK1/2. Ex. 1002 at ¶¶103-104; Ex. 1005 at 11:12-18 and 24:47-65; Ex. 1006 at 3163. This cell line also contains other proteins in the MAPK signaling pathway that help regulate the cell cycle, including MEK 1/2 and Ras. Ex. 1002 at ¶¶103-104; Ex. 1006 at 3163. As discussed above, one of skill in the art reading both Darzynkiewicz and Yen would be motivated to detect the activation state of two or more of these activatable proteins in order to better understand the correlation of the activation of these proteins as they regulate the cell cycle. Ex. 1002 at ¶103.

b. a) providing a population of cells comprising said first and said second activatable proteins, wherein said first and second activatable proteins are distinct proteins that each have at least an activated isoform, and a non-activated isoform;

Darzynkiewicz teaches his method can be applied to populations of HL-60 promyelocytic leukemic cells. Ex. 1005 at 11:12-18; Ex 1002 at ¶104. One of ordinary skill in the art understands that these cells lines contain many different activatable and distinct proteins including retinoblastoma, Ras, ERK1/2 and MEK 1/2. Ex. 1002 at ¶104; Ex. 1006 at 3163.

As a specific example, one of ordinary skill in the art would understand the first activatable protein could be RB and the second activatable protein could be ERK 1/2. Ex 1002 at ¶104. While this is one specific example, one of ordinary

skill in the art understands other sets of proteins that regulate cell cycle could also be measured based on the teachings of Darzynkiewicz and Yen, such as the first activateble protein being ERK 1/2 and the second activateble protein being MEK 1/2. Ex. 1002 at ¶108.

c. b) permeabilizing said population of cells;

Darzynkiewicz teaches that "[c]ells may, for example, be simultaneously fixed and permeabilized in HBSS" or "permeabilized using commercially available reagents." Ex. 1005 at 11:49-60. One of skill in the art understands this teaching of Darzynkiewicz is consistent with the knowledge that permeabilizing cells is an important step that enables antibodies to pass through cell membranes to come into contact with intracellular proteins such as RB, ERK 1/2, MEK 1/2, and Ras. Ex. 1002 at ¶¶31 and 105.

d. c) contacting said permeabilized population of cells with at least two distinguishably labeled activation state-specific antibodies,

Darzynkiewicz teaches permeabilizing the population of cells as discussed above. Darzynkiewicz then teaches contacting these cells with labeled antibodies. Ex. 1005 at 25:29-31 ("Next, the cells are labeled with antibodies conjugated to flow cytometrically detectable fluorophores."); Ex. 1002 at ¶106. The labels "are mutually distinguishable" and as "is well known in the art, there are a number of two-flourophore combinations that permit concurrent and discriminable detection."

id. at 26:3-10; *see also id.* 26:13-24 (giving examples of particular fluorophores that can be used in the invention, including FITC and PE-CY5 "tandem resonance energy transfer fluorophore"); *see also* Ex. 1001 at 31:9-53 (the '584 patent also cites to fluorescein [FITC] and PECY5 as preferred fluorophores).

Darzynkiewicz only expressly discloses one state-specific antibody, pRB^{P-} mAb, which detects the underphosphorylated state of retinoblastoma susceptibility gene protein (abbreviated pRB in Darzynkiewicz). Ex. 1005 at 3:2-26; Ex. 1002 at ¶107. However, as discussed above, Darzynkiewicz teaches his method can be used to investigate other proteins that participate in the regulation of the cell cycle. One of skill in the art understands Darzynkiewicz's method can employ other state-specific antibodies to detect other cell cycle regulation proteins of interest. Ex. 1002 at ¶103.

Yen teaches an assay that, like Darzynkiewicz, analyzes the effect of intracellular signaling proteins on cell cycle regulation. In particular, Yen teaches measuring the level of activated ERK2. To perform these measurements, Yen conjugates an activation state-specific antibody, V6671 rabbit polyclonal antibody, to activated ERK 1/2. Ex. 1006 at 3165; Ex. 1002 at ¶107. This antibody binds to phosphorylated Thr183 and Tyr185 sites of active ERK2 proteins. *Id*. Moreover, Yen teaches other proteins in the MAPK pathway effect cell cycle regulation including MEK and Ras. Ex. 1006 at 3163.

Therefore, it would have been obvious to one of skill in the art to use a second distinguishably labeled state-specific antibody in the method of Darzynkiewicz based on the teachings of Darzynkiewicz and Yen. For example, one of skill in the art could contact the cells with the activation state-specific antibody pRB^{P-} mAb taught by Darzynkiewicz and an activation state-specific V6671 rabbit polyclonal antibody for ERK 2 taught by Yen. Ex. 1002 at ¶107.

In addition, one of skill in the art would also understand that the teachings of Darzynkiewicz and Yen are not limited to these antibodies. It would have been obvious to contact the cells with other state-specific antibodies that were well known at the time of the invention which correspond to cell cycle regulation proteins in the MAPK pathway, such as anti-phospho-p44/42 MAPK (Thr202/Tyr204) to detect activated ERK 1/2 and anti-phospho-MEK1/2 (Ser217/221) to detect activated MEK1/2. Ex. 1002 at ¶¶22-24 and 108; *see also* Ex. 1008 at 5107-5108; Ex. 1010 at 39436.

At the time of the invention, utilizing appropriate activation state-specific antibodies with the distinguishable labels within the methods of Darzynkiewicz would only require routine application of standard laboratory techniques. Ex. 1002 at ¶¶108-109.

e. wherein a first of said at least two distinguishably labeled activation state-specific antibodies is specific for said activated isoform of said first activatable protein;

Darzynkiewicz teaches in one example distinguishably labeling pRB^{P-} mAb which is specific for unphosphorylated pRB (alternatively described in Darzynkiewicz as hypophosphorylated) with FITC. Ex. 1005 at 42:12-14. Unlike most kinases where the phosphorylation form is considered the active form, the unphosphorylated state of pRB is considered the active form of pRB. *Id*. at 3:2-26; Ex. 1002 at ¶ 107. As noted in the '584 patent, the active form of a protein is one that has a property not possessed by at least one other isoform, for example "protein binding activity." Ex. 1001 at 3:7-17. Here, the unphosphorylated form of pRB has the property of binding E2F transcription factors not possessed by the phosphorylated form of pRB. Ex. 1005 at 3:11-13.

Moreover, as discussed previously, the method of Darzynkiewicz is not limited to the specific example of using pRB^{P-} mAb to detect unphosphorylated pRB, but as stated in Darzynkiewicz is applicable to measuring the concurrent correlation of using antibodies to detect two or more proteins generally. Ex. 1005 at 10:13-16; Ex. 1002 at ¶¶102-103. In addition, Darzynkiewicz teaches using his method to detect "the concurrent levels of other proteins that participate in the regulation of the cell cycle." Ex. 1005 at 27:22-24; Ex. 1002 at ¶102.

As taught by Yen, other proteins of interest that regulate the cell cycle in connection with RB include ERK, MEK, and Ras. Ex. 1006 at 1363; Ex. 1002 at ¶¶103-104. For example, anti-phospho-p44/42 MAPK (Thr202/Tyr204) to detect activated ERK 1/2 and anti-phospho-MEK1/2 (Ser217/221) to detect activated MEK1/2 were commercially available by the time of the invention and could be used in the method of Darzynkiewicz to detect the proteins of interest taught by Yen. Ex. 1002 at ¶¶22-24 and 108; *see also* Ex. 1008 at 5107-5108; Ex. 1010 at 39436. Thus, it would have been obvious to one of skill in the art for either of these activation state-specific antibodies to be the claimed first antibody. Ex. 1002 at ¶¶108-109.

Finally, a multitude of labels existed at the time of the invention that could successfully conjugate to these state-specific antibodies and be used in concurrent multiparametric analysis, including FITC, PE, and PECY5, which are noted in Darzynkiewicz as suitable to use. Ex 1002 at ¶¶106 and 109; Ex. 1005 at 8:29-33 and 26:9-24.

Therefore, the first state-specific antibody would be obvious based on the choice of the first activatable protein. If the first protein is pRB then the first state specific antibody could be pRB^{P-} mAb and if the first protein is MEK then the first state specific antibody could be anti-phospho-MEK1/2 (Ser217/221), either of which could be distinguishably labeled with FITC.

f. and a second of said at least two distinguishably labeled activation state-specific antibodies is specific for said activated isoform of said second activatable protein; and

As discussed above, the method of Darzynkiewicz is not limited to the specific example of using pRB^{P-} mAb to detect unphosphorylated pRB, but as stated in Darzynkiewicz is applicable to measure the "concurrent levels of other proteins that participate in the regulation of the cell cycle." Ex. 1005 at 10:13-16; and 27:22-24; Ex. 1002 at ¶¶102-103.

As taught by Yen, another activation specific antibody is V6671 rabbit polyclonal antibody to detect activated ERK2. Ex. 1006 at 3165; Ex. 1002 at ¶107. In addition, Yen teaches that other proteins of interest that regulate the cell cycle in connection with RB include ERK, MEK, and Ras. Ex. 1006 at 1363; Ex. 1002 at ¶¶103-104. For example, anti-phospho-p44/42 MAPK (Thr202/Tyr204) to detect activated ERK 1/2 and anti-phospho-MEK1/2 (Ser217/221) to detect activated MEK1/2 were commercially available by the time of the invention and could be used in the method of Darzynkiewicz. Ex. 1002 at ¶¶22-24 and 108; *see also* Ex. 1008 at 5107-5108; Ex. 1010 at 39436. Thus, it would have been obvious to one of skill in the art for either of these activation state-specific antibodies to be the claimed second antibody. Ex. 1002 at ¶¶108-109.

Finally, a multitude of labels existed at the time of the invention that could successfully conjugate to these state-specific antibodies and be used in concurrent

multiparametric analysis, including FITC, PE, and PECY5, which are noted in Darzynkiewicz as suitable to use. Ex 1002 at ¶¶106 and 109; Ex. 1005 at 8:29-33 and 26:9-24.

Therefore, the second state-specific antibody would be obvious based on the choice of the second activatable protein. If the second protein is ERK 1/2 then the second state specific antibody could be either V6671 rabbit polyclonal antibody or anti-phospho-p44/42 MAPK (Thr202/Tyr204), either of which could be distinguishably labeled with PE.

g. d) using flow cytometry to detect binding of said first and said second distinguishably labeled activation state-specific antibodies to their corresponding activated isoform of said first and second activatable proteins in single cells of said population of cells,

Darzynkiewicz teaches that the knowledge and technology to concurrently detect two or more antibodies using flow cytometry was known in the art. Ex. 1005 at 27:22-24 ("Present multiparametric flow cytometric techniques permit more than two antibodies to be discriminably detected in a single assay"); *see also id.* at 23:23-34 ("[f]low cytometric techniques are by now well established").

In addition, the '584 patent acknowledges at the time of the invention that detecting labeled antibodies was well known in the art as well as noting the existence of commercially available FACS device to perform flow cytometric detecting and sorting. *Id*. at 37:10-20. It would have been obvious to one of skill in

the art to use a flow cytometer as taught by Darzynkiewicz to detect the distinguishably labeled antibodies that correspond to active forms of the corresponding proteins in single cells. Ex. 1002 at ¶¶103 and 109.

h. wherein said binding of said first distinguishably labeled activation state-specific antibody is indicative of the activation state of said first activatable protein, and said binding of said second distinguishably labeled activation state-specific antibody is indicative of the activation state of said second activatable protein.

One of skill in the art understands the binding of a labeled activation state specific antibody is indicative of and can be used to detect the activated state of the associated protein. Ex. 1002 at ¶¶25-26. In particular, the binding of labeled pRB^{P-} mAb is indicative of the activation state of pRB, the binding of labeled V6671 rabbit polyclonal antibody is indicative of the activation state of ERK 1/2, the binding of labeled anti-phospho-p44/42 MAPK (Thr202/Tyr204) is indicative of the active state of ERK1/2, and the binding of labeled anti-phospho-p44/42 MAPK (Thr202/Tyr204) is indicative of the active state of MEK1/2. Ex. 1005 at 10:54-64; Ex. 1002 at ¶¶107-109.

Claim 2

2. The method according to claim 1, wherein said first activatable protein is a kinase.

Claim 1 is rendered obvious as discussed above. Darzynkiewicz in view of Yen teaches the first activatable protein can be MEK 1/2, which is a kinase. Ex. 1002 at ¶110.

Claim 4

4. The method according to claim 1, wherein said first activatable protein is a first kinase and said second activatable protein is a second kinase.

Claim 1 is rendered obvious as discussed above Darzynkiewicz in view of Yen teaches the first activatable protein can be MEK 1/2 and the second activatable protein can be ERK 1/2, both of which are a kinases. Ex. 1002 at ¶¶110-111; Ex. 1006 at 3163.

Claim 5

5. The method according to claim 4, wherein said activated isoform of said first kinase is a phosphorylated kinase, and said activated isoform of said second kinase is a second phosphorylated kinase.

Claim 4 is obvious as discussed above. One of skill in the art understands that both ERK1/2 and MEK1/2 are kinases activated by phosphorylation which means their activated forms would be phosphorylated kinases. Ex. 1002 at ¶112.

Claim 6

6. The method according to claim 5, wherein said first distinguishably labeled activation state-specific antibody is specific for said

first phosphorylated kinase, and said second distinguishably labeled activation state-specific antibody is specific for said second phosphorylated kinase.

Claim 5 is obvious as discussed above. To detect phosphorylated ERK1/2 and MEK 1/2, one of skill in the art could use FITC labeled anti-phospho-p44/42 MAPK (Thr202/Tyr204) and use PE labeled anti-phospho-MEK1/2 (Ser217/221). Ex 1002 at ¶113. These antibodies are specific for ERK 1/2 and MEK 1/2, respectively. *id*.

Claim 10

10. The method according to any one of claims 1 to 9, wherein step a) further comprises contacting said population of cells with an agent that induces the activation of at least said first activatable protein.

Claims 1, 2, and 4-6 are obvious as discussed above. Both Darzynkiewicz and Yen teach contacting the cells with retinoic acid (RA) to induce activation of proteins including pRB, ERK 1/2, and MEK 1/2. Ex. 1002 at ¶114; Ex. 1005 at 35:22-24; Ex. 1006 at 3163.

Claim 11

11. The method according to any one of claims 1 to 9, wherein step a) further comprises contacting said population of cells with an agent that induces the activation of said first and said second activatable proteins.

Claims 1, 2, and 4-6 are obvious as discussed above. Both Darzynkiewicz and Yen teach contacting the cells with retinoic acid (RA) to induce activation of proteins including pRB, ERK 1/2, and MEK 1/2. Ex. 1002 at ¶114; Ex. 1005 at 35:22-24; Ex. 1006 at 3163.

Claim 12

12. The method according to any one of claims 1 to 9, wherein said method further comprises sorting said single cells based on said activation state of said first activatable protein, and said activation state of said second activatable protein.

Claims 1, 2, and 4-6 are obvious as discussed above. Darzynkiewicz teaches using a "flow cytometer/cell sorter" with "an argon laser (emission at 488nm)" to detect labels such as FITC, PE, and Cy5PE attached to antibodies. Ex. 1005 at 44:25-29; Ex. 1002 at ¶¶115-116. In addition, one of skill in the art understood that multi-parametric flow cytometers were well known at the time of the invention and could distinguish between different types of fluorescent labels concurrently, including distinguishing between FITC and PE labels using a single argon laser. Ex. 1002 at ¶¶116-117; *see also* Ex. 1005 at 27:22-24; Ex. 1011 at 79 and 83.

Therefore, it would have been obvious to one of skill in the art at the time of the invention to use Darzynkiewicz's argon laser "flow cytometer/cell sorter" with FITC labeled phospho-p44/42 MAPK (Thr202/Tyr204) to detect activated ERK

1/2 and with PE labeled anti-phospho-MEK1/2 (Ser217/221) to detect activated MEK1/2 and to sort cells based on the detection of those signals. Ex. 1002 at ¶¶115, 118-119.

Claim 13

13. The method according to claim 12, wherein said first distinguishably labeled activation state-specific antibody comprises a first fluorescent label, wherein said second distinguishably labeled activation state-specific antibody comprises a second fluorescent label, and wherein said sorting is by fluorescent activated cell sorting (FACS).

Claim 12 is obvious as discussed above. Darzynkiewicz teaches using a "flow cytometer/cell sorter," which is a fluorescent activated cell sorter (FACs), and "an argon laser (emission at 488nm)" to detect fluorescent labels such as FITC and PE attached to antibodies. Ex. 1005 at 44:25-29; Ex. 1002 at ¶116. At the time of the invention, multicolor FACS devices were well known to concurrently analyze and sort based on the detection of multiple fluorescent signals. Ex. 1002 at ¶116; *see also* Ex. 1001 at 37:16-24 ("A variety of FACS systems are known in the art and can be used in the methods of the invention").

It would have been obvious to one of skill in the art use a second fluorescent label with the method of Darzynkiewicz to detect a second protein. The argon laser as disclosed in Darzynkiewicz could excite both FITC and PE. Ex. 1002 at ¶116;

see also Ex. 1011 at 79 and 83. Simultaneous detection and sorting based on detecting excited FITC and PE only required routine filters. *Id*.

Therefore, it would have been obvious to one of skill in the art at the time of the invention to use a FACS device as taught by Darzynkiewicz to detect and sort FITC labeled phospho-p44/42 MAPK (Thr202/Tyr204) and PE labeled anti-phospho-MEK1/2 (Ser217/221). Ex. 1002 at ¶¶118-119.

Claim 14

14. The method according to claim 12, wherein said first distinguishably labeled activation state-specific antibody comprises a first FRET label, wherein said second distinguishably labeled activation state-specific antibody comprises a second FRET label, and wherein said sorting is by fluorescent activated cell sorting (FACS).

Claim 12 is obvious as discussed above. Darzynkiewicz teaches using fluorescent labels to sort by FACS, and teaches some of his labels are "tandem" labels. Ex. 1005 at 8:29-33 and 26:9-24 (PE-CY5 "tandem resonance energy transfer fluorophore" and Per-CP-Cy5.5). One of skill in the art understands these tandem labels taught by Darzynkiewicz are examples of FRET labels that were known to be compatible with flow cytometry. Ex. 1002 at ¶¶117-118; *see also* Ex. 1012 at 168. Two tandem fluorophores known to be excitable by an argon laser

and used in flow cytometry for multiparametric analysis include PE-CY5 and PE-CY7. Ex. 1002 at ¶¶116 and 118; *see also* Ex. 1011 at 79 and 83.

Therefore, based on the teachings of Darzynkiewicz in view of Yen, it would have been obvious to one of skill in the art at the time of the invention to use a flow cytometer to simultaneously detect signals from Cy5PE labeled anti-phospho-p44/42 MAPK and Cy7PE labeled anti-phospho-MEK1/2 labeled and to sort cells based on the detection of those signals.

Claim 15

15. The method according to claim 12, wherein said first distinguishably labeled activation state-specific antibody comprises a FRET label, wherein said second distinguishably labeled activation state-specific antibody comprises a fluorescent label, and wherein said sorting is by fluorescent activated cell sorting (FACS).

Claim 12 is obvious as discussed above. As discussed above, both fluorescent and FRET labels are taught by Darzynkiewicz. Based on the teachings of Darzynkiewicz, it would have been obvious to one of skill in the art at the time of the invention to use a flow cytometer to simultaneously detect signals from anti-phospho-p44/42 MAPK labeled with either a fluorescent label (FITC) or a FRET label (Cy5PE) and anti-phospho-MEK1/2 labeled with a either fluorescent label (PE) or a FRET label (Cy7PE) and sort cells based on the detection of those

signals. Ex. 1002 at ¶¶118-119. Detecting combinations of the above labels (FITC, PE, Cy5PE, and Cy7PE) could be accomplished using Darzynkiewicz's argon laser. Ex. 1002 at ¶116; *see also* Ex. 1011 at ¶¶79 and 83.

Claim 16

16. The method according to any one of claims 1 to 9, wherein step a) further comprises fixing said cells.

Claims 1, 2, and 4-6 are obvious as discussed above. Darzynkiewicz teaches that "[c]ells may, for example, be simultaneously *fixed* and permeabilized in HBSS (HEPES buffered saline)." Ex. 1005 at 25:1-19; Ex. 1002 at ¶120.

Claim 17

17. The method according to any one of claims 1 to 9, wherein said cells are mammalian cells.

Claims 1, 2, and 4-6 are obvious as discussed above. Darzynkiewicz and Yen both teach using HL-60 cells, which are mammalian cells. Ex. 1002 at ¶120.

Claim 18

18. The method according to claim 1, wherein said first activatable protein is a protein within the MAPK, AKT, STAT, NFkB, PKC or WNT signaling pathways and said second activatable protein is a protein within the MAPK, AKT, STAT, NFkB, PKC or WNT signaling pathways.

Claim 1 is obvious as discussed above. It would have been obvious to one of skill in the art in light of the teachings of Darzynkiewicz and Yen to select ERK1/2 and MEK1/2 as the first and second protein to better understanding the effect of the activation of these proteins on the MAPK signaling pathway. Ex. 1002 at ¶¶121-122.

Claim 19

19. The method according to claim 18, wherein said first activatable protein and second activatable protein are independently selected from the group consisting of … p44/42 MAP kinase, …MEK1/2, …ERK1/2, ….

Claim 18 is obvious as discussed above. It would have been obvious to one of skill in the art in view of Darzynkiewicz and Yen to analyze the activated form of ERK1/2 and MEK1/2 to better understanding the effect of the activation of these proteins on the MAPK signaling pathway. Ex. 1002 at ¶¶121-122.

Claim 20

20. The method according to claim 19, wherein said first activatable protein and second activatable protein are independently selected from the group consisting of … ERK1/2, MEK1/2, ….

Claim 19 is obvious as discussed above. It would have been obvious to one of skill in the art in view of Darzynkiewicz and Yen to analyze the activated form

of ERK1/2 and MEK1/2 to better understanding the effect of the activation of these proteins on the MAPK signaling pathway. Ex. 1002 at ¶¶121-122.

Claim 21

21. The method according to claim 1, 4, 5 or 6, wherein said first distinguishably labeled activation state-state specific antibody is selected from the group consisting of: …, anti-phospho-p44/42 MAP kinase (Thr202/Tyr204), … anti-phosphoMEK1/2 (Ser217/221), ….

Claims 1 and 4 are obvious as discussed above. In light of the teachings of Darzynkiewicz in view of Yen, it would have been obvious to one of skill in the art to use Darzynkiewicz's method and to use anti-phospho-p44/42 MAPK (Thr202/Tyr204) that has been distinguishably labeled to detect activated ERK1/2 and anti-phospho-MEK1/2 (Ser217/221) that has been distinguishably labeled to detect activated MEK1/2. Ex. 1002 at ¶¶121-122.

Claim 23

23. The method according to claim 1, wherein said activated isoform of either the first or second activatable protein is created through a covalent addition, structural change, or conformational change.

Claim 1 is obvious as discussed above. Darzynkiewicz in view of Yen teaches that the activation via phosphorylation of proteins that regulate the cell cycle, such as retinoblastoma, ERK 1/2 and MEK 1/2, . Ex. 1002 at ¶123. One of

skill in the art understands that activation by phosphorylation requires the covalent addition of a phosphate group to a protein which causes a conformation change in the protein due to the addition of the phosphate group. Ex. 1002 at ¶123.

Claim 24

24. The method according to claim 23 wherein said conformational change causes said activated isoform of either the first or second activatable protein to present at least one epitope that is not present in a non-activated isoform of either the first or second activatable protein.

Claim 1 is obvious as discussed above. The conformational change of any of retinoblastoma, ERK 1/2, and MEK 1/2 due to activation results in at least one epitope being present that is not present prior to the activation. Ex. 1002 at ¶124. For example, anti-phospho-p44/42 MAPK (Thr202/Tyr204) specifically targets the phospho-Thr202/phospho-Tyr204 epitopes in ERK 1/2 that are not present in un-phosphorylated ERK 1/2. *Id*. Similarly, anti-phospho-MEK1/2 (Ser217/221) specifically targets the phospho-Ser217/phospho-Ser221 epitopes in MEK 1/2 that are not present in un-phosphorylated MEK 1/2. *Id*.

Claim 25

25. The method according to claim 1 wherein the activated isoform of either the first or second activatable protein is a phosphorylated or a cleaved product of either the first or second activatable protein.

Claim 1 is obvious as discussed above. The activation of retinoblastoma, ERK 1/2, and MEK 1/2 are all due to phosphorylation. Ex. 1002 at ¶123. Therefore, the activated isoforms of these proteins are the phosphorylated products of their respective proteins.

Claim 26

26. The method according to claim 1 wherein the activated isoform of either the first or second activatable protein is created through binding to a third protein or protein complex.

Claim 1 is obvious as discussed above. As discussed with respect to claim 1, the first or second activatable protein can be MEK 1/2. It was well known at the time of the invention that the active form of MEK 1/2 is created by binding un-activated MEK 1/2 with Raf or MEK Kinase proteins. Ex. 1002 at ¶125.

Claim 27

27. The method according to claim 1 wherein the activated isoform of either the first or second activatable protein is created through binding to a soluble small molecule entity.

Claim 1 is obvious as discussed above. One of skill in the art would recognize GTP as a soluble small molecule which activates Ras. Ex. 1002 at ¶126. Ras is a protein that is within the MAPK signaling pathway like MEK 1/2 and

ERK 1/2. *Id*. In addition, antibodies specific to the activated form of Ras were known at the time of the invention. *Id*. at ¶127; *see also* Ex. 1013 at 119-120.

Therefore, one of skill in the art would have considered it obvious to apply the method of Darzynkiewicz to analyze the activation state of Ras since an activation state specific antibody existed and Ras is part of the MAPK signaling pathway. Ex. 1002 at ¶128.

E. Claims 3, 7-9 and 22 are rendered obvious under 35 U.S.C. § 103 by Darzynkiewicz in view of Yen and Belloc

As discussed above, Darzynkiewicz and Yen teach a method of using flow cytometry to concurrently detect isoforms of multiple different activated proteins that regulate cellular activity, including retinoblastoma, ERK 1/2, and MEK 1/2, using activation state specific antibodies. Belloc adds the teaching that those of skill in the art were also interested in analyzing the active isoforms of caspases to better understand cell cycle regulation.

In particular, Belloc teaches that "[c]aspase activation is now well known as a key event in the apoptotic process." Ex. 1007 at 152. Belloc further teaches that "we found that this caspase 3 activation can be detected by the anti-cleaved caspase 3 antibodies" and "[t]his labeling can be used in multivariate analysis." Ex. 1007 at 152-53. Fluorescently labeled antibodies conjugated to multiple proteins of interest, including activated caspase 3, were concurrently detected using a flow cytometer. *See e.g.*, *id*. at 153 and 159.

Belloc also recognizes that other caspases regulate cellular activity, including caspase-1. Ex. 1007 at 152. In addition, researchers were actively investigating immunoassays that correlate the activation states of caspase-1 and -3 using activation state-specific antibodies for each. Ex. 1002 at ¶97; *see also* Ex. 1014 at 13902 and Ex. 1001 at 28:56-62.

Therefore, at the time of the invention one of skill in the art would have been motivated to use Darzynkiewicz's and Belloc's teachings to concurrently detect activated caspases-1 and -3 using activation state specific antibodies to gain further understanding of the mechanics of how a cell regulates itself, and in particular how caspases regulate aptoptosis within a cell. Ex. 1002 at ¶¶132 and 133

Claim 3

3. The method according to claim 1, wherein said first activatable protein is a caspase.

Claim 1 is obvious as discussed above. At the time of the invention, one of skill in the art would understand caspase-3 is an activatable caspase. Ex. 1007 at 152; Ex. 1002 at ¶¶15 and 130.

Therefore, in light of Darzynkiewicz's teaching that his method should be used to study "the concurrent levels of other proteins that participate in the regulation of the cell cycle," it would have been obvious to analyze activated caspase-3 using multivariate flow cytometry as taught by Belloc with the methods

taught by Darzynkiewicz to analyze apoptosis in a cell. Ex. 1002 at ¶132; Ex. 1005 at 27:31-33.

Claim 7

7. The method according to claim 1, wherein said first activatable protein is a first caspase and said second activatable protein is a second caspase.

Claim 1 is obvious as discussed above. At the time of the invention, one of skill in the art would understand caspase-3 is an activatable caspase. Ex. 1007 at 152; Ex. 1002 at ¶¶15 and 130. One of skill in the art that at the time of the invention knew researchers were actively investigating assays that correlate the activity of other different caspases as well, such as caspases-1 and -3. Ex. 1002 at ¶130; *see also* Ex. 1014 at 13902 and Ex. 1001 at 28:56-62.

Therefore, it would have been obvious to use caspase-3 for the claimed first activatable protein and another caspase, such as caspase-1, as the claimed second activatable protein to study the regulation of apoptosis. Ex. 1002 at ¶¶132.

Claim 8

8. The method according to claim 7, wherein said activated isoform of said first caspase is a cleaved product of a first pro-caspase, and said activated isoform of said second caspase is a cleaved product of a second pro-caspase.

Claim 7 is obvious as discussed above. One of ordinary skill in the art understands the active forms of caspases-1 and -3 are the cleaved products of their respective pro-caspases. Ex. 1002 at ¶131; Ex. 1007 at 152 ("Procaspase 3 is a constitutive proenzyme that is activated by cleavage during apoptosis.").

Claim 9

9. The method according to claim 8, wherein said at least two distinguishably labeled activation state-specific antibodies comprise a first distinguishably labeled activation state-specific antibody that is specific for said activated isoform of said first caspase, and a second distinguishably labeled activation state-specific antibody that is specific for said activated isoform of said second caspase.

Claim 8 is obvious as discussed above. One of skill in the art that at the time of the invention understood that activation state-specific antibodies for caspases-1 and -3 were available at the time of the invention. Ex. 1002 at ¶130; *see also* Ex. 1014 at 13902 and Ex. 1001 at 28:56-62. Therefore, it would have been obvious to distinguishably label those antibodies to analyze activated caspase-1 and -3 using multivariate flow cytometry. Ex. 1007 at 152 ("[t]his labeling can be used in multivariate analysis"); Ex. 1002 at ¶132.

IX. CONCLUSION

In view of the foregoing, claims 1-27 of the '584 patent are not patentable and Petitioner requests that IPR be instituted and claims 1-27 of the '584 patent be canceled.

October 4, 2016

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CERTIFICATE OF COMPLIANCE

I, the undersigned, certify that the above IPR Petition complies with the applicable type-volume limitations of 37 C.F.R. § 42.24(a)(i). Exclusive of the portions exempted by 37 C.F.R. §42.24(a), this IPR Petition, including footnotes, contains 13,999 words, as counted by the word count function of Microsoft Word. This is less than the limit of 14,000 words as specified by 37 C.F.R. §42.24(a)(i).

Date: October 4, 2016

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CERTIFICATE OF SERVICE UNDER 37 C.F.R. § 42.6 (e)(4)

The undersigned hereby certifies that a copy of the **PETITION FOR INTER PARTES REVIEW**, all accompanying exhibits, and the **POWER OF ATTORNEY** has been served via Federal Express to the patent owner on October 4, 2016, upon the following: Bozicevic, Field & Francis LLP, 1900 University Avenue, Suite 200, East Palo Alto, CA 94303.

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