ANTICANCER, INC.
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Attorney for Plaintiff AntiCancer, Inc.

UNITED STATES DISTRICT COURT

FOR THE SOUTHERN DISTRICT OF CALIFORNIA

ANTICANCER, INC., a California corporation,

Plaintiff,

v.

CELLSIGHT TECHNOLOGIES, INC., a Delaware corporation; and DOES 1-50,

Defendants.

Case No. 10 CV 2515 MMA BLM

COMPLAINT FOR:

(1) INFRINGEMENT OF U.S. PATENTS NOS. 6,759,038 AND 6,649,159
(2) COPYRIGHT INFRINGEMENT
(3) FALSE DESIGNATION OF ORIGIN AND FALSE DESCRIPTION
(4) UNFAIR COMPETITION

DEMAND FOR JURY TRIAL

AntiCancer, Inc., by and through its counsel, alleges for its Complaint against CellSight Technologies, Inc. and Does 1-50, inclusive as follows:

JURISDICTION AND VENUE

1. This Court has subject matter jurisdiction over AntiCancer's claims for patent infringement, copyright infringement, trademark infringement and related claims arises under Titles 35, 17, and 15 of the United States Code and under 28 U.S.C. §§ 1331 and 1338(a).

2. This Court has supplemental jurisdiction over AntiCancer's claims arising

under the laws of the State of California pursuant to 28 U.S.C. § 1367(a) because those claims are so related to AntiCancer's claims under federal law that they form part of the same case or controversy and derive from a common nucleus of operative fact.

3. Venue is proper in this judicial district under pertinent law, including, *inter alia*, 28 U.S.C. §§ 1391(b), (c).

THE PARTIES

4. Plaintiff AntiCancer, Inc. ("AntiCancer") is a corporation organized and existing under the laws of the State of California and having as its principal place of business San Diego, California. Via years of research and innovation (and large investments of time, capital, and effort by its scientists and researchers), AntiCancer has developed patented techniques to, among other things,

- track metastasis of tumor cells in live lab animals through the use of fluorescent proteins, including green fluorescent protein ("GFP"), a protein which occurs naturally in a species of jellyfish, *Aequorea victoria* (known as the crystal jelly);
- optically image gene expression in live animals; and
- evaluate candidate protocols or drugs for treating disease using fluorescent proteins.

5. GFP is understood by those skilled in the art to mean a protein which fluoresces green or any other color and includes fluorophores such as red fluorescent protein (RFP) and/or DsRed.

6. AntiCancer has both developed and practices groundbreaking methods of fluorescence optical imaging. AntiCancer's scientists engineer tumor cells encoded with GFP and other fluorophores, which glow when excited by blue light. These tumor cells are implanted into laboratory animals (such as live mice) via such means as subcutaneous injection and surgical orthotopic implantation. When the cells fluoresce, they glow green (or other colors, depending on the fluorescent protein used), enabling scientists to track their growth and spread in the living animal in real time by fluorescence imaging (or afterward under a microscope). These methods are highly useful for learning whether a given drug or treatment regimen is slowing, stopping, or having no effect on the tumor cells being looked at.

7. AntiCancer is widely recognized as a world leader in fluorescence optical imaging. For example, the National Cancer Institute (NCI) has recognized AntiCancer in its print publications as "a leader in small-animal imaging technology and mouse models" and the developer of "leading mouse models for cancer research." In these same publications NCI noted that AntiCancer's mouse models "are now used in contract research with pharmaceutical and biotechnology companies to support novel cancer drug discovery and evaluation." And, in announcing the 2008 award of the Nobel Prize in Chemistry to three pioneers in the field of GFP, the Nobel committee cited AntiCancer's methods of using GFP to watch cancer cells spread by stating:

> The remarkable brightly glowing green fluorescent protein, GFP, was first observed in the beautiful jellyfish, *Aequorea victoria*, in 1962. Since then, this protein has become one of the most important tools used in contemporary bioscience. With the aid of GFP, researchers have developed ways to watch processes that were previously invisible, such as the development of nerve cells in the brain **or how cancer cells spread**.

(Emphasis added.)

8. AntiCancer has trademarked it's fluorescence mouse models as, among other trademarks, "MetaMouse." (See Certificate of Registration, Exhibit 1). Optical images obtained through the use of MetaMouse models are extremely valuable to researchers, and a primary purpose of a customer or licensee purchasing AntiCancer's products and services is to obtain such quality images. Thus, AntiCancer's use of MetaMouse images in its marketing and advertising are seen in the public as both proof of the efficacy of AntiCancer's products and services, as well as the actual product and service itself.

9. Defendant CellSight Technologies, Inc. ("CellSight") is a corporation organized and existing under the laws of the State of Delaware and having as its principal places of business in San Francisco and Los Angeles, California. It is primarily a CRO offering molecular imaging services using "multiple imaging strategies in living subjects," including fluorescence optical imaging services. On information and belief, CellSight has extensive contacts with, advertises to, and provides services for customers in San Diego County's academic, biotech, and pharmaceutical industries.

10. The true names and capacities, whether individual, corporate, associate, representative or otherwise, of DOES 1 through 50, inclusive, are unknown to plaintiff, who therefore sues them by such fictitious names. Plaintiff will seek leave to amend this complaint to show the true names and capacities of said defendants when they are ascertained. Plaintiff is informed and believes, and thereupon alleges, that each of the defendants named as a Doe, along with the named defendants, is responsible in some manner for the occurrences herein alleged, and that plaintiff's injuries herein alleged were legally or proximately caused by said defendants. Wherever it is alleged that any act or omission was also done or committed by any specifically named defendant, or by defendants generally, plaintiff intends thereby to allege, and does allege, that the same act or omission was also done and committed by each and every defendant named as a Doe, and each named defendant, both separately and in concert or conspiracy with the named defendants.

11. At all times mentioned herein, defendants, and each of them, were the agents, servants, co-conspirators, or employees of one another, and the acts and omissions herein alleged were done or suffered by them, acting individually and through or by their alleged capacity, within the scope of their authority. Each of the defendants aided and abetted and rendered substantial assistance in the accomplishment of the acts complained of herein. In taking the actions, as particularized herein, to aid and abet and substantially assist in the commission of the misconduct complained of, each defendant acted with an awareness of his, her or its primary wrongdoing and realized that his, her or its conduct would substantially assist in the accomplishment of that misconduct and was aware of his, her or its overall contribution to, and furtherance of the conspiracy, common enterprise, and common course of conduct. Defendants' acts of aiding and abetting included, *inter alia*, all of the acts each defendant is alleged to have committed in furtherance of the conspiracy, common enterprise, and common course of conduct complained of herein.

THE PATENTS-IN-SUIT

12. U.S. Patent. No. 6,759,038 (the "'038 patent"). The '038 patent (Exhibit 2) relates to the study of tumor progression. Specifically, it concerns model systems for tracking

cancer cells and tumor metastasis in vertebrates and evaluating candidate drugs for treating the tumors. It claims methods for following metastasis by looking at GFP-expressing tumor cells in vertebrate animal organ tissues, including humans. The priority date of the '038 patent is March 27, 1998.

13. U.S. Patent. No. 6,649,159 (the "'159 patent"). The '159 patent (Exhibit 3) relates to the whole-body external optical imaging of gene expression. It claims methods for such imaging, and methods for evaluating candidate protocols or drugs for treating disease using fluorophores linked to the endogenous promoters of genes. These methods offer noninvasive and real-time means for recording and analyzing gene expression in animals and humans. The '159 patent does not limit the methods by which the images produced by fluorescence optical tumor imaging can be monitored. Instead, any suitable methods are encompassed by the claims of the '159 patent. (For example, Example 1 to the specification of the '159 patent provides that high resolution images can be captured by computer, or continuously through video output onto videotape.) The priority date of the '159 patent is March 17, 2000.

14. When a user uses AntiCancer's methods to image GFP-expressing tumor cells or gene expression in a lab animal, it infringes AntiCancer's patents unless done pursuant to a license with AntiCancer.

15. AntiCancer frequently practices these (and other) methods for its own customers as part of its business as a CRO, both for commercial customers (such as pharmaceutical companies) and non-commercial customers (such as universities). AntiCancer has also extensively licensed the '159 and '038 patents.

DEFENDANTS' WRONGFUL COURSE OF CONDUCT

16. CellSight's website offers various products and services that individually and/or when combined directly infringe on claims in AntiCancer's '038 and '159 patents, and/or induce CellSight's customers to so infringe, including but not limited to the following:

- "Cell Kinetics Services Offered[:] Track changes in transplanted-tissues at multiple desired time points . . . Analyze effect of drugs on cells" (Exhibit 4);

- "Gene Expression Services Offered[:] Monitor endogenous gene expression and effect of external factors on the expression" (Exhibit 4, Pages 1-2);

- "Custom Services Offered[:] Genetically engineer cells to express one or more imaging reporter genes of different modalities[;] Assay cells or tissues for imaging reporter gene expression[;] . . . Pre-clinical and clinical reporter gene imaging studies[;] Monitor cancer therapy efficacy through reporter gene imaging or general molecular imaging" (Exhibit 4, Page 2); and

- "Methods to perform reporter gene imaging in living subjects include: Fluorescence[;] Bioluminescence[;] Positron Emission Tomography (PET)[:] CellSight Technologies provides means to combine the three methods of gene imaging with a single tri-fusion multimodality reporter gene. You can then track implanted cells or genes from preclinical applications and then translate to clinical applications. . . .Therapy developers can use the same triple fusion gene during therapy development for imaging cells in vitro all the way to imaging living subjects." (Exhibit 5, Page 2).

- "Technology - Genetic Engineering[:] Reporter Gene Technique For Imaging Cell Kinetics[:] Cells desired to be monitored are genetically engineered in culture by transfection, transduction or electroporation/nucleofection to constitutively express the reporter gene and then administered into the living subject. At any desired time-point thereafter a specific imaging probe can be injected to determine cell kinetics. This technique can be used to image all aspects of cell kinetics." (Exhibit 6).

- "Technology - Fluorescence Optical Imaging[:] Fluorescence images are obtained by detecting light of a certain wavelength emitted from molecules that have been excited by light of another wavelength. Various sensitive instruments are available for fluorescence imaging." (Exhibit 7); and

- "Fluorescence Reporter Genes[:] Fluorescence reporter genes, such as those encoding green fluorescent protein (GFP) and red fluorescent protein offer many in vitro and in vivo molecular-genetic applications. . . . Fluorescence Imaging of Reporter genes Can be Used to Image the Following Non-Invasively

in Small Animals[:] Transgene expression (Gene therapy monitoring)[;] Endogenous gene expression[;] Cell kinetics (cell therapy, regenerative medicine and cancer therapy)" (Exhibit 7);

17. CellSight's "Technology - Fluorescence Optical Imaging" web page prominently features a copyrighted and trademarked AntiCancer MetaMouse image of an RFP glioma (brain tumor) in a living mouse (Exhibit 7). CellSight copied this image directly from AntiCancer's website. Exhibit 8 is a copy of the page from AntiCancer's Metamouse website, which shows the image, along with its associated copyright notice, MetaMouse trademark, and indications of origin, all of which were deleted by CellSight before being posted on the CellSight website. Notably, this image is the one and only fluorescence optical image of an experimental animal featured on the entire CellSight website.

18. Adjacent to AntiCancer's MetaMouse image in Exhibit 7 is an illustration that reproduces, in all pertinent respects, Figure 2B in AntiCancer's '159 patent (see Exhibit 3, page 3).

19. CellSight's website prominently features and distributes a book entitled *Molecular Imaging With Reporter Genes*, which is edited (and written in large part) by the Chairman of CellSight's Scientific Advisory Board, Sanjiv Sam Gambhir, MD, Ph.D. ("Dr. Gambhir") and CellSight's Chief Technology Officer, Shahriar S. Yaghoubi, Ph.D. (Dr. Yaghoubi") (Exhibit 9). CellSight's prominent distribution of this book on its website, directly alongside advertisements for CellSight's services, implies and represents to CellSight's past, current, and prospective customers that CellSight has practiced the methods described in the book and is offering its commercial services to perform such methods for customers.

20. *Molecular Imaging With Reporter Genes* contains numerous descriptions and detailed instructions for performing fluorescence optical imaging in ways that individually or when combined with each other or with other statements made by CellSight on its website, directly infringe on claims in AntiCancer's '038 and '159 patents, and/or induce CellSight's customers to so infringe, including but not limited to the following illustrative examples:

- "Monitoring Tumor Mass[:] Noninvasive reporter gene imaging offers excellent opportunities to understand cancer progression, metastasis, and therapy in whole animals. Individual animals can be visually monitored for tumor burden at primary sites, difference in tumor progression rates can be distinguished, the possibility of metastases can investigated, individual responses to alternative therapies can be repeatedly monitored, and therapies can be altered and the consequences of these alterations observed." (Exhibit 9, page 2); and

- "Optical fluorescence imaging (FLI) can image a variety of *in vivo* processes including gene expression occurring in cells located within tissues of live small laboratory animal subjects (mostly mice) by observing the body surface distribution of FL signal. Specific genes of interest can be linked with reporter genes in transgenic animals and their expression followed *in vivo* over the animal's lifetime. This approach has also ben used in important *in vivo* applications such as monitoring therapeutic gene delivery strategies, tracking infectious diseases, and following the proliferation of cancer cells and their progeny in xenograft and transplant tumor models. The FL proteins are isolated from living organisms and the gene that encodes for these proteins may be inserted into cells and used as a reporter gene. GFP has been widely used in biological research for cell culture and *ex vivo* study of tissue sections as well as *in vivo* studies." (Exhibit 9, page 3).

21. In or around 2009 Drs. Gambhir and Yaghoubi were interviewed about techniques used at CellSight in an article titled "Advances in Imaging Techniques Help Drive Stem Cell Research Forward." (Exhibit 10.) The article states "Gambhir founded a company, CellSight Technologies in Sunnyvale, California, to pursue reporter-based imaging techniques. They will develop methods to image 'anything about the status of the cell,' said Yaghoubi, who serves part-time as chief scientific officer." (Exhibit 10, page 2.)

22. According to an Internal Revenue Service list of companies who received grants under its Qualifying Therapeutic Discovery Project, CellSight will have spent at least $202,209.00 on "Imaging cell trafficking to advance clinical translation of cell and gene therapeutics." (Exhibit 11, page 2.)

23. At all relevant times, CellSight has engaged in a pattern of willful, direct

infringement, and inducement of third party infringement, of AntiCancer's intellectual property, including patents, copyrights, and trademarks, with the purpose and effect of unfairly competing with AntiCancer and depriving AntiCancer of licensing and contract research revenue.

FIRST CLAIM FOR RELIEF

(Infringement of '038 Patent)

24. Plaintiff realleges and incorporates by reference as though fully set forth preceding paragraphs 1 through 23.

25. The '038 Patent issued on July 6, 2004. A true and correct copy of the '038 Patent is attached hereto as Exhibit 2 and incorporated herein by this reference.

26. Plaintiff is the sole owner of the '038 Patent.

27. Plaintiff is informed and believes that CellSight has infringed, and still is infringing, the '038 patent by making, using, selling, offering for sale and/or licensing products and services covered by one or more claims of the '038 Patent without plaintiff's authorization or consent.

28. Plaintiff is informed and believes that CellSight has infringed the '038 Patent and encouraged others to do so, and will continue to do so unless enjoined by this Court.

29. Plaintiff is informed and believes, and on that basis, alleges that CellSight is aware of the '038 Patent and that its infringement has been willful.

30. Plaintiff is informed and believes that CellSight is actively inducing and/or contributing to infringement of the '038 Patent by others, all of whom are sued herein as DOES 1 through 50. Plaintiff will seek leave to amend this complaint to show the true names and capacities of said defendants when they are ascertained.

31. By reason of the foregoing, plaintiff has suffered damages in an amount to be proven at trial and, in addition, has suffered irreparable loss and injury.

32. The acts of infringement described above are willful, deliberate and in reckless disregard of plaintiff's patent rights.

SECOND CLAIM FOR RELIEF

(Infringement of '159 Patent)

33. Plaintiff realleges and incorporates by reference as though fully set forth preceding paragraphs 1 through 32.

34. The '159 Patent issued on November 18, 2003. A true and correct copy of the '159 Patent is attached hereto as Exhibit 3 and incorporated herein by this reference.

35. Plaintiff is the sole owner of the '159 Patent.

36. Plaintiff is informed and believes that CellSight has infringed, and still is infringing, the '159 patent by making, using, selling, offering for sale and/or licensing products and services covered by one or more claims of the '159 Patent without plaintiff's authorization or consent.

37. Plaintiff is informed and believes that CellSight has infringed the '159 Patent and encouraged others to do so, and will continue to do so unless enjoined by this Court.

38. Plaintiff is informed and believes, and on that basis, alleges that CellSight is aware of the '159 Patent and that its infringement has been willful.

39. Plaintiff is informed and believes that CellSight is actively inducing and/or contributing to infringement of the '159 Patent by others, all of whom are sued herein as DOES 1 through 50. Plaintiff will seek leave to amend this complaint to show the true names and capacities of said defendants when they are ascertained.

40. By reason of the foregoing, plaintiff has suffered damages in an amount to be proven at trial and, in addition, has suffered irreparable loss and injury.

41. The acts of infringement described above are willful, deliberate and in reckless disregard of plaintiff's patent rights.

THIRD CLAIM FOR RELIEF

(Copyright Infringement)

42. Plaintiff realleges and incorporates by reference as though fully set forth preceding paragraphs 1 through 41.

43. AntiCancer's website contains original proprietary material that is copyrightable subject matter under the Copyright Act, 17 U.S.C. § 101 *et seq.*, including without limitation a MetaMouse webpage composition containing an optical image of a RFP glioma in the brain of a mouse (see Exhibit 8). On or about December 6, 2010 AntiCancer submitted to the Register of Copyrights, and the Register received, a completed application for registration, deposit copy, and the applicable fee in order to register the copyright to the composition. AntiCancer will seek leave of court to amend its complaint to attach and incorporate by reference a copy of the certificate of registration when it receives it.

44. Without AntiCancer's consent, authorization, approval, or license, CellSight knowingly, willingly, and unlawfully copied, prepared, published, and distributed AntiCancer's copyrighted work, or portions thereof, and continues to do so, including without limitation AntiCancer's RFP glioma image published prominently on CellSight's website (see Exhibit 7). CellSight's website infringes AntiCancer's copyrights and is not licensed to do so.

45. On information and belief, CellSight's infringement is and has been knowing and willful within the meaning of the Copyright Act, and the award of statutory damages should be enhanced in accordance with 17 U.S.C. § 504(c)(2).

46. By this unlawful copying, use, and distribution, CellSight has violated AntiCancer's exclusive rights under 17 U.S.C. § 106. CellSight has realized unjust profits, gains and advantages as a proximate result of its infringement. CellSight will continue to realize unjust profits, gains and advantages as a proximate result of its infringement as long as such infringement is permitted to continue. AntiCancer is entitled to an injunction restraining CellSight from engaging in any further such acts in violation of the United States copyright laws.

47. As a direct and proximate result of CellSight's direct and indirect willful copyright infringement, AntiCancer has suffered, and will continue to suffer, monetary loss to its business, reputation, and goodwill. AntiCancer is entitled to recover from CellSight, in amounts to be determined at trial, the damages sustained and will sustain, and any gains,

profits, and advantages obtained by CellSight as a result of CellSight's acts of infringement and CellSight's use and publication of the copied materials.

FOURTH CLAIM FOR RELIEF

(False Designation of Origin, False Description and Representation of AntiCancer's Product – 15 U.S.C. § 1125 *et seq.*)

48. Plaintiff realleges and incorporates by reference as though fully set forth preceding paragraphs 1 through 47.

49. Among AntiCancer's products and services are MetaMouse images which AntiCancer displays in its promotional and advertising material, including its website, along with AntiCancer's registered MetaMouse trademark.

50. CellSight's wrongful conduct includes without limitation, the removal of AntiCancer's indications of origin and/or trademark from AntiCancer's MetaMouse image/s and the use, advertising, marketing, offering, or distribution of the image/s as its own, including without limitation the image contained in Exhibit 7. CellSight's use, advertising, marketing, offering, or distribution of AntiCancer's MetaMouse image/s as its own misrepresents the source of the product/s and services and misleads the public into believing the image/s are CellSight's products or the result of CellSight's products and services. These acts constitute (a) false designation of origin, (b) false description, and (c) false representation that MetaMouse image/s originate from CellSight, all in violation of § 43(a) of the Lanham Trademark Act, set forth at 15. U.S.C. § 1125(a).

51. Upon information and belief, CellSight used, advertised, marketed, offered, or distributed AntiCancer's MetaMouse image/s with a willful and calculated purpose of misleading, deceiving, or confusing customers and the public as to the origin and authenticity of the image/s.

52. CellSight's use, advertising, marketing, offering, and distribution of AntiCancer's MetaMouse image/s is likely to continue unless restrained and enjoined.

53. As a result of CellSight's use, marketing, offering, and distribution of AntiCancer's MetaMouse image/s, AntiCancer has suffered and will continue to suffer damages and losses, including, but not limited, to irreparable injury to its business reputation and goodwill. AntiCancer

is entitled to injunctive relief and to an order compelling the impounding of all AntiCancer's images being used, offered, marketed, or distributed by CellSight. AntiCancer has no adequate remedy at law for CellSight's wrongful conduct, because among other things, (a) AntiCancer's images are unique and valuable property which have no readily determinable market value, (b) CellSight's use, marketing, or distribution of these images constitutes harm to AntiCancer's business reputation and goodwill such that AntiCancer could not be made whole by any monetary award, and (c) CellSight's wrongful conduct and the resulting damage to AntiCancer is continuing.

FIFTH CLAIM FOR RELIEF

(Unfair Competition – California Common Law)

54. Plaintiff realleges and incorporates by reference as though fully set forth preceding paragraphs 1 through 53.

55. CellSight's acts, as set forth above, constitute unfair competition under the common law of the State of California, all to the damage of AntiCancer as previously alleged.

SIXTH CLAIM FOR RELIEF

(Unfair Competition – California Business and Professions Code § 17200 *et seq.*)

56. Plaintiff realleges and incorporates by reference as though fully set forth preceding paragraphs 1 through 55.

57. CellSight took the actions described hereinabove for the twin purposes of injuring competition and harming AntiCancer. In so doing, CellSight committed unlawful, unfair and fraudulent business acts and practices, thus violating the provisions of the Unfair Competition Law, California Business and Professions Code § 17200 *et seq.*

58. As a legal and proximate result of defendants' wrongful course of conduct, AntiCancer is entitled to decrees enjoining CellSight from all further unfair competition, ordering it to cease and desist therefrom, and enjoining it to disgorge and/or make restitution of all ill-gotten gains received heretofore to AntiCancer, all pursuant to the provisions of California Business and Professions Code § 17203.

PRAYER FOR RELIEF

WHEREFORE, Plaintiff AntiCancer prays for relief as follows:

(1) That the Court enter a judgment against CellSight as indicated below:

(a) that CellSight has infringed the '038 and '159 patents, under 35 U.S.C. § 271(a), (b), (c), and (g);

(b) that CellSight has willfully infringed the '038 and '159 patents under 35 U.S.C. § 271(a), (b), (c), and (g);

(c) that CellSight has willfully infringed AntiCancer's copyright under 17 U.S.C. § 501;

(d) that CellSight has willfully committed and is committing acts of false designation of origin, false or misleading description of fact, and false or misleading representation against AntiCancer as defined in 15 U.S.C. § 1125(a);

(e) that CellSight has engaged in unfair competition in violation of the common law of the State of California; and

(f) that CellSight has engaged in unfair competition in violation of California Business and Professions Code § 17200 *et seq.*

(2) That the Court issue injunctive relief against CellSight, as well as its respective officers, agents, servants, employees and attorneys, and those persons in active concert or participation with them be preliminarily and permanently restrained and enjoined from:

(a) directly or indirectly infringing the '038 and '159 patents;

(b) imitating, copying, or making any other infringing use of AntiCancer's copyrighted material and any other works now or hereafter protected by any AntiCancer copyright;

(c) using any false designation of origin or false description which can or is likely to lead the trade or public or individuals, erroneously to believe that AntiCancer's products or services are actually CellSight's products or services; and

(d) assisting, aiding, or abetting any other person or business entity in engaging in or performing any of the activities referred to in subparagraphs (a) through (c) above.

(3) That the Court order CellSight to pay AntiCancer's damages as follows:

(a) AntiCancer's damages, including enhanced damages, and CellSight's profits pursuant to 35 U.S.C. § 284 for CellSight's willful infringement of the '038 and '159 patents;

(b) AntiCancer's damages and CellSight's profits pursuant to 17 U.S.C. § 504(b), or in the alternative, enhanced statutory damages pursuant to 17 U.S.C. § 504(c)(2), for CellSight's willful infringement of AntiCancer's copyrights;

(c) AntiCancer's damages and CellSight's profits pursuant to 15 U.S.C.§ 1117(a), trebled pursuant to 15 U.S.C. § 1117(c), for willful violation of AntiCancer's registered trademark and service mark;

(d) AntiCancer's damages and CellSight's profits pursuant to California common law; and

(e) AntiCancer's damages and CellSight's profits pursuant to California Business and Professions Code § 17206.

(4) That the Court award AntiCancer its attorney's fees incurred by it in prosecuting this action.

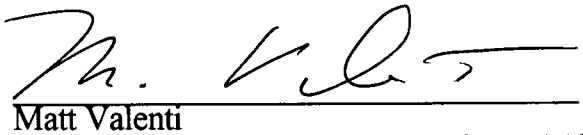
(5) That the Court assess pre-judgment and post-judgment interest and costs of suit against CellSight, and award such interest and costs to AntiCancer.

(6) That AntiCancer have such other and further relief as this Court may deem just and proper.

Respectfully submitted,

Dated: December 8, 2010

ANTICANCER, INC.

By: 

Matt Valenti
Attorney for Plaintiff ANTICANCER, INC.

DEMAND FOR TRIAL BY JURY

AntiCancer hereby demands a trial by jury as to all issues triable by jury.

Respectfully submitted,

Dated: December 8, 2010

ANTICANCER, INC.

By: _____
Matt Valenti
Attorney for Plaintiff ANTICANCER, INC.

INDEX OF EXHIBITS

Exhibit No.	Description
1.	AntiCancer's Certificate of Registration for Trademark MetaMouse dated July 4, 1995
2.	U.S. Patent No. 6,759,038
3.	U.S. Patent No. 6,649,159
4.	CellSight "Services & Products" webpage as of November 18, 2010
5.	CellSight "Technology" webpage as of November 18, 2010
6.	CellSight "Technology - Genetic Engineering" webpage as of November 18, 2010
7.	CellSight "Fluorescence Optical Imaging" webpage as of November 18, 2010
8.	AntiCancer MetaMouse Brain Cancer Models webpage
9.	*Molecular Imaging With Reporter Genes* (cover and excerpts)
10.	"Advances in Imaging Techniques Help Drive Stem Cell Research Forward"
11.	Internal Revenue Service Qualifying Therapeutic Discovery Project Grants List (excerpt)

EXHIBIT 1

EXHIBIT 1

Int. Cl.: 42

Prior U.S. Cl.: 100

Reg. No. 1,903,157

United States Patent and Trademark Office

Registered July 4, 1995

SERVICE MARK
PRINCIPAL REGISTER

METAMOUSE

ANTICANCER, INC. (CALIFORNIA CORPORATION)
7917 OSTROW STREET
SAN DIEGO, CA 92111

FOR: CONDUCTING BIOLOGICAL ASSAYS TO MEASURE BIOLOGICAL RESPONSE OF TEST AGENTS, IN CLASS 42 (U.S. CL. 100).

FIRST USE 2-23-1993; IN COMMERCE 2-23-1993.

SER. NO. 74-454,603, FILED 11-5-1993.

MICHAEL J. BURKE, EXAMINING ATTORNEY

EXHIBIT 2

EXHIBIT 2

US006759038B2

(12) **United States Patent**
Tan et al.

(10) **Patent No.: US 6,759,038 B2**
(45) **Date of Patent: *Jul. 6, 2004**

(54) **METASTASIS MODELS USING GREEN FLUORESCENT PROTEIN (GFP) AS A MARKER**

(75) Inventors: **Yuying Tan**, San Diego, CA (US); **Takashi Chishima**, Yokohama (JP)

(73) Assignee: **AntiCancer, Inc.**, San Diego, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **09/870,268**

(22) Filed: **May 29, 2001**

(65) **Prior Publication Data**

US 2002/0026649 A1 Feb. 28, 2002

Related U.S. Application Data

(63) Continuation of application No. 09/226,856, filed on Jan. 7, 1999, now Pat. No. 6,251,384, which is a continuation-in-part of application No. 09/067,734, filed on Apr. 28, 1998, now Pat. No. 6,235,968, which is a continuation-in-part of application No. 09/049,544, filed on Mar. 27, 1998, now Pat. No. 5,235,967, which is a continuation-in-part of application No. 08/848,539, filed on Apr. 28, 1997, now Pat. No. 6,232,523.

(51) **Int. Cl.**[7] **A61K 48/00**; C12N 5/00; C12N 15/63

(52) **U.S. Cl.** **424/93.21**; 800/8; 800/9; 800/10; 435/320.1; 435/325; 435/455; 514/44

(58) **Field of Search** 435/325, 320.1, 435/455; 800/8, 9, 10; 514/44; 424/93.2, 93.21

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,650,135	A	7/1997	Contag et al.	424/9.1
5,874,304	A	2/1999	Zolotukhin et al.	435/366

FOREIGN PATENT DOCUMENTS

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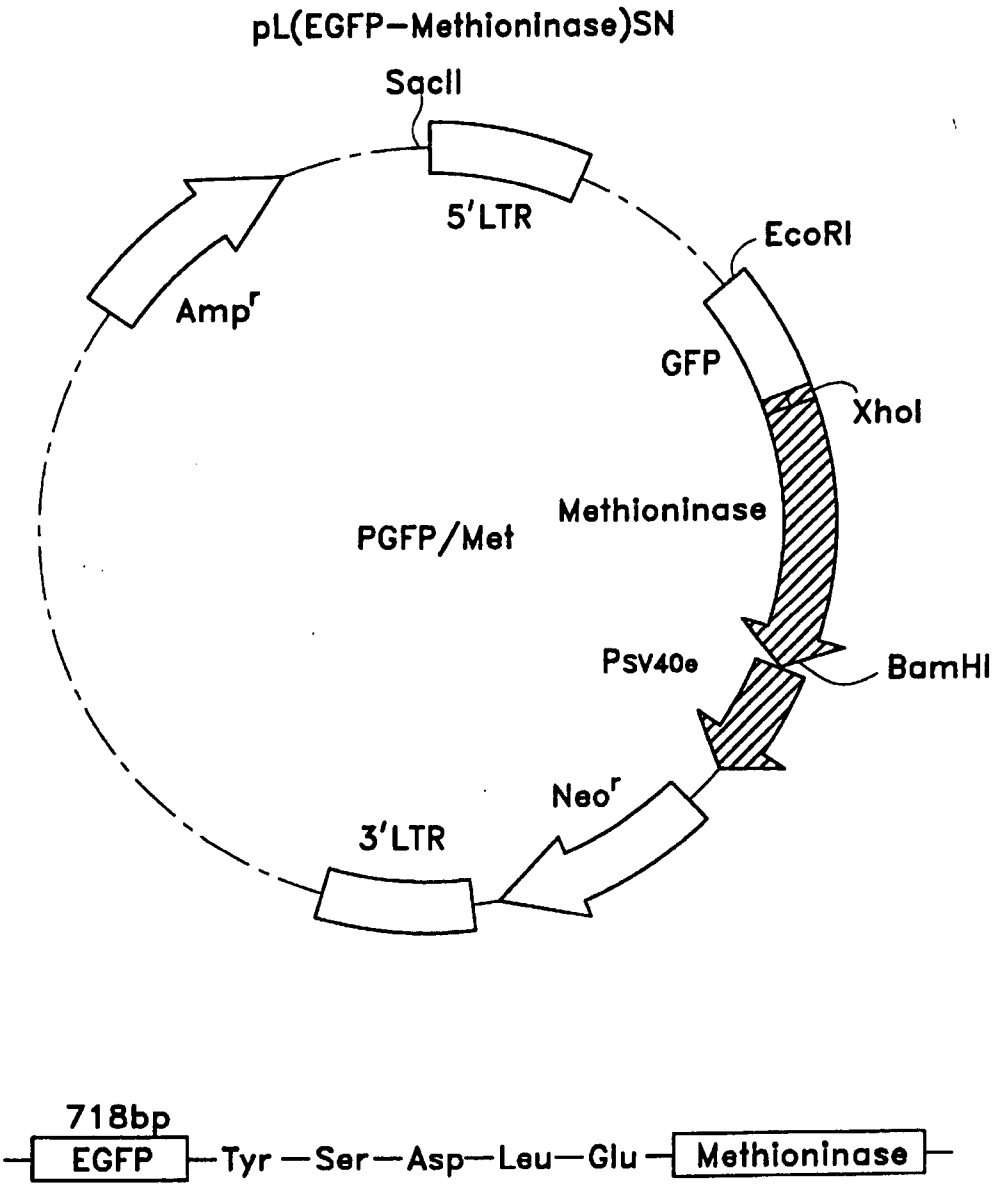
(57) **ABSTRACT**

A method to follow the progression of metastasis of a primary tumor, which method comprises removing fresh organ tissues from a vertebrate subject which has been modified to contain tumor cells that express GFP and observing the excised tissues for the presence of fluorescence is disclosed. The fluorescence can also be monitored by observing the tissues in situ. Vertebrate subjects which contain GFP producing tumors are useful models to study the mechanism of metastasis, as well as to evaluate candidate protocols and drugs. In addition, subjects already harboring tumors can be treated so as to modify the endogenous tumors to contain GFP. This permits clinical applications. Finally, by injecting a contrast dye into a subject harboring a GFP-labeled tumor, angiogenesis in the tumor can be observed directly.

12 Claims, 2 Drawing Sheets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SacII
5'LTR
EcoRI
Amp^r
GFP
XhoI
Methioninase
PGFP/Met
Psv40e
BamHI
Neo^r
3'LTR
718bp
EGFP
Tyr
Ser
Asp
Leu
Glu
Methioninase

Fig. 1a

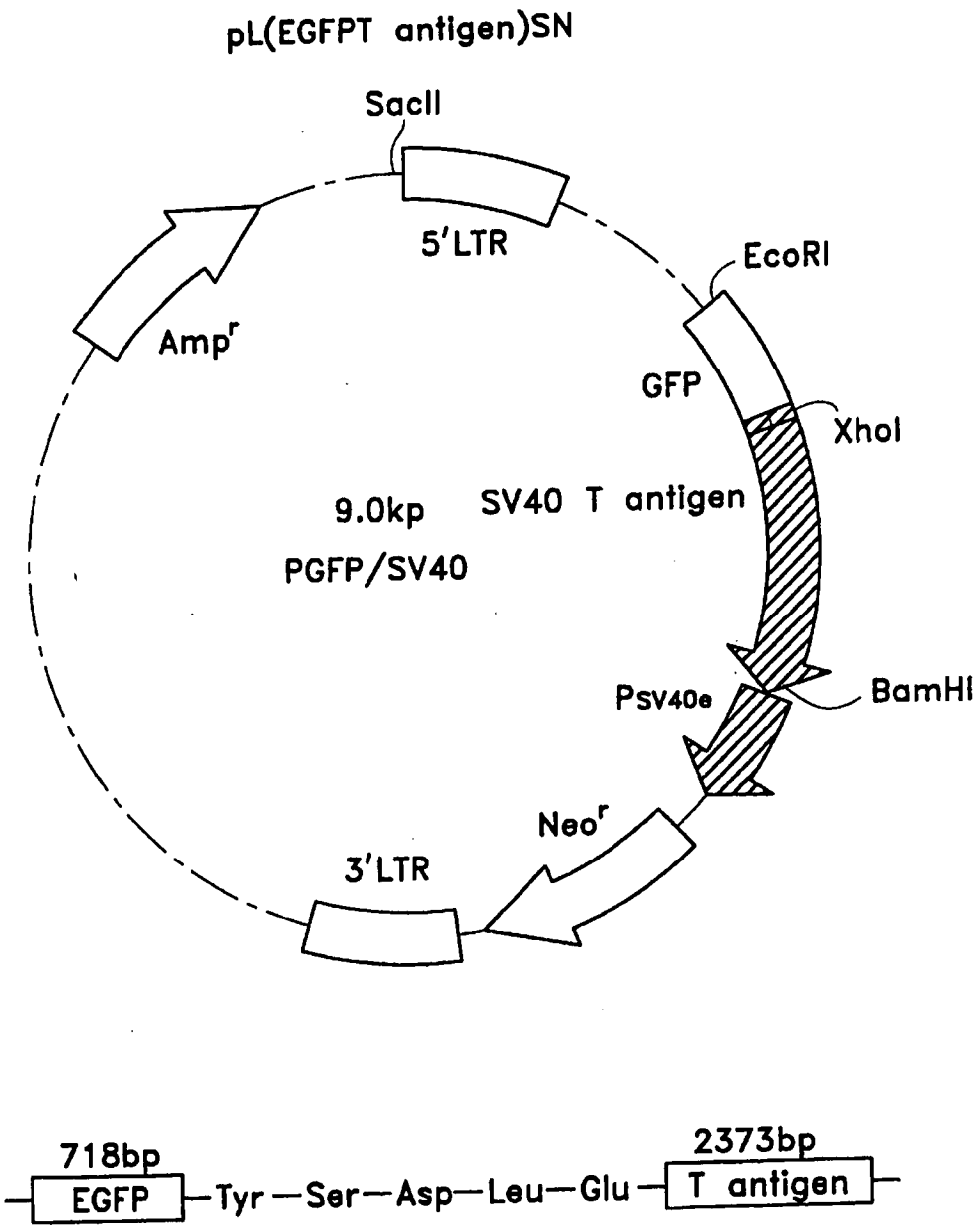
pL(EGFPT antigen)SN
SacII
5'LTR
EcoRI
Amp^r
GFP
XhoI
9.0kp
SV40 T antigen
PGFP/SV40
Psv40e
BamHI
Neo^r
3'LTR
718bp
EGFP
Tyr—Ser—Asp—Leu—Glu
2373bp
T antigen

Fig. 1b

METASTASIS MODELS USING GREEN FLUORESCENT PROTEIN (GFP) AS A MARKER

This application is a Continuation of U.S. Ser. No. 09/226,856, filed Jan. 7, 1999, now U.S. Pat. No. 6,251,384, which is a Continuation-in-part of U.S. Ser. No. 09/067,734, filed Apr. 28, 1998, now U.S. Pat. No. 6,235,968, which is a Continuation-in-part of U.S. Ser. No. 09/049,544, filed March 27, 1998, now U.S. Pat. No. 5,235,967, which is a Continuation-in-part of U.S. Ser. No. 08/848,539, filed April 28, 1997, now U.S. Pat. No. 6,232,523, the contents of which are incorporated by reference.

TECHNICAL FIELD

The invention relates to the study of tumor progression. Specifically, it concerns model systems for studying the metastasis of tumors in vertebrate systems and to models and methods for evaluating candidate drugs.

BACKGROUND ART

It has long been recognized that the ability of tumor tissues to metastasize constitutes a major portion of the life-threatening aspects of malignancy. Metastasis is the growth of secondary tumors at sites different from the primary tumor. Thus, despite surgical removal of the primary tumor, it may not be possible to arrest the progress of this condition. An understanding of the mechanism whereby metastasis occurs will be crucial to the development of protocols whereby the growth of secondary tumors can be controlled. In order to understand the mechanism of metastasis, it will be necessary to provide a model which permits identification of small numbers of tumor cells against a background of many host cells so that secondary tumor emboli and micrometastases can be observed over the course of real time.

Others have demonstrated extravasation and initial seeding steps in tumor metastasis in vitro using externally fluorescently labeled tumor cells. Khokha, R. et al., *Cancer Metastasis Rev* (1995) 14:279–301; Koop, S. et al., *Cancer Res* (1995) 55:2520–2523. Further, Margolis, L. B. et al., *In Vitro Cell Dev Biol* (1995) 31:221–226 was able to visualize the migration of externally fluorescently labeled lung tumor cells in host mouse lung in histoculture. In all cases, however, long-term observation was not possible due to the limitation of exogenous fluorescent labels. Retroviral transfer of a green fluorescent protein (GFP) gene has been shown to result in stable transfectants of human cancer cells in vitro (Levy, J. P. et al., *Nature Biotechnol* (1996) 14:610–614), as well as of hematopoietic cells (Grignani, F. et al. *Cancer Res* (1998) 58:14–19 and by Cheng, L. et al. *Gene Therapy* (1997) 4:1013–1022).

Attempts have been made to provide such a model using the β-galactosidase gene as a marker (Lin, W. C. et al., *Cancer Res* (1990) 50:2808–2817; Lin, W. C. et al., *Invasion and Metastasis* (1992) 12:197–209). However, this marker has not proved satisfactory, as fresh or processed tissue cannot be used. The present invention provides a marker which permits visualization of tumor invasion and micrometastasis formation in viable fresh tissue. In addition, by providing suitable contrast media, the method of the invention can be adapted to visualize angiogenesis in established and growing tumors. The methods of the invention can be applied not only to models of tumor growth and metastasis, but, through the use of retroviral vectors, can be employed to obtain clinical data in human subjects bearing tumors.

The present invention utilizes green fluorescent protein (GFP) as a marker. Heterologous expression of this protein, principally to monitor expression of fused DNA, was disclosed in U.S. Pat. No. 5,491,084. This document describes the expression of GFP in *E. coli* and *C. elegans* and postulates that cells in general can be modified to express GFP. Such expression, according to this document, permits not only a method to monitor expression of fused DNA, but also a means of monitoring protein localization within the cell.

The aspect of the invention which provides a metastatic model has been reported and described in a series of publications. Chishima, T. et al. *Cancer Research* (1997) 57:2042–2047 describe the construction of a dicistronic vector containing the gene for humanized green fluorescent protein (GFP) and dihydrofolate reductase (DHFR). This vector was transfected into CHO-K1 cells to obtain clone-38. Clone-38 showed stable GFP expression which was maintained in the presence of methotrexate (MTX). Clone-38 cells were injected into mice to obtain tumor fragments which were then implanted by surgical orthotopic implantation (SOI) on the ovarian serosa in nude mice. Metastasis could be followed in this model.

Chishima, T. et al. *Proc Natl Acad Sci USA* (1997) 94:11573–11576 describe the preparation of clone-26 by transfection of Anip 973 human lung adenocarcinoma cells with the codon optimized hGFP-S65T clone obtained from Clontech. Clone-26 was injected intravenously into nude mice and the resulting tumors were followed in histoculture.

Chishima, T. et al. *Clin Exp Metastasis* (1997) 15:547–552 and Chishima, T. et al. *Anticancer Res* (1997) 17:2377–2384 describe similar work with clone-26 wherein the cells were inoculated subcutaneously into nude mice resulting in a visualizable tumor which was then implanted into the visceral pleura of nude mice by SOI. Metastases were observed in this model as well.

Chishima, T. et al. *In Vitro Cell Dev Biol* (1997) 33:745–747 describe histoculture of clone-26 and visualization of growth using the fluorescence emitted by GFP.

Yang, M., et al., *Cancer Res* (1998) 58:4217–4221 describe transduction of the human lung cancer cell line H460 with a retroviral expression vector containing enhanced GFP to obtain a stable high-GFP-expressing clone. Cells from this cell line were injected into nude mice and the resulting subcutaneously growing labeled tumors were transplanted by SOI into the left lung of nude mice. Fluorescence could then be observed from the metastases in the collateral lung, pleural membrane and throughout the skeletal system.

Yang, M., et al., *Cancer Res* (In Press) report similar studies using a model for prostate tumor and showing fluorescence throughout the skeletal system in nude mice.

The contents of the foregoing publications are incorporated herein by reference.

In addition to the foregoing, Cheng, L., et al., *Gene Therapy* (1997) 4:1013–1022, describe the modification of hematopoietic stem cells using the GFP gene under control of a retroviral promoter. Although the authors state that human stem cells are transfected with this system only with difficulty, by using an enhanced form of the GFP, satisfactory brightness could be achieved.

In addition, Grignani, F., et al., *Cancer Res* (1998) 58:14–19, report the use of a hybrid EBV/retroviral vector expressing GFP to effect high-efficiency gene transfer into human hematopoietic progenitor cells.

Vectors containing various modified forms of GFP to provide various colors are marketed by Clontech. The Clon-

tech vectors intended for mammalian cell expression place the GFP under control of the cytomegalovirus (CMV) promoter.

DISCLOSURE OF THE INVENTION

The invention provides models which permit the intimate study of formation of metastases from primary tumors in a realistic and real-time setting. By using green fluorescent protein (GFP) as a stable and readily visualized marker, the progression of such metastasis can be modeled and the mechanism elucidated.

Thus, in one aspect, the invention is directed to a method to follow the progression of metastasis of a primary tumor, which method comprises removing fresh organ tissues from a vertebrate subject which has been modified to contain tumor cells that express GFP and observing the excised tissues for the presence of fluorescence.

In one embodiment, however, it is unnecessary to remove organ tissues; rather, the fluorescence can be visualized in the whole animal by real-time fluorescence optical tumor imaging (FOTI).

In another aspect, the invention is directed to a vertebrate subject which has been modified to contain tumor cells expressing GFP.

In these aspects, the vertebrate subject may constitute a model system, such as an immunocompromised mouse wherein tumor cells or a tumor, modified to express green fluorescent protein has been introduced into the subject. The model system may be used to evaluate candidate drugs for their capacity to inhibit metastasis. Alternatively, the subject may be a human or other vertebrate which natively contains the tumor, but wherein the tumor has been subjected to viral infection or to transfection with a retroviral vector so as to produce said GFP. The efficacy of drugs administered to such patients can be evaluated by following the course of metastasis in the subject.

In still other aspects, the invention is directed to tumor cells modified to produce GFP under control of heterologous control elements, to cells that are immortalized to provide stable cell lines as well as comprising visible amounts of GFP, to tissues containing metastatic tumors that produce GFP, and to histocultures of tissues which contain such metastasized tumors.

The invention also includes a method to observe and follow angiogenesis in solid tumors which method comprises (usually) exposing and observing said tumors. The tumors will have been modified to express GFP, and the subject will have been administered a contrast dye to permit this observation.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1*a* and 1*b* show the construction of expression vectors useful in the invention.

MODES OF CARRYING OUT THE INVENTION

The invention provides model systems for the study of the mechanism of metastasis of tumors generally, as well as to study angiogenesis in solid tumors. Advantage is taken of the visible marker green fluorescence protein (GFP) to label the tumor cells so that their migration and colonization in tissues distal to the tumor can be followed as the migration and colonization progresses. Further, by administering to the subject a contrast dye, such as rhodamine, the growth of blood vessels in solid tumors which have been labeled with GFP can also be observed.

Since sufficient intensity can be achieved to observe the migration of fluorescent cells in the intact animal, in addition to determining the migration of the cells by excising organs, the progression of metastasis can be observed in the intact subject. Either or both methods may be employed to observe metastasis in evaluating, in model systems, the efficacy of potential antimetastatic drugs. The success or failure of treatments provided to patients with potentially metastatic cancers can also be followed using the materials and methods of the invention.

The label used in the various aspects of the invention is green fluorescent protein (GFP). The native gene encoding this protein has been cloned from the bioluminescent jellyfish *Aequorea victoria* (Morin, J. et al., *J Cell Physiol* (1972) 77:313–318). The availability of the gene has made it possible to use GFP as a marker for gene expression. GFP itself is a 283 amino acid protein with a molecular weight of 27 kD. It requires no additional proteins from its native source nor does it require substrates or cofactors available only in its native source in order to fluoresce. (Prasher, D.C. et al., *Gene* (1992) 111:229–233; Yang, F. et al., *Nature Biotechnol* (1996) 14:1252–1256; Cody, C. W. et al., *Biochemistry* (1993) 32:1212–1218.) Mutants of the GFP gene have been found useful to enhance expression and to modify excitation and fluorescence. GFP-S65T (wherein serine at 65 is replaced with threonine) is particularly useful in the invention method and has a single excitation peak at 490 nm. (Heim, R. et al., *Nature* (1995) 373:663–664); U.S. Pat. No. 5,625,048. Other mutants have also been disclosed by Delagrade, S. et al., *Biotechnology* (1995) 13:151–154; Cormack, B. et al., *Gene* (1996) 173:33–38 and Cramer, A. et al. *Nature Biotechnol* (1996) 14:315–319. Additional mutants are also disclosed in U.S. Pat. No. 5,625,048. By suitable modification, the spectrum of light emitted by the GFP can be altered. Thus, although the term "GFP" is used in the present application, the proteins included within this definition are not necessarily green in appearance. Various forms of GFP exhibit colors other than green and these, too, are included within the definition of "GFP" and are useful in the methods and materials of the invention. In addition, it is noted that green fluorescent proteins falling within the definition of "GFP" herein have been isolated from other organisms, such as the sea pansy, *Renilla reriformis*. Any suitable and convenient form of the GFP gene can be used to modify the tumor cells useful in the models of the invention, and for retroviral transformation of endogenous tumors. The particular humanized hGFP-S65T clone is used in the examples set forth below for illustration.

Techniques for labeling cells in general using GFP are disclosed in U.S. Pat. No. 5,491,084 (supra).

In one application, the method of the invention provides a model system for studying the effects of various therapeutic candidate protocols and substances on metastatic growth of tumors.

In general, the model involves modifying a vertebrate, preferably a mammal, so as to contain tumor tissue, wherein the tumor cells have, themselves, been modified to contain an expression system for GFP. The tumor cells may arise from cell lines of the invention wherein tumor cells have been modified to contain expression systems for GFP and SV40 T-antigen. Tumors can be formed in such vertebrate systems by administering the transformed cells containing the GFP expression system and permitting these transformed cells to form tumors. Typically such administration is subcutaneous and the tumors are formed as solid masses. The tumors thus formed can be implanted in any suitable host tissue and allowed to progress, metastasize and develop.

Suitable procedures for growing the initial tumor, thus, involve transcutaneous injection of the tumor cells producing GFP, such as CHO cells, HeLa cells, carcinoma and sarcoma cell lines, well established cell lines such as the human lung adenocarcinoma line Anip 973, or lung cancer cell line H460 as well as GFP-containing human breast cancer lines MDA-MB468 and MDA-MB435; human prostate cancer lines PC3 and DU-145, human glioblastoma line 324, mouse melanoma B16 and others that may become available in the art, including the immortalized cells of the invention. The administered cells will have been modified to contain an expression system for GFP. After administration, solid tumors generally develop, typically at the site of subcutaneous injection. These tumors, which are themselves fluorescent, can then be removed and used for implantation in the model vertebrate.

Techniques for implantation of the solid tumors, now labeled with GFP, into vertebrates include direct implantation by surgical orthotopic implantation (SOI) at the desired site, typically the site from which the tumor cells were derived. Suitable sites include lung, liver, pancreas, stomach, breast, ovary, prostate, bone marrow, brain, and other tissues susceptible to malignancy. Once the solid tumors have been implanted, the vertebrate becomes a model system for studying metastasis. The tumor is thus allowed to progress and develop and the vertebrate is monitored for appearance of the GFP labeled cells at sites distal from the original implantation site. The monitoring can occur either on the whole vertebrate by opening the animal and observing the organs directly with a fluorescent microscope, or the tissues may be excised and examined microscopically. In some cases the tumors are sufficiently bright that opening the animal is unnecessary—they can be seen directly through the skin. In any case, as GFP is visible to the naked eye, no development systems to stain the tissue samples are required. Tissue samples are simply properly processed as fresh samples in slices of suitable size, typically 1 mm thick, and placed under a microscope for examination. Even colonies of less than 10 cells are thus visible. A variety of microscopic visualization techniques is known in the art and any appropriate method can be used.

It is particularly convenient to visualize the migration of tumor cells in the intact animal through fluorescent optical tumor imaging (FOTI). This permits real-time observation and monitoring of progression of metastasis on a continuous basis, in particular, in model systems, in evaluation of potential anti-metastatic drugs. Thus, the relative lack of metastasis observed directly in test animals administered a candidate drug in comparison to controls which have not been administered the drugs indicates the efficacy of the candidate and its potential as a treatment. In subjects being treated for cancer, the availability of FOTI permits those devising treatment protocols to be informed on a continuous basis of the advisability of modifying or not modifying the protocol.

In addition, the development of the tumor can be studied in vitro in histological culture. Suitable systems for such study include solid supported cultures such as those maintained on collagen gels and the like.

Suitable vertebrate subjects for use as models are preferably mammalian subjects, most preferably convenient laboratory animals such as rabbits, rats, mice, and the like. For closer analogy to human subjects, primates could also be used. Particularly useful are subjects that are particularly susceptible to tumor development, such as subjects with impaired immune systems, typically nude mice or SCID mice. Any appropriate vertebrate subject can be used, the choice being dictated mainly by convenience and similarity to the system of ultimate interest.

Any suitable expression system operable in the tumor cells to be implanted may be used. A number of vectors are commercially available that will effect expression in tumor cells of various types. The nature of the vector may vary with the nature of the tumor and the vertebrate in which it finds its origin. However, when GFP is used to visualize metastasis in a model system, it is preferred to utilize vectors which do not use retroviral or other viral promoters which may complicate the nature of the model.

In order to provide cell lines that are helpful in establishing tumors for these model systems, it is also advantageous to employ expression vectors which provide the cells with the SV40 T-antigen. The presence of this antigen ensures immortality of the culture. Thus, particularly useful in the invention are vectors which comprise expression systems that result in the production both of GFP and SV40 T-antigen.

In order to transfect and modify the transformed cells which are effective in generating tumors, any suitable transfection method may be used, such as liposomes, calcium phosphate precipitation, electroporation and use of a gene gun. Lipofection is preferred.

In contrast, when the method of the invention is used to visualize metastasis in tumors that natively occur in a subject such as a human cancer patient, vectors that employ retroviral or other viral promoters are preferred. The use of such vectors permits the insertion of an expression system for GFP into the already existent tumor. In addition, the expression system may contain nucleotide sequence encoding other useful proteins such as therapeutic proteins which permit simultaneous diagnosis of metastasis and treatment. Among such suitable proteins are included methioninase (see, for example, PCT/US93/11311 and PCT/US96/09935). Such proteins may be produced either as fusions with the GFP, or independently either using a dicistronic expression system or independent expression systems, one for the therapeutic protein and the other for the GFP.

Retroviral based expression systems for GFP have already been described by Grignani, F. et al. *Cancer Res* (1998) 58:14–19 and by Cheng, L. et al. *Gene Therapy* (1997) 4:1013–1022. In these reports, the retroviral expression system itself was used to transfect hematopoietic progenitor cells or packaging cells were employed to provide virus-containing supernatants which can be used directly for infection of the mammalian cells. Thus, in the method of the invention, the tumor contained in the vertebrate subject is typically infected with virus which has been modified and packaged to contain the expression system for GFP. In situ infection with virus results in the ability of the tumor to produce GFP and, in effect, label itself.

Various retroviral systems useful in producing proteins in mammalian cells are known in the art. Examples include commercially available vector and packaging systems such as those sold by Clontech, San Diego, Calif., including their Retro-X vectors pLNCX and pLXSN which permit expression of GFP under a variety of promoters by insertion into the multiple cloning site. These vectors contain ψ^* (the extended viral packaging signal) and antibiotic resistance genes for selection. A number of these systems have been developed for use in gene therapy, including vectors which provide a multiple cloning site sandwiched between 5' and 3' LTR derived from retroviral sources, and thus would be useful in labeling the tumors of human patients.

Thus, retroviral based vectors such as those set forth in FIGS. 1*a*–1*b* can be transfected into packaging cells and

transferred directly to targeted cancer cells or supernatants from the packaging cells can be used to infect tumor cells with the retrovirus. Preferred combinations of retrovirus and packaging cells include the GFP-retrovirus vector pLEIN in PT-67 packaging cells. Co-culture of the packaging cells with colon cancer cells results in transfer of the GFP-retrovirus to the cancer cells.

Using histoculture techniques, and supernatants from PT-67 packaging cells generating GFP-pLEIN virus, the successful modification of a human cancer tissue to display the fluorescence associated with GFP has been demonstrated. For use in vivo, the virus is administered, preferably locally to the tumor, which can be observed within hours after injection either of packaging cells or of the viral containing supernatants. The malignant cells can be identified by their green color, sometimes sufficiently bright so that the tumors can be seen through the skin.

In addition to direct observation of tumor metastasis and growth either in a model system or in a vertebrate, typically mammalian and more typically a human subject which is already afflicted by a tumor, the methods of the invention can be adapted to observe angiogenesis in solid tumors. The tumor is itself labeled with GFP as described above. The subject is then administered a contrast dye, typically by injection, preferably intravenous injection, which allows blood vessels in the tumor to be observed. Suitable dyes include rodamine and other contrast dyes. Any dye which forms a contrasting color with the green color of the GFP can be used. Preferably, the dye is coupled to an inert polymer such as polyethylene glycol to increase the length of time the dye will remain in the blood vessel. A sufficient amount of dye is provided to permit ready visualization; the amount of dye required will depend on the choice of dye, the location of the tumor, the nature of the background GFP, and the method used for observation. Within a few minutes, vessels growing into the solid tumors in such areas as the mesentery, colon wall, and omentum can be observed. Observations can be continued over substantial periods; for example, angiogenesis after several hours is still observed by using this method.

The following examples are intended to illustrate but not to limit the invention.

EXAMPLE 1

Preparation of Tumor Cells that Produce GFP

The humanized hGFP-S65T clone described by Zolotukhin, S. et al., *J Virol* (1996) 70:4646–4654 was used as the green fluorescent protein coding sequence. This codon-optimized gene was purchased from Clontech Laboratories, Inc. (Palo Alto, Calif.) and ligated into the dicistronic expression vector (pED-mtx[1]) obtained from Genetics Institute, Cambridge, Mass. and described in Kaufman, R. J. et al., *Nucleic Acids Res* (1991) 19:4485–4490. hGFP-S65T was digested with HindIII and blunted; the entire hGFP coding region was excised with XbaI and then unidirectionally subcloned into pED-mtx[1] which had been digested with PstI, blunted and then further digested with XbaI.

CHO-K1 cells were cultured in DMEM containing 10% fetal calf serum, 2 mM L-glutamine and 100 μM nonessential amino acids. Near confluent cells were incubated with a precipitated mixture of LipofectAMINE™ reagent (GIBCO) and saturating amounts of plasmids for six hours and then replenished with fresh medium. The cells were harvested by trypsin/EDTA 48 hours later and subcultured at 1:15 into selective medium containing 1.5 μM methotrexate (MTX). Cells with stably integrated plasmids were selected in MTX-containing medium and isolated with cloning cylinders (Bel-Art Products, Pequannock, N.J.) by EDTA. After amplification and transfer, Clone-38 was selected because of its high-intensity GFP fluorescence and stability.

In a similar manner, Anip 973 cells, a human lung cancer cell line obtained from Harbin Medical University, China, were cultured as described above for CHO-K1 cells except using RPMI1640 (GIBCO) in place of DMEM. Transfection, selection and amplification and transfer were conducted as described above. Clone-26 was chosen because of its high-intensity GFP fluorescence and stability.

EXAMPLE 2

Mouse Model Using Modified CHO Cells

Clone-38, which was stable at 1.5 μM MTX and which proliferated at the same rate as the parental CHO-K1 cells as ascertained by comparing doubling times, was used in this model.

Three six-week old Balb/C nu/nu female mice were injected subcutaneously with a single dose of 10^7 Clone-38 cells that had been harvested by trypsinization and washed three times with cold serum-containing medium and then kept on ice. The cells were injected in a total volume of 0.4 ml within 40 minutes of harvesting and the nude mice sacrificed three weeks after injection. All of the mice had a subcutaneous tumor ranging in diameter from 13.0 mm to 18.5 mm (mean=15.2 mm±2.9 mm). The tumor tissue was strongly fluorescent. It was shown by extracting GFP from cultured Clone-38 cells in comparison to Clone-38 cells prepared from the tumor that the levels of production of GFP were the same in both.

To construct the model, tumor fragments (1 mm^3) derived from the nude mouse subcutaneous Clone-38 tumor grown as described above, were implanted by surgical or surgical orthotopic implantation (SOI) on the ovarian serosa in six nude mice as described by Fu, X. et al., *Anticancer Res* (1993) 13:283–286, incorporated herein by reference. Briefly, the mice were anesthetized by isofluran inhalation and an incision was made through the left lower abdominal pararectal line and peritoneum to expose the left ovary and part of the serosal membrane, which was scraped with a forceps. Four 1 mm^3 tumor pieces were fixed on the scraped site with an 8–0 nylon suture and the ovary then returned to the peritoneal cavity. The abdominal wall and skin were closed with 6–0 silk sutures.

Four weeks later, the mice were sacrificed and lung and various other organs were removed. The fresh samples were sliced at approximately 1 mm thickness and observed directly under fluorescent and confocal microscopy. Samples were also processed for histological examination for fluorescence and conventional staining. Frozen sections were prepared wherein the slides were rinsed with phosphate buffer saline and fixed for 10 minutes at 4° C.; 10% formaldehyde plus 0.2% glutaraldehyde and PBS were added and the slides were then washed with PBS. The fixed tissue was stained with hematoxylin and eosin using standard techniques.

Light and fluorescence microscopy were carried out using a Nikon microscope equipped with a Xenon lamp power supply and a GFP filter set (Chromotechnology Corp., Brattleboro, Vt.). Confocal microscopy was with an MRC-600 Confocal Imaging System (Bio-Rad) mounted on a Nikon microscope with an argon laser.

The mice, at sacrifice, had tumors in the ovaries ranging in diameter from 18.7 mm–25.3 mm (mean 21.9±3.1 mm). The fresh organ tissues examined under fluorescence microscopy with no treatment of the tissues showed seeding of the tumor throughout the peritoneal cavity, including the colon (6/6 mice), cecum (5/6), small intestine (4/6), spleen (1/6), and peritoneal wall (6/6). Numerous micrometastases were detected in the lungs of all mice and multiple micrometastases were also detected on the liver (1/6), kidney (1/6), contralateral ovary (3/6), adrenal gland (2/6), para-aortic lymph node (5/6) and pleural membrane (5/6). Single-cell micrometastases could not be detected by the standard histological techniques described above and even the multiple cell colonies were difficult to detect using them. As the colonies developed, the density of tumor cells decreased markedly in the center.

In an additional experiment, 5×10^6 Clone-38 cells were injected into a nude mouse through the tail vein and the mouse sacrificed after two minutes. Fresh visceral organs were analyzed by fluorescence microscopy and showed the presence of fluorescent cells in peritoneal wall vessels which formed emboli in the capillaries of the lung, liver, kidney, spleen, ovary, adrenal gland, thyroid gland and brain.

Thus, using these techniques, progression of micrometastasis can be observed as seeded cells develop into colonies within the relevant target organs. Further, screening for micrometastases can be done easily and quickly in all systemic organs.

EXAMPLE 3

Murine Model Using Human Lung Cancer Cells

The procedures are generally those set forth in Example 2 except that Clone-26 cells as prepared in Example 1 were used instead of Clone-38 CHO cells.

A. As in Example 2, tumors were grown in six-week-old Balb/C nu/nu male mice injected subcutaneously with a single 0.4 ml dose of 10^7 Clone-26 cells within 40 minutes of harvesting by trypsinization and washing three times with cold serum-containing medium. The cells were kept on ice prior to injection. The animals were sacrificed when the tumors had reached approximately 1.2 cm diameters. The 1.2 cm tumors formed after about 5 weeks.

B. The tumor pieces, 1 mm^3, were implanted by SOI into the left visceral pleura of 8 mice as described by Astoul, P. et al., *Anticancer Research* (1994) 14:85–92; Astoul, P. *J Cell Biochem* (1994) 56:9–15, both incorporated herein by reference. Briefly, the mice were anesthetized by isofluoran inhalation an a small 1 cm transverse incision made on the left lateral chest, via the fourth intercostal space, resulting in total lung collapse. Five tumor pieces were sewn together with a 7–0 nylon surgical suture and fixed by making one knot. The lung was taken up by forceps and the tumor sewn into the lower part of the lung with one suture, after which the lung was returned to the chest cavity and the muscles and skin closed with a single layer of 6–0 silk sutures. The lung was reinflated by withdrawing air from the chest cavity with a 23-gauge needle.

C. Four of the mice were sacrificed at 4 weeks and another 4 at 8 weeks. Pleural tumors for the 4-week group ranged from 244.40 mm^3–522.88 mm^3; those from the 8 week group from 1279.08 mm^3–2714.40 mm^3. This represented mean volumes of 371 mm^3 and 1799 mm^3. Specimens of tissue were sliced at 1 mm thickness and observed directly under fluorescent microscopy using a Nikon microscope equipped with a Xenon lamp power supply and a Leica stereo fluorescence microscope equipped with a mercury lamp power supply and GFP filter sets. All of the animals showed chest wall invasion and local and regional spread of the tumor, but in the 8-week mice, all tumors involved the mediastinum and contralateral pleural cavity as well as metastases on the visceral and parietal pleura. Pulmonary hilum lymph nodes were involved in 3 of 4 mice of the 4-week group and all of the mice in the 8-week group. Cervical node involvement was detected in one of the mice of the 8-week group, but no other metastases were observed. The animals were also observed directly before the tissues were excised. The margin of the invading tumor in normal lung tissue could be detected by GFP fluorescence and a small vessel could be seen developing at the margin of the tumor.

D. In an additional experiment, 8 nude mice were injected in the tail vein with a single dose of 1×10^7 Clone-26 cells that had been harvested by trypsinization and washed 3 times with cold serum-containing medium. The injection contained a total volume of 0.8 ml within 40 min. of harvesting. Again, 4 mice were sacrificed at 4 weeks and another 4 at 8 weeks and tissue specimens were obtained and studied by microscopy as described above. Numerous micrometastatic colonies were detected in whole lung tissue in both groups ranging from 5.2 μm to 32.5 μm in the 4-week group and 5.5 μm–178.3 μm in the 8-week group. The colonies from the 8-week group did not appear further developed as compared with those from the 4-week group. Numerous small colonies ranging in number to less than 10 cells were detected at the lung surface in both groups and brain metastases were detected in 1 mouse of the 4-week group and 2 from the 8-week group. One mouse in the 8-week group had systemic metastases in the brain, the submandibular gland, the whole lung, the pancreas, the bilateral adrenal glands, the peritoneum and the pulmonary hilum lymph nodes.

E. In an additional experiment, similar to that set forth in the previous paragraph, the mice injected in a tail vein with 10^7 Clone-26 cells were sacrificed at 4, 8 and 12 weeks and the tissues examined as described. Most of the colonies and mice sacrificed at 8 weeks were not obviously further developed compared with those sacrificed at 4 weeks, but numerous small quantities ranging in number down to less than 10 cells and ranging in size from 5.5 μm–110 μm were detected at the lung surface. At 12 weeks, there were many small metastatic colonies which appeared dormant, although other colonies grew extensively by this time, reaching a size up to 1100 μM, suggesting a heterogeneity of dormant and active tumor colonies in the lung.

EXAMPLE 4

Growth of Clone-26 Tumor Cells in Histoculture

Six-week old SCID/SCID mice were injected intravenously with a single dose of 7.5×10^7 Clone-26 cells which had been harvested by trypsinization and washed 3 times with cold serum-containing medium and kept on ice as described above. The cells were injected in a total volume of 0.5 ml within 40 minutes of harvesting. After 3 weeks, numerous micrometastatic colonies were detected in whole lung tissue up to approximately 550 μm. After 5 weeks, the mice were sacrificed and the Clone-26 seeded mouse lungs were removed and histocultured on spun gels using the histoculture methods developed by Leighton, J. *Cancer Res* (1957) 17:929–941; Leighton, J. et al., *Cancer Res* (1960) 20:575–597; Hoffman, R. M. *Cancer Cells* (1991) 3:86–92. Tumor colonies spread rapidly in the lung tissue over time

and after 1 week the tumor cells started to invade and colonize supporting collagen sponge-gel. After 2 weeks, tumor cells formed satellite colonies in the sponge-gel distant from the primary colonies in the lung tissue, thus growing faster in histoculture than in SCID mice. Tumor colonies could grow in histoculture for more than 1 month.

EXAMPLE 5

Construction of a Retroviral Expression Vector for GFP and Preparation of Labeled Tumor Cell Lines

FIGS. 1*a* and 1*b* show the construction of expression vectors for GFP under control of the SV40 promoter. The constructs employ commercially available pEGFP series vectors available from Clontech. Both bacterial and mammalian expression vectors are available which permit production of additional proteins, as well as GFP, either as fusions or in dicistronic systems. FIG. 1*a* shows the construction of an expression vector, pGFP/Met, for a fusion of GFP with methioninase; FIG. 1*b* shows the construction of a vector pGFP/SV40 for production of a fusion protein of GFP with the SV40 T-antigen.

Commercial vectors containing the GFP coding sequence of the desired spectral characteristics using the pLEIN system described in Example 6 were transfected into cell lines originating from tumors, such as human breast cancer, human prostate cancer, human glioblastoma and mouse melanoma. In this manner, human breast cancer cell lines MF-7, MDA-MB468 and MDA-MB435, human prostate cancer cell lines PC3 and DU145, human glioblastoma cell line 324, human lung cancer cells Anip-73 and H460, human colon cancer cells lines Colo-205, HCT-15 and WiDr, human gastric cancer cell line NVGC-4, human kidney cancer cell line SN12C, human tongue cancer cell line SCC-25, human melanomas LOX and SK-mel-5, labeled Chinese hamster ovary cells from cell line CHO-K1 and mouse melanoma cell line B16 labeled with green fluorescent protein were established.

The SV40 T-antigen protein is useful to immortalize cultured cells so as to establish permanent cell lines. Accordingly, the vector pGFP/SV40 is transfected into a series of tumor cell cultures to provide fluorescent immortalized cell lines.

EXAMPLE 6

In Vivo Labeling of Established Tumors

Unlabeled tumors derived from the human lung cancer cell line Anip973 were established in mice using the procedure set forth in Example 3, paragraphs A and B, but substituting unlabeled Anip973 cells for clone 26. The mice were then injected with 1×10^7 packaging cells containing the retroviral vector GFP-retrovirus pLEIN contained in PT67 cells. This virus packaging system is available from Clontech, San Diego, Calif. pLEIN contains an insert of the coding sequence for EGFP, a red-shifted variant of wild-type GFP that has been optimized for brighter fluorescence and higher expression in mammalian cells. It has an excitation maximum of 488 nm and an emission maximum at 507 nm. This mutant contains a double amino acid substitution at position 64 from Phe to Leu and at position 65 from Ser to Thr. It is described by Comack, B. et al. *Gene* (1996) 173:31–38. There are more than 190 silent base changes to maximize human codon usage preferences as described by Haas, J. et al. *Curr Biol* (1996) 6:315–324. Thus, pLEIN contains the above-described GFP coding sequence inserted into the multiple cloning site of pLXIN to obtain a dicistronic expression system which permits coordinated translation of the GFP and neomycin resistance. Three days after injection of the cells into the peritoneal cavity of the mice, the tumor cells could be seen in the seminal vesicles under bright-field microscopy and under fluorescent microscopy.

EXAMPLE 7

Observation of Angiogenesis

A suspension containing 1×10^7 clone-38 cells, described in Example 1, were injected into the peritoneal cavity of a mouse. Five days later, the mouse was injected in the tail with rhodamine and the mouse was then put under anesthesia and the abdominal cavity opened sufficiently to visualize the tumor. Recovery from this surgery is straightforward. In some cases, abdominal opening is unnecessary as the intraperitoneal tumors can be visualized through intact skin. Tumors were visible in the abdominal cavity and angiogenesis was apparent as identified by the rhodamine fluorescence. Similar results were found in tumors growing in the omentum in the wall of the small intestine, and in the mesentery.

In an analogous experiment, a suspension containing 1×10^7 cells of clone-26, described in Example 1, were injected into the peritoneal cavity of a mouse. After one day, tumors appeared in the mesentery and in the colon wall. These were observed by anesthetizing the mouse and a minimal opening of the abdomen. Observations on day 3 of a similarly treated mouse showed tumors in the wall of the small intestine and in the omentum as well as in the colon wall and mesentery. On day 5, a similarly treated mouse was injected in the tail with 100 μl of 2×10^3 M rhodamine and a few vessels could be seen in the tumor growing in the mesentery. After day 60, numerous vessels were seen in the tumor growing in the colon wall.

EXAMPLE 8

Construction of Metastatic Models

Using the labeled human cancer cell lines described in Example 5, murine models are established for various types of cancer. The cell lines are implanted into 6-week-old nu/nu female mice with a single dose of 107 GFP expressing human tumor cells which had been harvested by trypsinization and washed three time with cold, serum-containing medium and then kept on ice. The cells are injected in subcutaneous space in the flank of the animal at a total volume of 0.4 ml within 40 min of harvesting. The nude mice are sacrificed to harvest the tumor fragments 3 weeks after tumor cell injection. These tumor fragments are then used for surgical implantation into the corresponding tissue (surgical orthotopic implantation (SOI)) in nude mice as recipients.

The recipient mice are first anesthetized and then implanted using established SOI techniques with fragments of the subcutaneously grown colon cancer, lung cancer, breast cancer, prostate cancer or melanoma. In all cases, except for melanoma, the size of the fragment is 1 mm^3; for melanoma, 0.025 mm^3 fragments are prepared from the human melanoma LOX-GFP subcutaneous tumor and 5–6 fragments are implanted. The progress of metastasis is then observed using FOTI with a Leica Stereomicroscope MZ12 with a mercury lamp source. GFP is excited with a D425/60 bandpass filter and a 470DCXR dichroic mirror; fluorescence is emitted through a GG475 longpass filter (Chroma

Technology, Brattle-boro, Vt.) and collected by a thermo-electrically cooled ST-133 Micromass High-Speed Controlled Camera—TEA/CCD-1317K1 (Princeton Instruments, Trenton, N.J.) with a 17×1035 pixels chip. The images are processed and analyzed with ImagePro+3.1 Software (Media Cybernetics, Silver Spring, Md.). High resolution images are captured by computer, or continuously through video output onto video tape.

In the colon cancer model, a small midline incision is made in the abdomen and the colorectal part of the intestine is exteriorized. The serosa is removed and 8–15 pieces of tumor fragments are implanted. An 8–0 surgical suture is used to penetrate the small tumor pieces and suture them to the wall of the intestine. The intestine is returned to the abdominal cavity and abdominal wall is closed. The animals are then observed for metastases.

For lung cancer models, a small 1 cm transverse incision is made on the left lateral chest via the fourth intercostal space; total lung collapse results. Five tumor pieces sewn together with 8–0 nylon surgical suture are fixed by making one knot; the lung is taken out by forceps and the tumor sewn into the lower part of the lung with one suture. After returning the lung to the chest cavity, the chest muscles and skin are closed. The lung is reinflated by withdrawing air from the chest cavity with a 23-gauge needle. The animals can then be observed for metastasis either by FOTI or by excising various tissues.

For breast cancer, an incision of 1.5 cm is made along the medial side of the nipple and after blunt dissection, the fat pad is exposed. A small incision is made and a small pocket formed to accommodate 2 fragments of the tumor tissue; an 8–0 suture is made to close the pocket. The skin layer is then closed. The animals are then observed by FOTI or by tissue excision.

For prostate cancer, an opening is made above the pubic symphysis to expose the prostate gland. The fascia surrounding the dorsal portion of the prostate and the dorsal lateral lobes of the gland are separated by a small incision. Five randomized fragments are sutured into the incision using a 8–0 nylon suture. The two parts of the separated lobes are sutured together and the surrounding fascia used to wrap this portion of the gland to consolidate the incision. The abdomen is then closed and the animals maintained for observation.

For melanoma, 5–6 fragments are transplanted subdermally into the flank with a 13×¼ cancer implant needle (Popper & Sons, New Hyde Park, N.Y.).

Images can be obtained as described above showing metastases to various locations in the animal.

The animals treated as described above, can then be used to evaluate potential protocols for treatment of cancer and metastasis inhibition. The metastatic progress of the fluorescent tumors in animals administered the protocols is compared to similar animals lacking treatment. The efficacy of the protocols can then be directly observed.

What is claimed is:

1. A method to evaluate a candidate protocol or drug for the inhibition of metastasis of a primary tumor which method comprises:

administering said protocol or drug to a subject which is a mouse, rat or rabbit which contains a primary tumor that stably expresses green fluorescent protein (GFP) in cells of said tumor when said tumor metastasizes and monitoring the progression of metastasis by observing the presence, absence or intensity of the fluorescence at various locations in the treated subject;

wherein said subject contains said tumor that expresses GFP and wherein said subject is a genetically immunocompromised mouse, rat or rabbit, or a mouse, rat or rabbit which is syngeneic to said tumor;

monitoring the progression of metastasis in a control, which contains a similar tumor that expresses green fluorescent protein;

wherein said control subject contains said tumor that expresses GFP wherein said control subject is an immunocompromised mouse, rat or rabbit, or a mouse, rat or rabbit which is syngeneic to said tumor; and

comparing the progression of metastasis in said treated subject with the progression of metastasis in said control subject wherein the control subject and treated subject are intact;

whereby a diminution of the progression of metastasis in said treated subject as compared to said control subject identifies the protocol or drug as effective in inhibiting metastasis.

2. The method of claim **1** wherein the progression of metastasis is monitored by fluorescent optical tumor imaging in the intact subject.

3. The method of claim **1** wherein said subject contains said tumor by virtue of surgical orthotopic implantation of said tumor.

4. The method of claim **1** wherein said subject contains said tumor by virtue of injecting cells of a stably transformed tumor cell line which has been transfected with an expression vector containing a first nucleotide sequence encoding green fluorescent protein (GFP) and a second nucleotide sequence encoding a selection marker, both said first and second nucleotide sequences being under control of a viral promoter and wherein said cell line stably effects high level expression of said GFP in the absence of selection agent and maintains a high level expression of GFP when said cell line proliferates through multiple passages of said cell line.

5. A method to monitor metastasis of a primary tumor in a subject which is a mouse, rat or rabbit which contains said primary tumor, and wherein said tumor stably expresses green fluorescent protein (GFP) in cells of said tumor when said tumor metastasizes,

wherein said subject contains said tumor that expresses GFP and wherein said subject is a genetically immunocompromised mouse, rat or rabbit, or a mouse, rat or rabbit which is syngeneic to said tumor;

which method comprises monitoring the progression of metastasis by observing the presence, absence or intensity of the fluorescence as a function of time at various locations in said subject wherein the subject is intact.

6. The method of claim **5** wherein the progression of metastasis is monitored by fluorescent optical tumor imaging in the intact subject.

7. The method of claim **5** wherein said subject contains said tumor by virtue of surgical orthotopic implantation of said tumor.

8. The method of claim **5** wherein said subject contains said tumor by virtue of injecting cells of a stably transformed tumor cell line which has been transfected with an expression vector in containing a first nucleotide sequence encoding green fluorescent protein (GFP) and a second nucleotide sequence encoding a selection marker, both said first and second nucleotide sequences being under control of a viral promoter and wherein said cell line stablely effects high level expression of said GFP in the absence of selection agent and maintains a high level expression of GFP when said cell line proliferates through multiple passages of said cell line.

9. A method to monitor metastasis of a primary tumor in a mammalian subject which contains said primary tumor, and wherein said tumor stably expresses green fluorescent protein (GFP) in cells of said tumor when said tumor metastasizes,

wherein said primary tumor is endogenous to said mammalian subject and expresses said GFP as a result of locally administering a retroviral vector to said subject in the vicinity of said tumor, said retroviral vector containing an expression system for said GFP;

which method comprises monitoring the progression of metastasis by observing the presence, absence or intensity of the fluorescence as a function of time at various locations in said subject.

10. The method of claim 9 wherein the subject is human.

11. The method of claim 9 wherein the progression of metastasis is monitored by excising fresh organ tissues from various locations in said subject.

12. The method of claim 11 wherein said excised tissues are observed by microscopic examination of fresh tissue slices.

* * * * *

EXHIBIT 3

EXHIBIT 3

US006649159B2

(12) **United States Patent**
Yang et al.

(10) **Patent No.: US 6,649,159 B2**
(45) **Date of Patent: Nov. 18, 2003**

(54) **WHOLE-BODY OPTICAL IMAGING OF GENE EXPRESSION AND USES THEREOF**

(75) Inventors: **Meng Yang**, San Diego, CA (US); **Eugene Baranov**, San Diego, CA (US)

(73) Assignee: **AntiCancer, Inc.**, San Diego, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/812,710**

(22) Filed: **Mar. 19, 2001**

(65) **Prior Publication Data**

US 2002/0013954 A1 Jan. 31, 2002

Related U.S. Application Data

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(51) **Int. Cl.**[7] **A61K 48/00**; A61K 49/00; A01N 63/00; C12N 15/63; C12N 15/85

(52) **U.S. Cl.** **424/93.21**; 424/93.1; 424/93.2; 424/9.1; 435/320.1; 435/325

(58) **Field of Search** 800/8; 435/320.1, 435/325; 536/24.5; 424/93.1

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(57) **ABSTRACT**

The invention relates to the whole-body external optical imaging of gene expression. Specifically, methods for whole-body external optical imaging of gene expression and methods for evaluating a candidate protocol or drug for treating diseases or disorders using a fluorophore operatively linked to the promoter of a gene and external optical imaging are provided herein. Methods to screen for substances or genes that regulate target promoters are also provided.

13 Claims, 2 Drawing Sheets

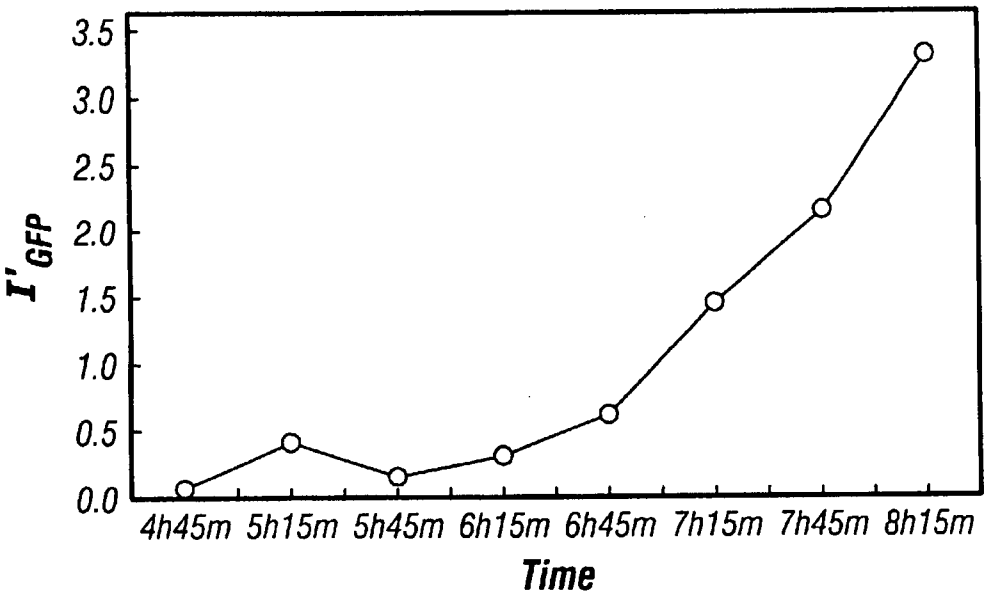
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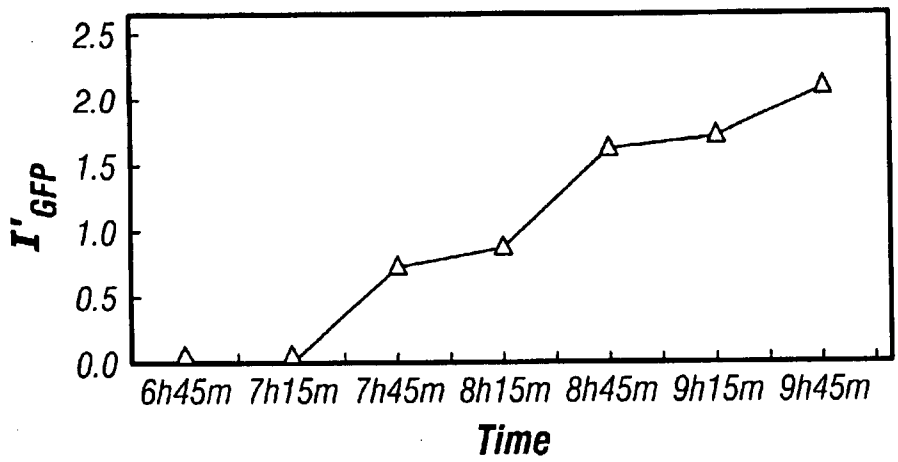
FIG. 1A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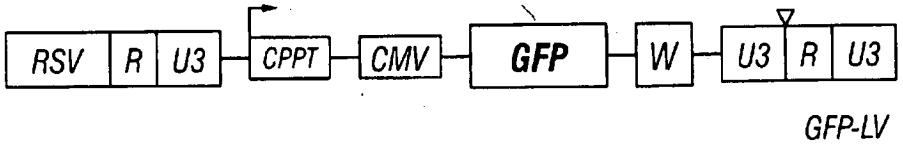
Time

FIG. 1B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R
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CPPT
CMV
GFP
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U3
R
U3
GFP-LV

FIG. 2A

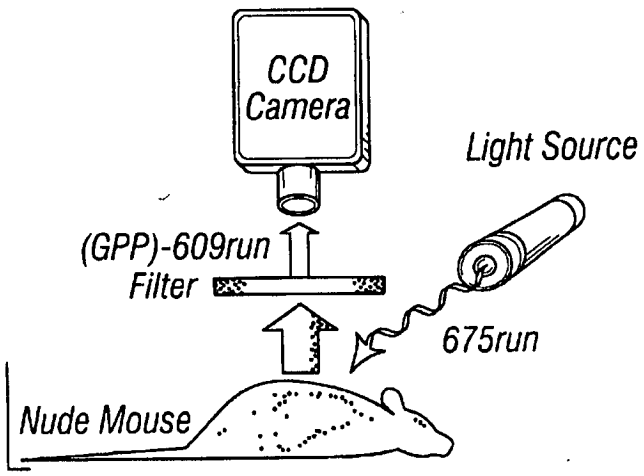
CCD Camera
Light Source
(GPP)-609run Filter
675run
Nude Mouse

FIG. 2B

WHOLE-BODY OPTICAL IMAGING OF GENE EXPRESSION AND USES THEREOF

This application claims priority under 35 U.S.C. 119 from provisional application U.S. Ser. No. 60/190,196 filed Mar. 17, 2000, the contents of which are incorporated herein by reference.

TECHNICAL FIELD

The invention relates to the whole-body external optical imaging of gene expression. Specifically, methods for whole-body external optical imaging of gene expression and methods for evaluating a candidate protocol or drug for treating diseases or disorders using a fluorophore operatively linked to the promoter of a gene and external optical imaging are provided herein. Methods to screen for substances or genes that regulate target promoters are also provided.

BACKGROUND ART

Whole-body imaging technology has been used to monitor "tracer molecules" in the intact body. For example, Brenner et al. studied the diagnostic value of iodine-123-2-hydroxy-3-iodo-6-methoxy-N-[(1-ethyl-2-pyrrolidinyl) methyl] benzamide (IBZM) whole-body imaging in comparison to thallium-201 scintigraphy in patients with metastatic malignant melanoma (Brenner et al., *Eur. J. Nucl. Med.,* 26(12):1567–71 (1999)). Benard et al. conducted clinical evaluation of processing techniques for attenuation correction with ^{137}Cs in whole-body PET imaging (Benard et al., *J. Nucl. Med.,* 40(8):1257–63 (1999)). Jerusalem et al. showed that whole-body positron emission tomography using ^{18}F-fluorodeoxyglucose for posttreatment evaluation in Hodgkin's disease and non-Hodgkin's lymphoma has higher diagnostic and prognostic value than classical computed tomography scan imaging (Jerusalem et al., *Blood,* 94(2):429–33 (1999)). Eustace et al. discussed practical issues, clinical applications, and future directions of whole-body MR imaging (Eustace et al., *Magn. Reson. Imaging Clin. (N. Am),* 7(2):209–36 (1999)). Engelson et al. studied fat distribution in HIV-infected patients reporting truncal enlargement quantified by whole-body magnetic resonance imaging (Engelson et al., *Am. J. Clin. Nutr.,* 69(6 :1162–9 (1999)). Valk et al. used whole-body positron emission tomography (PET) imaging with [^{18}F]fluorodeoxyglucose in management of recurrent colorectal cancer (Valk et al., Arch. Surg., 134(5):503–11 (1999)). Saunders et al. evaluated fluorine-18-fluorodeoxyglucose whole body positron emission tomography imaging in the staging of lung cancer (Saunders et al., *Ann. Thorac. Surg.,* 67(3):790–7 (1999)).

U.S. Pat. No. 5,650,135 discloses a noninvasive method for detecting the localization of an entity under study from within a mammalian subject, which method comprises: (a) administering to the subject a conjugate of the entity and a light-generating moiety or a transformed cell expressing the light-generating moiety; (b) after a period of time in which the conjugate or transformed cell can achieve localization in the subject, immobilizing the subject within the detection field of a photodetector device; (c) maintaining the subject in an immobilized condition, (d) during said maintaining, measuring photon emission from the light-generating moiety, localized in the subject, with the photodetector device until an image of photon emission can be constructed; and (e) detecting said image through an opaque tissue of said mammal. U.S. Pat. No. 5,650,135 also discloses a noninvasive method for detecting the level of an entity under study in a mammalian subject over time, which

method comprises: (a) administering to the subject a conjugate of the entity and a light-generating moiety or a transformed cell expressing the light-generating moiety; (b) placing the subject within the detection field of a photodetector device; (c) maintaining the subject in the detection field of the device; (d) during said maintaining, measuring photon emission from the light-generating moiety, in the subject, with the photodetector device; and (e) repeating steps (b) through (d) at selected intervals, wherein said repeating is effective to detect changes in the level of the entity in the subject over time.

Recently, Yang et al. conducted whole-body optical imaging of green fluorescent protein-expressing tumors and metastases (Yang et al., *Proc. Natl. Acad. Sci. (USA),* 97(3): 1206–11 (2000)). Yang et al. have imaged, in real time, fluorescent tumors growing and metastasizing in live mice. The whole-body optical imaging system is external and noninvasive. It affords unprecedented continuous visual monitoring of malignant growth and spread within intact animals. Yang et al. have established new human and rodent tumors that stably express very high levels of the Aequorea victoria green fluorescent protein (GFP) and transplanted these to appropriate animals. B16F0-GFP mouse melanoma cells were injected into the tail vein or portal vein of 6-week-old C57BL/6 and nude mice. Whole-body optical images showed metastatic lesions in the brain, liver, and bone of B 16F0-GFP that were used for real time, quantitative measurement of tumor growth in each of these organs. The AC3488-GFP human colon cancer was surgically implanted orthotopically into nude mice. Whole-body optical images showed, in real time, growth of the primary colon tumor and its metastatic lesions in the liver and skeleton. Imaging was with either a trans-illuminated epifluorescence microscope or a fluorescence light box and thermoelectrically cooled color charge-coupled device camera. The depth to which metastasis and micrometastasis could be imaged depended on their size. A 60-micrometer diameter tumor was detectable at a depth of 0.5 mm whereas a 1, 800-micrometer tumor could be visualized at 2.2-mm depth. The simple, noninvasive, and highly selective imaging of growing tumors, made possible by strong GFP fluorescence, enables the detailed imaging of tumor growth and metastasis formation. This should facilitate studies of modulators of cancer growth including inhibition by potential chemotherapeutic agents.

Methods for monitoring gene expression are known in the art (see generally, Ausubel et al. (Ed.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc.). However, whole-body external optical imaging of gene expression, which offers simple, noninvasive, highly selective, and real-time recording and analysis of gene expression in an intact multi-cellular organisms, e.g., animals, is not available currently. The present invention addresses this and other related needs in the art.

DISCLOSURE OF THE INVENTION

The invention provides for whole-body external optical imaging of gene expression and methods for evaluating a candidate protocol or drug for treating diseases or disorders. The method uses a fluorophore operatively linked to the promoter of a gene and external optical imaging. Methods to screen for substances or genes that regulate target promoters are also provided.

In a specific embodiment, a method to monitor the expression of a gene is provided, which method comprises: a) delivering to a multi-cellular organism a nucleic acid encod-

ing a fluorophore operatively linked to the promoter of a gene whose expression is to be analyzed or delivering a cell containing said nucleic acid; and b) observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said organism by whole-body external fluorescent optical imaging, whereby the expression of said gene is monitored.

In a preferred embodiment, a nucleic acid encoding a fluorophore operatively linked to the promoter of the gene is delivered directly to the organism. Also preferably, the nucleic acid encoding a fluorophore operatively linked to the promoter of the gene is in a viral vector such as a viral vector derived from adenovirus or a lentivirus.

In another preferred embodiment, a cell containing a nucleic acid encoding a fluorophore operatively linked to the promoter of the gene is delivered to the organism. More preferably, the cell is delivered to the organism via a surgical procedure such as direct implantation by surgical orthotopic implantation (SOI) at a desired site.

In still another preferred embodiment, the fluorophore operatively linked to the promoter of a gene is a humanized fluorophore. Also preferably, the fluorophore is a green fluorescent protein (GFP), a blue fluorescent protein (BFP) or a red fluorescent protein (RFP). More preferably, the GFP is the humanized hGFP-S65T.

In yet another preferred embodiment, the multi-cellular organism to be analyzed is a plant or an animal, including a transgenic animal. More preferably, the animal is a mammal. A human can also be analyzed by the present method.

In yet another preferred embodiment, the gene to be analyzed is expressed in a tissue or organ specific manner. More preferably, the gene is expressed in connective, epithelium, muscle or nerve tissue. Also more preferably, the gene is expressed in an internal animal organ such as brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, gland, internal blood vessels, etc. Yet more preferably, the gene to be analyzed is a tumor or cancer associated gene such as an oncogene or a tumor suppressor gene.

In yet another preferred embodiment, the expression of more than one gene is monitored simultaneously.

In another specific embodiment, a method to evaluate a candidate protocol or drug for treating a disease or disorder is provided, which method comprises: a) administering said protocol or drug to a non-human mammalian subject which expresses a fluorophore under the direction of a promoter of a gene associated with a disease or disorder, and determining the expression of said promoter via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said mammalian subject by whole-body external fluorescent optical imaging; b) determining the expression of said promoter, via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said mammalian subject by whole-body external fluorescent optical imaging, in a control non-human mammalian subject which expresses said fluorophore under the direction of said promoter of said gene; and c) comparing the expression of said promoter determined in steps a) and b), wherein the expression determined in step a) is different from that in step b) identifies said protocol or drug as effective in treating the disease or disorder.

If overexpression of the gene is associated with the disease or disorder, the expression determined in step a) is lower than that in step b) when said protocol or drug is effective in treating the disease or disorder.

If underexpression of the gene is associated with the disease or disorder, the expression determined in step a) is higher than that in step b) when said protocol or drug is effective in treating the infection.

Preferably, the disease or disorder is a cancer, an immune system disease or disorder, a metabolism disease or disorder, a muscle and bone disease or disorder, a nervous system disease or disorder, a signal disease or disorder, or a transporter disease or disorder.

Preferably, the non-human mammalian subject which expresses a fluorophore under the direction of a promoter of the gene is produced by delivering a nucleic acid encoding the fluorophore operatively linked to the promoter of the gene, or a cell containing the nucleic acid, to the non-human mammalian subject. Alternatively, the non-human mammalian subject used in the screen is a transgenic animal.

The non-human mammalian subject used in the screening is preferably a well established laboratory animal such as a mice, a rabbit or a non-human primate.

The fluorophore used in the screening is preferably a green fluorescent protein (GFP), a blue fluorescent protein (BFP) or a red fluorescent protein (RFP).

More than one candidate protocol or candidate drug is preferably screened for simultaneously.

If the non-human mammalian subject expresses a fluorophore under the direction of a promoter of an infectious organism, the expression determined in step a) is lower than that in step b) when said protocol or drug is effective in treating infection caused by the infectious organism.

The non-human mammalian subject used in the screening is preferably an infectious disease animal model.

The infectious organism screened against is preferably a fungus such as a yeast, a bacterium such as an eubacteria or an archaebacteria, or a virus such as a Class I virus, a Class II virus, a Class III virus, a Class IV virus, a Class V virus or a Class VI virus.

If the infection is caused by a bacterium, the candidate drug to be screened is preferably an antibiotic.

In still another specific embodiment, a method to screen for a modulator of the expression of a gene in a non-human multi-cellular organism is provided, which method comprises: a) administering a test substance to a non-human multi-cellular organism which expresses a fluorophore under the direction of a promoter of a gene, and determining the expression of said promoter via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said multi-cellular organism by whole-body external fluorescent optical imaging; b) determining the expression of said promoter, via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations by whole-body external fluorescent optical imaging, in a control multi-cellular organism which expresses said fluorophore under the direction of said promoter of said gene; and c) comparing the expression of said promoter determined in steps a) and b), wherein the expression determined in step a) is different from that in step b) identifies said test substance as a modulator of said gene expression. Preferably, the promoter is an endogenous promoter of the multi-cellular organism.

In yet another specific embodiment, a method to screen for a non-human multi-cellular organism that expresses a gene at an altered level is provided, which method com-

prises: a) administering a mutation-inducing agent or treatment to a non-human multi-cellular organism which expresses a fluorophore under the direction of a promoter of a gene, and determining the expression of said promoter via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said multi-cellular organism by whole-body external fluorescent optical imaging; b) determining the expression of said promoter, via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations by whole-body external fluorescent optical imaging in an untreated control multi-cellular organism which expresses said fluorophore under the direction of said promoter of said gene; and c) comparing the expression of said promoter determined in steps a) and b), wherein the expression determined in step a) is different from that in step b) identifies a multi-cellular organism that expresses said gene at the altered level. Preferably, the mutation-inducing agent or treatment causes a mutation in germ-line cells of the multi-cellular organism so that the desired mutation is stably-transferable to offspring of the multi-cellular organism.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1A** and **1B** show the time course of expression of adenoviral-administered GFP in brain and liver respectively. Fluorescence first becomes visible in the brain within six (6) hours after local delivery and liver fluorescence became detectable at about seven (7) hours after injection into the tail vein.

FIGS. **2A** and **2B** art pertinent to administration of lentiviral vectors. FIG. **2A** is a diagram of lentiviral vector GFP-LV. FIG. **2B** is a diagram of a control observation method; whole body measurement involved use of a light box.

MODES FOR CARRYING OUT THE INVENTION

A. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. All patents, applications, published applications and other publications and sequences from GenBank and other data bases referred to herein are incorporated by reference in their entirety.

As used herein, "delivering a nucleic acid to a multi-cellular organism" refers to a process in which the nucleic acid is either administered directly into the body of the multi-cellular organism, or the nucleic acid is administered into a cell first, and then the cell containing the nucleic acid is administered into the body of the multi-cellular organism. After delivery into the organism, the nucleic acid may exist independently from the genome of the host organism or may be integrated into the genome of the host organism. If the nucleic acid is integrated into a germline cell of the host organism, such nucleic acid may be transmitted into the host organism's offspring.

As used herein, "whole-body external fluorescent optical imaging" refers to an imaging process in which the presence, absence or intensity of the fluorescence generated by the fluorophore at various locations in the host organism is monitored, recorded and/or analyzed externally without any procedure, e.g., surgical procedure, to expose and/or to excise the desired observing site from the host organism. To achieve the whole-body external fluorescent optical imaging, it is necessary that the intensity of the fluorescence generated by the fluorophore is sufficiently high so that, even when the fluorescence site is an internal one within the host organism body, the fluorescence signal can be analyzed externally without exposing or excising the site from the host body, or while the animal is not controlled.

As no invasive procedures are required and the intensity of the signal is sufficiently great for direct observation, the animal may remain completely mobile and need not be restrained. The ability to provide a completely non-invasive observation protocol is highly significant. If the animal is traumatized either by, e.g., incision or by physical restraint, e.g., straps or pins, the alteration in metabolism may affect the expression of the genes in organs or tissues.

Since whole-body external fluorescent optical imaging are quick and easily amenable to automation, it can be used for monitoring large number of gene expression simultaneously. In addition, it can be employed in high-throughput screening methods for identifying protocols, substances including candidate drugs, and cis-acting regulators that regulate the expression of a target gene. Using the whole-body external fluorescent optical imaging provided in this application, multiple candidate protocols, substances, drugs, and cis-acting regulators can be screened for, either against a single target gene or against multiple target genes, in either a single animal or in multiple animals, simultaneously.

As used herein, "fluorophore" refers to a protein that is auto-fluorescent such that no other substrates or co-factors are needed for it to fluoresce. Non-limiting examples of such fluorophores include green fluorescent proteins (GFPs), blue fluorescent proteins (BFPs) and red fluorescent protein (RFPs), and functional fragments, derivatives and analogues thereof.

As used herein, "a promoter region or promoter element" refers to a segment of DNA or RNA that controls transcription of the DNA or RNA to which it is operatively linked. The promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of RNA polymerase. These sequences may be cis acting or may be responsive to trans acting factors. Promoters, depending upon the nature of the regulation, may be constitutive or regulated.

As used herein, "operatively linked or operationally associated" refers to the functional relationship of DNA with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA. In order to optimize expression and/or in vitro transcription, it may be necessary to remove, add or alter 5' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation (i.e., start) codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, e.g., Kozak, *J. Biol. Chem.*, 266:19867–19870 (1991)) can be inserted immediately 5' of the start codon and may enhance expression. The desirability of (or need for) such modification may be empirically determined.

As used herein, "humanized fluorophore" refers to a fluorophore whose codon is modified according to the codon usage pattern in human genome to enhance its expression while substantially maintaining its fluorescent characteristics.

As used herein, "multi-cellular organism" refers to an organism with certain cell numbers, mass, and internal structure so that internal sites of such multi-cellular organism are not externally detectable by non-fluorescent optical imaging without exposing the internal sites. Sufficiently high intensity of internal fluorescence is needed for external fluorescent optical imaging of the internal site.

As used herein, "plant" refers to any of various photosynthetic, eucaryotic multi-cellular organisms of the kingdom Plantae, characteristically producing embryos, containing chloroplasts, having cellulose cell walls and lacking locomotion.

As used herein, "animal" refers to a multi-cellular organism of the kingdom of Animalia, characterized by a capacity for locomotion, nonphotosynthetic metabolism, pronounced response to stimuli, restricted growth and fixed bodily structure. Non-limiting examples of animals include birds such as chickens, vertebrates such fish and mammals such as mice, rats, rabbits, cats, dogs, pigs, cows, ox, sheep, goats, horses, monkeys and other non-human primates.

As used herein, "expressed in a tissue or organ specific manner" refers to a gene expression pattern in which a gene is expressed, either transiently or constitutively, only in certain tissues or organs, but not in other tissues or organs.

As used herein, "tissue" refers to a collection of similar cells and the intracellular substances surrounding them. There are four basic tissues in the body: 1) epithelium; 2) connective tissues, including blood, bone, and cartilage; 3) muscle tissue; and 4) nerve tissue.

As used herein, "organ" refers to any part of the body exercising a specific function, as of respiration, secretion or digestion.

As used herein, "disease or disorder" refers to a pathological condition in an organism resulting from, e.g., infection or genetic defect, and characterized by identifiable symptoms.

As used herein, neoplasm (neoplasia) refers to abnormal new growth, and thus means the same as tumor, which may be benign or malignant. Unlike hyperplasia, neoplastic proliferation persists even in the absence of the original stimulus.

As used herein, cancer refers to a general term for diseases caused by any type of malignant tumor.

As used herein, "oncogene" refers to a mutated and/or overexpressed version of a normal gene of animal cells (the proto-oncogene) that in a dominant fashion can release the cell from normal restraints on growth, and thus alone, or in concert with other changes, convert a cell into a tumor cell. Exemplary oncogenes include, but are not limited to, abl, erbA, erbB, ets, fes (fps), fgr, fms, fos, hst, int1, int2, jun, hit, B-lym, mas, met, mil (raf), mos, myb, myc, N-myc, neu (ErbB2), ral (mil), Ha-ras, Ki-ras, N-ras, rel, ros, sis, src, ski, trk and yes.

As used herein, "tumor suppressor gene" (or anti-oncogene, cancer susceptibility gene) refers to a gene that encodes a product which normally negatively regulates the cell cycle, and which must be mutated or otherwise inactivated before a cell can proceed to rapid division. Exemplary tumor suppressor genes include, but are not limited to, p16, p21, p53, RB (retinoblastoma), WT-1 (Wiln's tumor), DCC (deleted in colonic carcinoma), NF-1 (neurofibrosarcoma) and APC (adenomatous polypospis coli).

As used herein, "an immune system disease or disorder" refers to a pathological condition caused by a defect in the immune system. The immune system is a complex and highly developed system, yet its mission is simple: to seek and kill invaders. If a person is born with a severely defective immune system, death from infection by a virus, bacterium, fungus or parasite will occur. In severe combined immunodeficiency, lack of an enzyme means that toxic waste builds up inside immune system cells, killing them and thus devastating the immune system. A lack of immune system cells is also the basis for DiGeorge syndrome: improper development of the thymus gland means that T cell production is diminished. Most other immune disorders result from either an excessive immune response or an 'autoimmune attack'. For example, asthma, familial Mediterranean fever and Crohn disease (inflammatory bowel disease) all result from an over-reaction of the immune system, while autoimmune polyglandular syndrome and some facets of diabetes are due to the immune system attacking 'self' cells and molecules. A key part of the immune system's role is to differentiate between invaders and the body's own cells—when it fails to make this distinction, a reaction against 'self' cells and molecules causes autoimmune disease.

As used herein, "a metabolism disease or disorder" refers to a pathological condition caused by errors in metabolic processes. Metabolism is the means by which the body derives energy and synthesizes the other molecules it needs from the fats, carbohydrates and proteins we eat as food, by enzymatic reactions helped by minerals and vitamins. There is a significant level of tolerance of errors in the system: often, a mutation in one enzyme does not mean that the individual will suffer from a disease. A number of different enzymes may compete to modify the same molecule, and there may be more than one way to achieve the same end result for a variety of metabolic intermediates. Disease will only occur if a critical enzyme is disabled, or if a control mechanism for a metabolic pathway is affected.

As used herein, "a muscle and bone disease or disorder" refers to a pathological condition caused by defects in genes important for the formation and function of muscles, and connective tissues. Connective tissue is used herein as a broad term that includes bones, cartilage and tendons. For example, defects in fibrillin—a connective tissue protein that is important in making the tissue strong yet flexible—cause Marfan syndrome, while diastrophic dysplasia is caused by a defect in a sulfate transporter found in cartilage. Two diseases that originate through a defect in the muscle cells themselves are Duchenne muscular dystrophy (DMD) and myotonic dystrophy (DM). DM is another 'dynamic mutation' disease, similar to Huntington disease, that involves the expansion of a nucleotide repeat, this time in a muscle protein kinase gene. DMD involves a defect in the cytoskeletal protein, dystrophin, which is important for maintaining cell structure.

As used herein, "a nervous system disease or disorder" refers to a pathological condition caused by defects in the nervous system including the central nervous system, i.e., brain, and the peripheral nervous system. The brain and nervous system form an intricate network of electrical signals that are responsible for coordinating muscles, the senses, speech, memories, thought and emotion. Several diseases that directly affect the nervous system have a genetic component: some are due to a mutation in a single gene, others are proving to have a more complex mode of

inheritance. As our understanding of the pathogenesis of neurodegenerative disorders deepens, common themes begin to emerge: Alzheimer brain plaques and the inclusion bodies found in Parkinson disease contain at least one common component, while Huntington disease, fragile X syndrome and spinocerebellar atrophy are all 'dynamic mutation' diseases in which there is an expansion of a DNA repeat sequence. Apoptosis is emerging as one of the molecular mechanisms invoked in several neurodegenerative diseases, as are other, specific, intracellular signaling events. The biosynthesis of myelin and the regulation of cholesterol traffic also figure in Charcot-Marie-Tooth and Neimann-Pick disease, respectively.

As used herein, "a signal disease or disorder" refers to a pathological condition caused by defects in the signal transduction process. Signal transduction within and between cells mean that they can communicate important information and act upon it. Hormones released from their site of synthesis carry a message to their target site, as in the case of leptin, which is released from adipose tissue (fat cells) and transported via the blood to the brain. Here, the leptin signals that enough has been eaten. Leptin binds to a receptor on the surface of hypothalamus cells, triggering subsequent intracellular signaling networks. Intracellular signaling defects account for several diseases, including cancers, ataxia telangiectasia and Cockayne syndrome. Faulty DNA repair mechanisms are also invoked in pathogenesis, since control of cell division, DNA synthesis and DNA repair all are inextricably linked. The end-result of many cell signals is to alter the expression of genes (transcription) by acting on DNA-binding proteins. Some diseases are the result of a lack of or a mutation in these proteins, which stop them from binding DNA in the normal way. Since signaling networks impinge on so many aspects of normal function, it is not surprising that so many diseases have at least some basis in a signaling defect.

As used herein, "a transporter disease or disorder" refers to a pathological condition caused by defects in a transporter, channel or pump. Transporters, channels or pumps that reside in cell membranes are key to maintaining the right balance of ions in cells, and are vital for transmitting signals from nerves to tissues. The consequences of defects in ion channels and transporters are diverse, depending on where they are located and what their cargo is. For example, in the heart, defects in potassium channels do not allow proper transmission of electrical impulses, resulting in the arrhythmia seen in long QT syndrome. In the lungs, failure of a sodium and chloride transporter found in epithelial cells leads to the congestion of cystic fibrosis, while one of the most common inherited forms of deafness, Pendred syndrome, looks to be associated with a defect in a sulphate transporter.

As used herein, "infection" refers to invasion of the body of a multi-cellular organism with organisms that have the potential to cause disease.

As used herein, "infectious organism" refers to an organism that is capable to cause infection of a multi-cellular organism. Most infectious organisms are microorganisms such as viruses, bacteria and fungi.

As used herein, "bacteria" refers to small prokaryotic organisms (linear dimensions of around 1 μm) with non-compartmentalized circular DNA and ribosomes of about 70S. Bacteria protein synthesis differs from that of eukaryotes. Many anti-bacterial antibiotics interfere with bacteria proteins synthesis but do not affect the infected host.

As used herein, "eubacteria" refers to a major subdivision of the bacteria except the archaebacteria. Most Gram-positive bacteria, cyanobacteria, mycoplasmas, enterobacteria, pseudomonas and chloroplasts are eubacteria. The cytoplasmic membrane of eubacteria contains ester-linked lipids; there is peptidoglycan in the cell wall (if present); and no introns have been discovered in eubacteria.

As used herein, "archaebacteria" refers to a major subdivision of the bacteria except the eubacteria. There are 3 main orders of archaebacteria: extreme halophiles, methanogens and sulphur-dependent extreme thermophiles. Archaebacteria differs from eubacteria in ribosomal structure, the possession (in some case) of introns, and other features including membrane composition.

As used herein, "virus" refers to obligate intracellular parasites of living but non-cellular nature, consisting of DNA or RNA and a protein coat. Viruses range in diameter from about 20 to about 300 nm. Class I viruses (Baltimore classification) have a double-stranded DNA as their genome; Class II viruses have a single-stranded DNA as their genome; Class III viruses have a double-stranded RNA as their genome; Class IV viruses have a positive single-stranded RNA as their genome, the genome itself acting as mRNA; Class V viruses have a negative single-stranded RNA as their genome used as a template for mRNA synthesis; and Class VI viruses have a positive single-stranded RNA genome but with a DNA intermediate not only in replication but also in mRNA synthesis. The majority of viruses are recognized by the diseases they cause in plants, animals and prokaryotes. Viruses of prokaryotes are known as bacteriophages.

As used herein, "fungi" refers to a division of eucaryotic organisms that grow in irregular masses, without roots, stems, or leaves, and are devoid of chlorophyll or other pigments capable of photosynthesis. Each organism (thallus) is unicellular to filamentous, and possess branched somatic structures (hyphae) surrounded by cell walls containing glucan or chitin or both, and containing true nuclei.

As used herein, "antibiotic" refers to a substance either derived from a mold or bacterium or organically synthesized, that inhibits the growth of certain microorganisms without substantially harming the host of the microorganisms to be killed or inhibited.

As used herein, "test substance" refers to a chemically defined compound (e.g., organic molecules, inorganic molecules, organic/inorganic molecules, proteins, peptides, nucleic acids, oligonucleotides, lipids, polysaccharides, saccharides, or hybrids among these molecules such as glycoproteins, etc.) or mixtures of compounds (e.g., a library of test compounds, natural extracts or culture supernatants, etc.) whose effect on the promoter to be analyzed is determined by the disclosed and/or claimed methods herein.

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections that follow.

B. Methods of Whole-Body External Optical Imaging of Gene Expression

In a specific embodiment, a method to monitor the expression of a gene is provided herein, which method comprises: a) delivering to a multi-cellular organism a nucleic acid encoding a fluorophore operatively linked to the promoter of a gene whose expression is to be analyzed or a cell containing said nucleic acid; and b) observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said organism by whole-body external fluorescent optical imaging, whereby the expression of said gene is monitored.

The present methods can be used to monitor gene expression for any suitable purposes including prognostic, diag-

nostic and screening purposes. For example, if abnormal gene expression is associated with a disease or disorder in a multi-cellular organism such as a plant or an animal, the present method can be used in prognosis or diagnosis by monitoring the abnormal gene expression. The present monitoring methods are advantageous over the currently available gene expression monitoring methods in several aspects. First, the present monitoring methods can avoid any invasive procedures and this is particularly advantageous for human clinical uses. Second, the present monitoring methods offer in vivo, real-time and continuous monitor and analysis of gene expression in plants or animals, which cannot be accomplished using the currently available monitoring methods. Third, the present monitoring methods are quick and easily amenable to automation, which are important for monitoring large number of gene expression simultaneously. Since many diseases or disorders involve abnormal gene expression of more than gene, the present monitoring methods are particularly suitable for the prognosis and diagnosis of these diseases or disorders. Besides prognosis or diagnosis, if expression of certain genes is a good indicator of tissue or organ health or functionality, the present monitoring methods can also be used in monitoring the health or functionality of these tissues or organs without any invasive procedures.

1. Methods for Delivering the Nucleic Acids into the Multi-Cellular Organism

The nucleic acids encoding a fluorophore operatively linked to the promoter of a gene whose expression is to be analyzed can be a DNA or a RNA. Such nucleic acids can be delivered into the body of the multi-cellular organism by any methods known in the art.

For example, if the host multi-cellular organism is an animal, the DNA or RNA sequence can be delivered to the interstitial space of tissues of the animal body, including those of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular, fluid, mucopolysaccharide matrix among the reticular fibers or organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation of the lymph fluid of the lymphatic channels.

The DNA or RNA sequence can be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression can be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts.

In a specific embodiment, the DNA or RNA sequence is delivered directly to a tissue of the host animal. Preferably, the DNA or RNA sequence is delivered directly to muscle, skin or mucous membrane. Delivery to the interstitial space of muscle tissue is preferred because muscle cells are particularly competent in their ability to take up and express polynucleotides.

The DNA or RNA sequence can be delivered directly to a tissue of the host animal by injection, by gene gun technology or by lipid mediated delivery technology. The injection can be conducted via a needle or other injection devices. The gene gun technology is disclosed in U.S. Pat. No. 5,302,509 and the lipid mediated delivery technology is disclosed in U.S. Pat. No. 5,703,055.

In still another specific embodiment, the DNA or RNA sequence is delivered to a cell of host animal and said cell containing the DNA or RNA sequence is delivered to a suitable tissue of the host animal. Preferably, the DNA or RNA sequence is delivered to tail or portal vein of the host animal.

The DNA or RNA sequence can be delivered to the cells of the host animal by a number of methods (see generally Koprowski & Weiner, DNA vaccination/genetic vaccination, 1998. Springer-verlag Berlin Heidelberg) including $Ca_3(PO_4)_2$-DNA transfection (Sambrook et al., *Molecular Cloning,* 2nd Edition, Plainview, N.Y. Cold Spring Harbor Press, 1989), DEAE dextran-DNA transfection (Sambrook et al., *Molecular Cloning,* 2nd Edition, Plainview, N.Y. Cold Spring Harbor Press, 1989), electroporation (e.g., protocols from Bio-Rad), transfection using "LIPOFECTIN"™ reagent (e.g., protocols from BRL-Life Science), gene gun technology (U.S. Pat. No. 5,302,509), or viral gene delivery system (Kaplitt et al., *Viral Vectors, Academic Press, Inc.,* 1995).

Gold-particle based gene gun delivery is disclosed in U.S. Pat. No. 5,302,509. In a specific embodiment, Bio-Rad helios gene gun system is used in the DNA delivery. (BIO-RAD Inc. New England). The helios gene gun is a convenient, hand-held device that provides rapid and direct gene transfer in vivo. The device employs an adjustable, helium pulse to sweep DNA coated gold microcarriers from the inner wall of a small plastic cartridge directly into the target cells. The tubing prepstation and tubing cutter provide a simple way to prepare 50 cartridge "bullets" at a time.

In a preferred embodiment, a nucleic acid encoding a fluorophore operatively linked to the promoter of the gene is delivered directly to the organism. More preferably, the nucleic acid encoding a fluorophore operatively linked to the promoter of the gene is delivered to the organism, or to a cell to be delivered to the organism, in a viral vector such as a viral vector derived from adenovirus or a lentivirus.

Any viral vectors known in the art can be used. For example, vectors derived from a parvovirus (U.S. Pat. Nos. 5,252,479 and 5,624,820), a paramyxovirus such as simian virus 5 (SV5) (U.S. Pat. No. 5,962,274), a retrovirus such as HIV (U.S. Pat. Nos. 5,753,499 and 5,888,767), and a baculovirus such as a nuclear polyhedrosis virus (U.S. Pat. No. 5,674,747) can be used. Preferably, a vector derived from adenovirus can be used (U.S. Pat. Nos. 5,670,488, 5,817,492, 5,820,868, 5,856,152, 5,981,225).

U.S. Pat. No. 5,670,488 discloses an adenoviral vector comprising an adenovirus genome from which one or more of the E4 open reading frames has been deleted, but retaining sufficient E4 sequences to promote virus replication in vitro, and additionally comprising a DNA sequence of interest operably linked to expression control sequences and inserted into said adenoviral genome.

U.S. Pat. No. 5,817,492 discloses a recombinant adenoviral vector comprising: two DNA sequences which serve as a substrate for a recombinase enzyme, an origin of replication which is operable in an animal cell, a promoter, a foreign gene and a poly(A) sequence, wherein said origin of replication, promoter, foreign gene and poly(A) sequence are located between the two DNA sequences, and wherein said vector contains an E1A gene region deletion.

U.S. Pat. No. 5,820,868 discloses a live recombinant bovine adenovirus vector (BAV) wherein a part or all of the E3 multiple gene coding region is replaced by a heterolo-

gous nucleotide sequence encoding a foreign gene or fragment thereof. It also discloses a live recombinant bovine adenovirus vector (BAV) wherein part or all of the E3 multiple gene coding region is replaced by a heterologous nucleotide sequence encoding a foreign gene or fragment thereof and wherein said heterologous nucleotide sequence is optionally under the control of a promoter not normally associated with either said foreign gene or the bovine adenovirus genome.

U.S. Pat. No. 5,856,152 discloses a hybrid viral vector comprising: (a) adenovirus sequences comprising the adenovirus 5'and 3' cis-elements necessary for replication and virion encapsidation; and (b) adeno-associated virus sequences comprising the 5' and 3' ITRs of an adeno-associated virus, said adeno-associated virus sequences flanked by the adenoviral sequences of (a); and (c) a selected gene operatively linked to regulatory sequences which direct its expression in a target cell, said gene and regulatory sequences flanked by the adeno-associated virus sequences of (b).

U.S. Pat. No. 5,981,225 discloses a gene transfer vector consisting essentially of, in 5' to 3' orientation, the following elements: (i) a first adenovirus inverted terminal repeat, (ii) an adenoviral VAI gene and/or VAII gene, (iii) a gene foreign to adenovirus, wherein said gene is operably linked to a promoter functional in adenovirus target cells, and (iv) a second adenovirus inverted terminal repeat, wherein the order of elements (ii) and (iii) may be reversed; and wherein one or both of element (i) and element (iv) additionally comprise an adenovirus packaging signal, and wherein said vector is incapable of producing, in vitro, recombinant adenovirus virus particles which have encapsidated therein said vector unless said vector is co-transfected or co-infected into adenovirus host cells with adenovirus genomic DNA or adenovirus particles containing adenovirus genomic DNA, respectively.

In another preferred embodiment, cells containing a nucleic acid encoding a fluorophore operatively linked to the promoter of the gene are delivered to the organism. More preferably, the cells are delivered to the organism via a surgical procedure such as direct implantation by surgical orthotopic implantation (SOI) at a desired site (see e.g., Chang et al., *Anticancer Res.,* 19(5B):4199 (1999); and An et al., *Prostate,* 34(3 :169–74 (1998)).

It will be understood, that by introducing a nucleic acid molecule wherein a promoter is coupled to a nucleotide sequence encoding a fluorescent reporter gene, the introduced nucleic acid molecule can be used as a surrogate for the endogenous promoter. Thus, if the endogenous gene is over-expressed or under-expressed in the context of a particular condition, the behavior of the introduced construct will mimic that of the endogenous promoter. It is not necessary that the reporter-encoding nucleotide sequence be operably linked only to a promoter; the nucleotide sequence encoding reporter may be introduced into the nucleotide sequence encoding the protein normally under control of the promoter or coupled to another protein. Any method of operably linking the nucleotide sequence encoding reporter to the control sequences for the gene whose expression is to be monitored falls within the scope of the invention.

It will be seen that there are a number of ways to introduce this construct. First, the nucleic acid comprising the reporter encoding nucleotide sequence operably linked to the control sequences/promoter of interest can be introduced to the multicellular organism by direct injection, but preferably using a viral vector, such as a adenoviral vector or a antiviral vector. Since the introduced construct is not endogenous, the expression of this construct essentially functions as a surrogate for the endogenous gene. That is, the same influences which influence the endogenous gene will also influence the introduced construct. Thus, the conclusions reached by observing the expression of the construct, including the effects of various treatments on such expression, can be extrapolated to, and are equally valid for, the counterpart endogenous gene.

Second, the reporter encoding nucleotide sequence could be introduced into the cells of a particular tissue by targeting to the promoter to be studied and inserted using position-specific techniques, such as homologous recombination. When this method is used, the expression of the endogenous promoter can be observed directly as well as can the effect of various treatments thereon.

Third, a construct such as those described for the first method can be provided to embryonic tissue to obtain transgenic organisms where the reporter construct is itself endogenous, see, for example, Fukumura, D., et al., *Cell* (1998) 94:715–725, incorporated herein by reference, which describes transgenic mice which use GFP as a reporter for VEGF promoter activity.

Techniques for all three methods are well known in the art.

2. Fluorophores

Any fluorophores known in the art can be used in the present methods. In a preferred embodiment, the fluorophore operatively linked to the promoter of a gene is a humanized fluorophore. Also preferably, the fluorophore is a green fluorescent protein (GFP), a blue fluorescent protein (BFP) and a red fluorescent protein (RFP). More preferably, the GFP is the humanized hGFP-S65T.

The native gene encoding GFP has been cloned from the bioluminescent jellyfish Aequorea victoria (Morin et al., *J. Cell Physiol.,* 77:313–318 (1972)). The availability of the gene has made it possible to use GFP as a marker for gene expression. GFP itself is a 283 amino acid protein with a molecular weight of 27 kD. It requires no additional proteins from its native source nor does it require substrates or cofactors available only in its native source in order to fluoresce (Prasher et al., *Gene,* 111:229–233 (1992); Yang et al., *Nature Biotechnol.,* 14:1252–1256 (1996); and Cody et al., *Biochemistry,* 32:1212–1218 (1993)). Mutants of the GFP gene have been found useful to enhance expression and to modify excitation and fluorescence. GFP-S65T (wherein serine at 65 is replaced with threonine) is particularly useful in the invention method and has a single excitation peak at 490 nm. (Heim et al., *Nature,* 373:663–664 (1995)); and U.S. Pat. No. 5,625,048). Other mutants have also been disclosed by Delagrade et al., *Biotechnology,* 13:151–154 (1995); Cormack et al., *Gene,* 173:33–38 (1996); and Cramer et al. *Nature Biotechnol.,* 14:315–319 (1996). Additional mutants are also disclosed in U.S. Pat. No. 5,625,048. By suitable modification, the spectrum of light emitted by the GFP can be altered. Thus, although the term "GFP" is used in the present application, the proteins included within this definition are not necessarily green in appearance. Various forms of GFP exhibit colors other than green and these, too, are included within the definition of "GFP" and are useful in the methods and materials of the invention. In addition, it is noted that green fluorescent proteins falling within the definition of "GFP" herein have been isolated from other organisms, such as the sea pansy, *Renilla reriformis.* Any suitable and convenient form of the GFP gene can be used in the invention. Techniques for labeling cells in general using GFP are disclosed in U.S. Pat. No. 5,491,084 (supra).

Other GFP, BFP and RFP can be used in the present methods. For instances, the green fluorescent proteins encoded by nucleic acids with the following GenBank accession Nos. can be used: U47949 (AGP1); U43284; AF007834 (GFPuv); U89686 (*Saccharomyces cerevisiae* synthetic green fluorescent protein (cox3::GFPm-3) gene); U89685 (*Saccharomyces cerevisiae* synthetic green fluorescent protein (cox3::GFPm) gene); U87974 (Synthetic construct modified green fluorescent protein GFP5-ER (mgfp5-ER)); U87973 (Synthetic construct modified green fluorescent protein GFP5 (mgfp5)); U87625 (Synthetic construct modified green fluorescent protein GFP-ER (mfgp4-ER)); U87624 (Synthetic construct green fluorescent protein (mgfp4) mRNA)); U73901 (Aequorea victoria mutant 3); U50963 (Synthetic); U70495 (soluble-modified green fluorescent protein (smGFP)); U57609 (enhanced green fluorescent protein gene); U57608 (enhanced green fluorescent protein gene); U57607 (enhanced green fluorescent protein gene); U57606 (enhanced green fluorescent protein gene); U55763 (enhanced green fluorescent protein (egfp); U55762 (enhanced green fluorescent protein (egfp); U55761 (enhanced green fluorescent protein (egfp); U54830 (Synthetic *E. coli* Tn3-derived transposon green fluorescent protein (GF); U36202; U36201; U19282; U19279; U19277; U19276; U19281; U19280; U19278; L29345 (Aequorea victoria); M62654 (Aequorea victoria); M62653 (Aequorea victoria); AAB47853 ((U87625) synthetic construct modified green fluorescent protein (GFP-ER)); AAB47852 ((U87624) synthetic construct green fluorescent protein).

Similarly, the blue fluorescent proteins encoded by nucleic acids with the following GenBank accession Nos. can be used: U70497 (soluble-modified blue fluorescent protein (smBFP); 1BFP (blue variant of green fluorescent protein); AAB16959 (soluble-modified blue fluorescent protein).

Also similarly, the red fluorescent proteins encoded by nucleic acids with the following GenBank accession Nos. can be used: U70496 (soluble-modified red-shifted green fluorescent protein (smRSGFP); AAB16958 (U70496) soluble-modified red-shifted green fluorescent protein).

A fluorophore that changes color with time is reported by Teiskikh, A., et al., *Science* (2000) 290:1585–1588, incorporated herein by reference. This permits tracing time dependent expression.

3. Multi-Cellular Organisms

The present methods can be used in monitoring gene expression in any suitable multi-cellular organisms. In a preferred embodiment, the multi-cellular organism to be analyzed is a plant or an animal, including a transgenic animal. More preferably, the animal is a mammal including a human. Animals that can be analyzed with the present monitoring emthods include, but are not limited to, mice, rats, rabbits, cats, dogs, pigs, cows, ox, sheep, goats, horses, monkeys and other non-human primates.

4. Tissue or Organ Specific Gene Expression

The present methods can be used in monitoring expression of genes that are expressed in a tissue or organ specific manner. The present methods can be used in monitoring health and/or functionality of tissues and/or organs if expression pattern of certain genes are associated with health and/or functionality of these tissues and organs. Preferably, the gene to be monitored is expressed in connective, epithelium, muscle or nerve tissue. Also preferably, the gene to be monitored is expressed in an accessory organ of the eye, annulospiral organ, auditory organ, Chievitz organ, circumventricular organ, Corti organ, critical organ, enamel organ, end organ, external female gential organ, external male genital organ, floating organ, flower-spray organ of Ruffini, genital organ, Golgi tendon organ, gustatory organ, organ of hearing, internal female genital organ, internal male genital organ, intromittent organ, Jacobson organ, neurohemal organ, neurotendinous organ, olfactory organ, otolithic organ, ptotic organ, organ of Rosenmüller, sense organ, organ of smell, spiral organ, subcommissural organ, subfornical organ, supernumerary organ, tactile organ, target organ, organ of taste, organ of touch, urinary organ, vascular organ of lamina terminalis, vestibular organ, vestibulocochlear organ, vestigial organ, organ of vision, visual organ, vomeronasal organ, wandering organ, Weber organ and organ of Zuckerkandl. More preferably, the gene to be monitored is expressed in an internal animal organ such as brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, gland, internal blood vessels, etc.

In other embodiments, the fluorophore, e.g., GFP, BFP or RFP, can be operatively linked to the following animal transcriptional control regions that exhibit tissue specificity to monitor these tissue specific gene expressions in animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., *Cell* 38:639–646 (1984); Ornitz et al., *Cold Spring Harbor Symp. Quant. Biol.* 50:399–409 (1986); MacDonald, *Hepatology* 7:425–515 (1987)); insulin gene control region which is active in pancreatic beta cells (Hanahan et al., *Nature* 315:115–122 (1985)), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., *Cell* 38:647–658 (1984); Adams et al., *Nature* 318:533–538 (1985); Alexander et al., *Mol. Cell Biol.* 7:1436–1444 (1987)), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., *Cell* 45:485–495 (1986)), albumin gene control region which is active in liver (Pinckert et al., *Genes and Devel.* 1:268–276 (1987)), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., *Mol. Cell. Biol.* 5:1639–1648 (1985); Hammer et al., *Science* 235:53–58 1987)), alpha-1 antitrypsin gene control region which is active in liver (Kelsey et al., *Genes and Devel.* 1:161–171 (1987)), beta globin gene control region which is active in myeloid cells (Mogram et al., *Nature* 315:338–340 (1985); Kollias et al., *Cell* 46:89–94 (1986)), myelin basic protein gene control region which is active in oligodendrocyte cells of the brain (Readhead et al., *Cell* 48:703–712 (1987)), myosin light chain-2 gene control region which is active in skeletal muscle (Sani, *Nature* 314:283–286 (1985)), and gonadotrophic releasing hormone gene control region which is active in gonadotrophs of the hypothalamus (Mason et al., *Science* 234:1372–1378 (1986)).

5. Tumor or Cancer Associated Gene Expression

The present methods can be used in monitoring expression of genes that are specifically expressed in tumors or cancers. Preferably, the gene to be analyzed is a tumor or cancer associated gene such as an oncogene or a tumor suppressor gene. For instance, the expression of the oncogenes listed in the following Table 1 can be monitored by the present methods.

TABLE 1

Oncogenes and tumor viruses

Acronym	Virus	Species	Tumor origin	Comments
abl	Abelson leukemia	Mouse	Chronic myelogenous leukemia	TyrPK(src)
erbA	Erythroblastosis	Chicken		Homology to human glucocorticoid receptor
erbB	Erythroblastosis	Chicken		TryPK EGF/TGFc receptor
ets	E26 myeloblastosis	Chicken		Nuclear
fes (fps)[a]	Snyder-Thellen sarcoma Gardner-Arnstein sarcoma	Cat		TryPK(src)
fgr	Gardner-Rasheed sarcoma	Cat		TyrPK(src)
fms	McDonough sarcoma	Cat		TyrPK CSF-1 receptor
fps (fes)[a]	Fujinami sarcoma	Chicken		TyrPK(src)
fos	FBJ osteosarcoma	Mouse		Nuclear, TR
hst	NVT	Human	Stomach tumor	FGF homologue
intl	NVT	Mouse	MMTV-induced carcinoma	Nuclear, TR
int2	NVT	Mouse	MMTV-induced carcinoma	FGF homologue
jun	ASV17 sarcoma	Chicken		Nuclear, TR
hit	Hardy-Zuckerman 4 sarcoma	Cat		TyrPK GFR L
B-lym	NVT	Chicken	Bursal lymphoma	
mas	NVT	Human	Epidermoiod carcinoma	Potentiates response to angiotensin II
met	NVT	Mouse	Osteosarcoma	TyrPK GFR L
mil (raf)[b]	Mill Hill 2 acute leukemia	Chicken		Ser/ThrPK
mos	Moloney sarcoma	Mouse		Ser/ThrPK
myb	Myeloblastosis	Chicken	Leukemia	Nuclear, TR
myc	MC29 myelocytomatosis	Chicken	Lymphomas	Nuclear TR
N-myc	NVT	Human	Neuroblastomas	Nuclear
neu (ErbB2)	NVT	Rat	Neuroblastoma	TryPK GFR L
ral (mil)[b]	3611 sarcoma	Mouse		Ser/ThrPK
Ha-ras	Harvey murine sarcoma	Rat	Bladder, mammary and skin carcinomas	GTP-binding
Ki-ras	Kirsten murine sarcoma	Rat	Lung, colon carcinomas	GTP-binding
N-ras	NVT	Human	Neuroblastomas leukaemias	GTP-binding
rel	Reticuloendothe-liosis	Turkey		
ros	UR2	Chicken		TyrPK GFR L
sis	Simian sarcoma	Monkey		One chain of PDGF
src	Rous sarcoma	Chicken		TyrPK
ski	SKV770	Chicken		Nuclear
trk	NVT	Human	Colon carcinoma	TyrPK GFR L
yes	Y73, Esh sarcoma	Chicken		TyrPK(src)

Similarly, the expression of the following tumor suppressor genes can be monitored by the present methods: p16, p21, p27, p53, RB, WT-1, DCC, NF-1 and APC.

Since abnormally high level of oncogene expression and abnormally low expression of tumor suppressor gene are often good indicators of oncogenesis, the present methods can be used in prognosis or diagnosis of cancer, in monitoring the development of oncogenesis and in evaluating the efficacy of the cancer therapy.

C. Methods to Evaluate a Candidate Protocol or Drug for Treating Disease or Disorder

Since the method of the invention evaluates gene expression with regard to particular control sequences, the effect of various compounds, treatments (such as irradiation) or other perturbations of the genetic environment can be evaluated for their effect on expression using the methods of the invention. Thus, gene toxic agents, for example, can be identified.

In a specific embodiment, a method to evaluate a candidate protocol or drug for treating a disease or disorder is provide herein, which method comprises: a) administering said protocol or drug to a non-human mammalian subject which expresses a fluorophore under the direction of a promoter of a gene associated with a disease or disorder, and determining the expression of said promoter via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said mammalian subject by whole-body external fluorescent optical imaging; b) determining the expression of said promoter, via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said mammalian subject by whole-body external fluorescent optical imaging, in a control non-human mammalian subject which expresses said fluorophore under the direction of said promoter of said gene; and c) comparing the expression of said promoter determined in steps a) and b), wherein the expression determined in step a) is different from that in step b) identifies said protocol or drug as effective in treating the disease or disorder.

In a preferred embodiment, overexpression of the gene is associated with the disease or disorder and the expression determined in step a) is lower than that in step b) identifies said protocol or drug as effective in treating the disease or disorder.

In another preferred embodiment, underexpression of the gene is associated with the disease or disorder and the expression determined in step a) is higher than that in step b) identifies said protocol or drug as effective in treating the infection.

In still another preferred embodiment, the non-human mammalian subject which expresses a fluorophore under the direction of a promoter of a gene associated with a disease or disorder is produced by delivering a nucleic acid encoding the fluorophore operatively linked to the promoter, or a cell containing the nucleic acid, to the non-human mammalian subject (see Section B supra).

Any non-human mammalian subject can be used in the present screening methods. Preferably, the non-human mammalian subject used in the screening is a well established laboratory animal such as a mice, a rabbit or a non-human primate. Also preferably, the non-human mammalian subject used in the screening is an infectious disease animal model. Still preferably, the non-human mammalian subject used in the screen is a transgenic animal.

Any fluorophores known in the art, including the ones described in Section B, can used in the present screening methods. In a preferred embodiment, the fluorophore used in the screening is s a green fluorescent protein (GFP), a blue fluorescent protein (BFP) or a red fluorescent protein (RFP).

The present methods can be used to screen candidate protocols or drugs for treating any known diseases or disorders. In a preferred embodiment, the diseases or disorders to be screened against are cancers, immune system diseases or disorders, metabolism diseases or disorders, muscle and bone diseases or disorders, nervous system diseases or disorders, signal diseases or disorders and transporter diseases or disorders.

In yet another preferred embodiment, the non-human mammalian subject expresses a fluorophore under the direction of a promoter of an infectious organism and the expression determined in step a) is lower than that in step b) identifies said protocol or drug as effective in treating infection caused by the infectious organism.

The non-human mammalian subject used in the screening may be an infectious disease animal model.

The infectious organism screened against may be a fungus such as a yeast, a bacterium such as an eubacteria or an archaebacteria, or a virus such as a Class I virus, a Class II virus, a Class III virus, a Class IV virus, a Class V virus or a Class VI virus.

Any substances can be screened using the present screening methods for finding drug candidates for treating infection. In a preferred embodiment, a combinatorial library is used in the screening assays. Methods for synthesizing combinatorial libraries and characteristics of such combinatorial libraries are known in the art (See generally, *Combinatorial Libraries: Synthesis, Screening and Application Potential* (Cortese Ed.) Walter de Gruyter, Inc., 1995; Tietze and Lieb, *Curr. Opin. Chem. Biol.,* 2(3):363–71 (1998); Lam, *Anticancer Drug Des.,* 12(3 :145–67 (1997); Blaney and Martin, *Curr. Opin. Chem. Biol.,* 1(1):54–9 (1997); and Schultz and Schultz, *Biotechnol. Prog.,* 12(6 :729–43 (1996)).

If the infection is caused by bacteria, known antibiotics can be screened using the present screening methods for finding a suitable drug candidate. Preferably, the antibiotics to be screened are aminoglycosides (e.g., streptomycin, gentamicin, sisomicin, tobramycin, amicacin), ansamycins (e.g., rifamycin), antimycotics polyenes (e.g., nystatin, pimaricin, amphotericin B., pecilocin), benzofuran derivatives (e.g., griseofulvin), β-lactam antibiotics penicillins (e.g., penicillin G and its derivatives, oral penicillins, penicillinase-fixed penicillin broad-spectrum penicillins, penicillins active against Proteus and Pseudomonas), cephalosporins (e.g., cephalothin, cephaloridine, cephalexin, cefazolin, cefotaxime), chloramphenicol group (e.g., chloramphenicol, thiamphenicol, azidamphenicol), lmidazole fluconazole, itraconazole, linosamides (e.g., lincomycin, clindamycin), macrolides (e.g., azithromycin, erythromycin, oleandomycin, spiramycin, clarithromycin), peptides, peptolides, polypeptides (e.g., polymyxin B and E, bacitracin, tyrothricin, capreomycin, vancomycin), quinolones (e.g., nalidixic acid, ofloxacin, ciprofloxacin, norfioxin), tetracyclines (e.g., tetracycline, oxytetracycline, minocycline, doxycycline) and other antibiotics (e.g., phosphomycin, fusidic acid).

D. Methods to Screen for Gene Expression Modulators and Regulators

The above-described screening methods can also be used to identify gene expression modulators, i.e., trans-acting substances that modulate the expression of a target gene in a multi-cellular organism, or regulators, i.e., cis-acting genes of a multi-cellular organism that regulate the expression of the target gene. Besides for identifying disease or disorder treatment protocols or drugs, the screening methods described herein have wide applications in industrial, agricultural, environmental protection and many other fields. For example, transgenic animals such as transgenic cows are commercially used. It is desirable to find a suitable substance that increases the expression of the transgene and such substance can be added to the animal feed. Similarly, it is desirable to find and modify gene(s) within the transgenic cow that enhances the expression of the target transgene.

Once it is decided that alteration of the expression level of a target gene is desirable, a fluorophore can be operatively linked to the promoter, or other transcriptional control region, of the target gene and be expressed in a multi-cellular organism. Then, the multi-cellular organism expressing the fluorophore can be treated with a test substance to identify which substance modulates the fluorophore expression. Alternatively, the multi-cellular organism expressing the fluorophore itself can be mutagenized to identify genes within itself that alter the fluorophore expression. These screening principles have long been used to identify cis- or trans-acting regulators of gene expression in unicellular organisms such as bacteria or yeast. However, due to the lack of quick and simple screening methods, such screening are impractical for multi-cellular organisms such as plants and animals. The whole-body external optical imaging of gene expression disclosed herein makes such screening or mutant-haunt practical for multi-cellular organisms.

In a specific embodiment, a method to screen for a modulator of the expression of a gene in a multi-cellular organism is provided herein, which method comprises: a) administering a test substance to a non-human multi-cellular organism which expresses a fluorophore under the direction of a promoter of a gene, and determining the expression of said promoter via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said multi-cellular organism by whole-body external fluorescent optical imaging; b) determining the expression of said promoter, via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations by whole-body external fluorescent optical imaging, in a control multi-cellular organism which expresses said fluorophore under the direc-

tion of said promoter of said gene; and c) comparing the expression of said promoter determined in steps a) and b), wherein the expression determined in step a) is different from that in step b) identifies said test substance as a modulator of said gene expression. Preferably, the promoter is an endogenous promoter of the multi-cellular organism.

In another specific embodiment, a method to screen for a multi-cellular organism that expresses a gene at an altered level is provided herein, which method comprises: a) administering a mutation-inducing agent or treatment to a non-human multi-cellular organism which expresses a fluorophore under the direction of a promoter of a gene, and determining the expression of said promoter via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said multi-cellular organism by whole-body external fluorescent optical imaging; b) determining the expression of said promoter, via observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations by whole-body external fluorescent optical imaging, in an untreated control multi-cellular organism which expresses said fluorophore under the direction of said promoter of said gene; and c) comparing the expression of said promoter determined in steps a) and b), wherein the expression determined in step a) is different from that in step b) identifies a multi-cellular organism that expresses said gene at said altered level. Preferably, the mutation-inducing agent or treatment causes a mutation in germ-line cells of the multi-cellular organism so that the desired mutation is stably-transferable to offspring of the multi-cellular organism.

In addition, the various protocols described in the art for "Big Blue" transgenic mice can be utilized in the system of the invention.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLE 1

Visualization of Gene Expression in Various Tissues using Adenovirus

Four six-week-old male of female nude/nude, nude/+, or C57BL/6 mice were used. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals under assurance number A3873-1. Mice were fed with autoclaved laboratory rodent diet (Tecklad LM-485, Western Research Products, Orange, Calif.).

The vector employed was adenorival (vAd) vector AdCMV5GFPAE1/AE3 [vAd-green fluorescent protein (GFP)] (Quantum, Montreal, Canada), which expresses enhanced GFP and the ampicillin resistance gene.

This vector was provided to various tissues to visualize expression of the CMV promoter in these tissues. Expression of reporter under control of any desired promoter can be visualized by suitable modification of this vector, as described above.

Liver: After exposure of the portal vein following an upper midline abdominal incision, total volume of 100 μl (8×10^{10} pfu/ml) vAd-GFP per mouse were injected in the portal vein using a 1 ml 39G1 latex-free syringe (Becton Dickinson, Franklin Lakes, N.J.). The puncture hole of portal vein was pressed for about 10 seconds with sterile cotton to stop any bleeding. The incision in the abdominal wall was closed with a 7-0 surgical suture in one layer. The animals were kept under Ketamine anesthesia during surgery. All procedures of the operation described above were performed with a 7× magnification microscope (Leica MZ6, Nussloch, Germany). Animals were kept in a barrier facility under HEPA filtration.

Brain: The parietal bone of the skull was exposed after an upper midline scalp incision. Twenty microliters containing 8×10^{10} plaque-forming units (pfu)/ml vAd-GFP per mouse was injected in the brain by using a 1-ml 27G1/2 latex-free syringe (Becton Dickinson). The puncture hole in the skull was plugged with bone wax. The incision in the scalp was closed with a 7-0 surgical suture in one layer. The animals were kept under isofluorane anesthesia during surgery.

Pancreas: The pancreas was exposed after an upper midline abdominal incision. One-hundred microliters containing 8×10^{10} pfu/ml vAd-GFP per mouse was injected in the pancreas by using a 1-ml $30G_{1/2}$ latex-free syringe (Becton Dickinson). The puncture hole was pressed for about 10 sec with sterile cotton for hemostasis. The incision was closed with a 7-0 surgical suture in one layer. The animals were kept under Kersel anesthesia during surgery. All procedures of the operation described above were performed with a ×7 magnification stereo microscope.

Prostate: The bladder and prostate were exposed after a lower midline abdominal incision. Thirty microliters containing 8×10^{10} pfu/ml vAd-GFP per mouse was injected in the prostate by using a 1-ml $30G_{1/2}$ latex-free syringe (Becton Dickinson). The puncture hole in the prostate was pressed for about 10 sec with sterile cotton for hemostasis. The incision in the abdominal wall was closed with a 6-0 surgical suture in one layer. The animals were kept under isofluorane anesthesia during surgery. All procedures of the operation described above were performed with a ×7 magnification stereo miscroscope.

Bone Marrow: For bone marrow injection, animals were anesthetized by inhalation of isofluorane. The skin on the hind leg was opened with a 1-cm incision to expose the tibia. A 27-gauge needle with latex-free syringe (Becton Dickinson) then was inserted in the bone marrow cavity. A total volume of 20 μl (8×10^{10} pfu/ml) vAd-GFP per mouse was injected into the bone marrow cavity. The puncture hole in the bone was plugged with bone wax, and the incision was closed with a 6-0 surgical suture.

Visualization: For visualization at high magnification, Leica fluorescence stereo microscope, model LZ12, equipped with a 50-W mercury lamp, was used. Selective excitation of GFP was produced through a D425/60 bandpass filter and 470 DCXR dichroic mirror. Emitted fluorescence was collected through a long-pass filter GG475 (Chroma Technology; Brattleboro, Vt.) on a Hamamatsu C5810—3-chip cooled color charge-coupled device camera (Hamamatsu Photonics Systems, Bridgewater, N.J.). Images were processed for contrast and brightness and analyzed with the use of IMAGE PRO PLUS 3.1 software (Media Cybernetics, Silver Springs, Md.). Images of 1,024×724 pixels were captured directly on an IBM PC or continuously through video output on a high-resolution Sony VCR model SLV-R-1000 (Sony, Tokyo).

Imaging at lower magnification that visualized the entire animal was carried out in a light box illuminated by blue light fiber optics (Lightools Research, Encinitas, Calif.) and imaged by using the thermoelectrically cooled color charge-coupled device camera, as described above.

Quantitation: The intensity of GFP fluorescence is measured to account for variations in the exciting illumination

with time and across the imaging area. These factors are corrected for by using the intrinsic red fluorescence of mouse skin as a base line to correct the increase over intrinsic green fluorescence caused by GFP. This can be done because there is relatively little red luminance in the GFP radiance. Consequently, the green fluorescence was calculated relative to red based on red and green channel composition in the skin image. A ratio (γ) of green to red channels was determined for each pixel in the image of skin without and with GFP. Values of γ for mouse skin throughout the image in the absence of GFP were fairly constant, varying between 0.7 and 1.0. The contribution of GFP fluorescence from within the animal increased the green component relative to red, which was reflected in higher γ values. The total amount of GFP fluorescence was approximated by multiplying the number of pixels in which value γ was higher than 1 times the γ value of each pixel. Such a product roughly corresponds to the integral GFP fluorescence [Γ_{GFP}] above the maximum value of γ for skin without GFP. The number of pixels in mouse skin images with γ value >1.0 without GFP was less than 0.02% and increased with GFP expression. The value of [Γ_{GFP}] is shown as a function of time after virus injection in FIGS. 1A and 1B for brain and liver respectively.

Images of the various organs were compared when taken at high magnification on live intact animals or similar organs viewed directly after death and dissection. The images show the distribution of gene expression in the various organs. In all cases, the images made externally are similar to those of the exposed organs.

When the live animal was viewed in a light box, it was also possible to monitor the expression of the gene, thus permitting a real time observation of the living animal and expression as it occurs in this animal. For example, a light box determination of expression of the GFP in nude mouse liver taken at 72 hours clearly shows this result. Similar results are observed in the nude mouse brain 24 hours after gene delivery. The method is quite sensitive in that the intensity of GFP fluorescence in the mouse liver at a depth of 0.8 mm under the skin was about 25% of that of the exposed organ. Gene expression is externally measurable if the average fluorescence of the GFP expressing organs is at least 20% above the average fluorescence of the surrounding skin, and at maximal level of GFP expression, the intensity in the liver exceeded more than 100 times the back dorsal and abdominal skin fluorescence.

EXAMPLE 2

Visualization of Genes Using Lentiviral Vectors

Lentiviral vectors have been shown to transduce a broad spectrum of non-dividing cells in vitro, such as neurons, retina, liver, muscle and hematopoietic stem cells (see, for example, Naldini, L. et al., *Science* (1996) 272:263–267; Kafri T. et al.; *Nat. Genet* (1997) 17:314–317; Takahashi, M. et al., *J. Virol* (1999) 73:7812–7816; Miyoshi, H. et al. *Science* (1999) 283:682–686). Although it has been reported that hepatocytes are refractory to lentiviral transductioni unless they progress into the cell cycle (Park, F. et al. *Nat. Genet* (2000) 24:49–52), it is shown below that lentiviral gene delivery to the liver for expression visualization is practical.

A lentiviral vector based on HIV1 designated GFP-LV was used. This vector contains a self-inactivating mutation in the U-3 region, a post-transcriptional element, and an internal CMV promoter. It also contains cppt, the central polypurine tract derived from HIV-pol and a woodchuck hepatitis virus post-transcriptional element (WPRE). A diagram of this vector is shown in FIG. 2A.

The vector GHP-LV at 1×10^9 IU was injected into the portal vein of nude mice; (Hsd:asymic nude-nu). Six (6) days after injection green fluorescence was testable in the liver using in-vivo fluorescence optical imaging, as shown in FIG. 2B. At day 21, all lobes of the liver of the mice injected with this vector exhibited a homogeneous green fluorescence.

GHP-LV at 1×10^9 IU was also injected intraperitoneally and this method also resulted in a high level of transduction of liver and spleen.

Western Blot demonstrated dose dependence of GFP expression in the range of $0.5–2.5\times10^9$ IU. Vector integration in the liver 3 weeks after injection was demonstrated by PCR.

Confirmation that the transduced cells were not rapidly dividing was achieved by administering 5' bromo-2' deoxyuridine (BrdU) 15 mgs/kg by daily IP injections in order to label dividing cells. While the cells in the duodenum showed high labelling, only about 3% of liver cells were BrdU positive in either control or lentiviral-treated livers.

EXAMPLE 3

Additional Applications

In addition to the procedures exemplified in Examples 1 and 2, the methods of the invention may be used to monitor expression of control sequences that are regulated by the unfolded protein response (UPR) as described, for example, by Niwa, M., et al., *Cell* (1999) 99:691–702, the contents of which are incorporated herein by reference. Another suitable target for study is the circadian rhythm controlling genes which were studied using less convenient techniques by Yamaguchi, S., et al, *Nature* (2001) 409:684, incorporated herein by reference.

Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

What is claimed is:

1. A method to monitor the ability of a promoter to promote expression in an animal of an endogenous gene that is controlled by said promoter, which method comprises:

a) delivering, to an animal, cells containing a nucleic acid encoding a fluorophore operatively linked to the promoter of said endogenous gene whose ability to promote expression is to be analyzed; and

b) observing the presence, absence or intensity of the fluorescence generated by said fluorophore at various locations in said animal by whole-body external fluorescent optical imaging,

whereby the ability of said promoter to promote expression is monitored, and

wherein said fluorophore is a protein that is autofluorescent such that no substrates or cofactors are needed for it to fluoresce.

2. The method of claim **1**, wherein the cells are delivered to the animal via a surgical procedure.

3. The method of claim **2**, wherein the cells are delivered to the animal via direct implantation by surgical orthotopic implantation (SOI) at a desired site.

4. The method of claim **1**, wherein the animal is a human and the fluorophore is a humanized fluorophore.

5. The method of claim 1, wherein the fluorophore is selected from the group consisting of a green fluorescent protein (GFP), a blue fluorescent protein (BFP) and a red fluorescent protein (RFP).

6. The method of claim 5, wherein the animal is a human and the GFP is the humanized hGFP-S65T.

7. The method of claim 1, wherein the animal is a mammal.

8. The method of claim 7, wherein the mammal is selected from the group consisting of a mouse, a rat, a rabbit, a cat, a dog, a pig, a cow, an ox, a sheep, a goat, a horse, a monkey and a non-human primate.

9. The method of claim 1, wherein the endogenous gene is normally expressed in a tissue or organ specific manner.

10. The method of claim 9, wherein the tissue is selected from the group consisting of connective, epithelium, muscle and nerve tissues.

11. The method of claim 9, wherein the organ is selected from the group consisting of brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, gland, and internal blood vessels.

12. The method of claim 1, wherein the endogenous gene is an endogenous tumor or cancer associated gene.

13. The method of claim 12, wherein the tumor or cancer associated gene is an oncogene or a tumor suppressor gene.

* * * * *

EXHIBIT 4

EXHIBIT 4

CellSight Technologies, Inc.

Imaging Therapeutics

- **Services**

- Cell Kinetics
- Gene Expression
- Custom Services

- **Products**

- Tri-fusion Reporter Gene

Order Molecular Imaging with Reporter Genes

Services

CellSight provides customized expert molecular imaging services for monitoring cell kinetics and gene expression in research animals and humans. We employ non-invasive molecular genetic imaging as well as *in vitro* detection techniques.

Cell Kinetics Services Offered

- Monitor pharmacokinetics of therapeutic cells in living subjects (research animals and humans) at multiple desired time-points following administration. Client therapeutic cells can vary
  - Therapeutic immune cells (NK, T Cells, DCs, Macrophages)
  - Stem cells (Messenchymal, Embryonic, iPS, Adult Stem Cells)
  - Other therapeutic cells (Pancreatic islets, etc...)
- Track changes in transplanted tissues at multiple desired time points:
  - Survival of tissue cells
  - Changes in characteristics of tissue cells
  - Migration of tissue cells
  - Proliferation of tissue cells
- Analyze effect of drugs on cells:
  - Cell killing by a therapeutic agent
  - Cell growth/proliferation induced by a therapeutic agent
  - Therapeutic agent causing cell migration or inhibiting cell migration
  - Therapeutic agent causing a change in cell characteristics (e.g. differentiation)

Gene Expression Services Offered

- Evaluate pharmacokinetics of therapeutic transgenes including magnitude of therapeutic transgene expression at each location

- Monitor endogenous gene expression and effect of external factors on the expression

Custom Services Offered

- Genetically engineer cells to express one or more imaging reporter genes of different modalities
- Assay cells or tissues for imaging reporter gene expression
- Pre-label cells with imaging probes
- Pre-clinical and clinical pharmacokinetic analysis of therapeutic cells
- Pre-clinical and clinical reporter gene imaging studies
- Monitor cell and gene therapy through molecular imaging
- Monitor cancer therapy efficacy through reporter gene imaging or general molecular imaging
- Pre-clinical and clinical molecular imaging consulting

Products

- Multi-modality Tri-fusion imaging reporter gene constructs
- Viral and non-viral kits for genetically engineering cells to express one or more imaging reporter genes
- Cell lines stably expressing one or more imaging reporter genes

CellSight Technologies, Inc. ï¿½ 185 Berry Street, Suite 350 ï¿½ San Francisco, CA 94107 ï¿½ Tel: 650-799-1589 ï¿½ Fax: 415-353-9423

EXHIBIT 5

EXHIBIT 5

CellSight Technologies, Inc.

Imaging Therapeutics

- **Cell Kinetics**
  - Direct Labeling
  - Genetic Engineering
- **Reporter Gene Imaging**
  - Fluorescence
  - Bioluminescence
  - Positron Emission Tomography (PET)
  - Clinical Imaging
  - References

Order Molecular Imaging with Reporter Genes

Technology

CellSight allows therapy developers to "barcode" their cells or genes prior to introduction into living subjects (including humans) and then view these "barcoded" cells or genes at various time points after treatment. Information can be gathered not only for the injected therapeutic cells but also their progeny in the living subject.

Cell Kinetics data that can be obtained at multiple time points

- Cell Locations
- Cell Quantity at each Location
- Cell Survival
- Cell Proliferation
- Cell Status and Characteristics

Proven methods to image Cell Kinetics

- Direct Labeling with an Imaging Probe
- Genetic engineering of cells to express imaging reporter genes

This video illustrates PET reporter gene based imaging of therapeutic cells in the brain of a glioma patient. The PET image is superimposed over the brain MRI, showing the location and intensity of the PET reporter imaging probe [18F]FHBG before and after injection of HSV1-tk expressing cytolyticT cells.

Methods to perform reporter gene imaging in living subjects include

- Fluorescence
- Bioluminescence
- Positron Emission Tomography (PET)

CellSight Technologies provides means to combine the three methods of gene imaging with a single tri-fusion multimodality reporter gene. You can then track implanted cells or genes from preclinical applications and then translate to clinical applications.

PET allows imaging in both small and large animals, a technique translatable to imaging in humans.

CellSight personnel have been granted FDA "Investigational New Drug" (IND) to image PET reporter gene expression in patients using [18F]FHBG. This is the only FDA approved molecular imaging probe to image cells in humans.

Therapy developers can use the same triple fusion gene during therapy development for imaging cells in vitro all the way to imaging living subjects.

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EXHIBIT 6

EXHIBIT 6

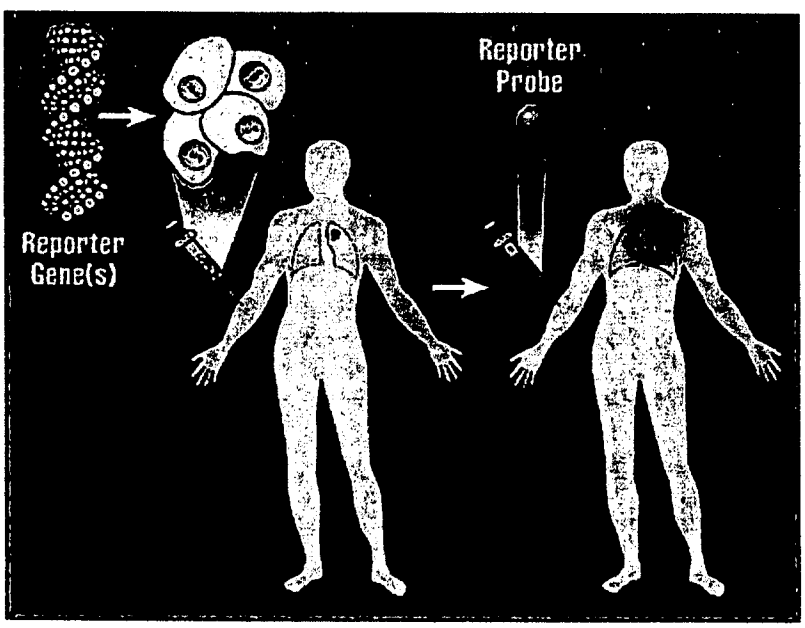
CellSight Technologies, Inc.

Imaging Therapeutics

- **Cell Kinetics**
  - Direct Labeling
  - Genetic Engineering
- **Reporter Gene Imaging**
  - Fluorescence
  - Bioluminescence
  - Positron Emission Tomography (PET)
  - Clinical Imaging
  - References

Order Molecular Imaging with Reporter Genes

Technology - Genetic Engineering



Reporter Gene Technique For Imaging Cell Kinetics

Cells desired to be monitored are genetically engineered in culture by transfection, transduction or electroporation/nucleofection to constitutively express the reporter gene and then administered into the living subject. At any desired time-point thereafter a specific imaging probe can be injected to image reporter gene expression to determine cell kinetics. This technique can be used to image all aspects of cell kinetics.

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EXHIBIT 7

EXHIBIT 7

CellSight Technologies, Inc.

Imaging Therapeutics

About Us Technology Services & Products Contact Us

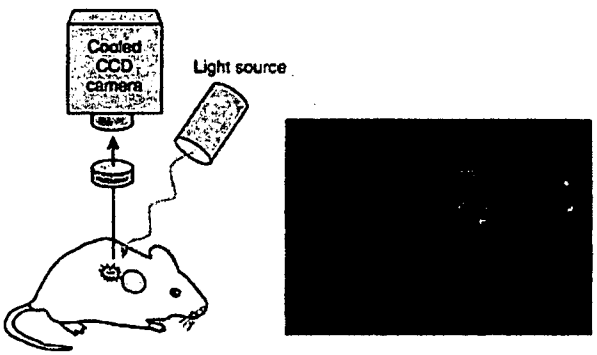
- **Cell Kinetics**
  - Direct Labeling
  - Genetic Engineering
- **Reporter Gene Imaging**
  - Fluorescence
  - Bioluminescence
  - Positron Emission Tomography (PET)
  - Clinical Imaging
  - References

Technology - Fluorescence Optical Imaging

Fluorescence images are obtained by detecting light of a certain wavelength emitted from molecules that have been excited by light of another wavelength. Various sensitive instruments are available for fluorescence imaging.

Fluorescence Reporter Genes

Fluorescence reporter genes, such as those encoding green fluorescent protein (GFP) and red fluorescent protein offer many in vitro and in vivo molecular-genetic applications. However, their sensitivity is much lower for imaging in living subjects due to significant auto-fluorescence and attenuation of both excitation and emission lights.



Fluorescence Imaging Reporter Genes Can be Used to Image the Following Non-invasively in Small Animals

- Transgene expression (Gene therapy monitoring)
- Endogenous gene expression
- Cell kinetics (Cell therapy, regenerative medicine and cancer therapy)
- Protein-protein interactions
- Signal transduction
- Pharmacodynamics, or molecular effects of external factors, such as administered therapeutic agents

Advantages of Fluorescent Reporter Genes

- Multi-spectral imaging (Ability to detect two or more molecular events at the same time)
- Single cell microscopic imaging
- Cell Sorting
- Intravital microscopy
- Tomographic imaging

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EXHIBIT 8

EXHIBIT 8

Organ-specific models-Metamouse

Brain cancer models

Example: Human U-87-RFP glioma growing in brain of nude mouse. Whole-body image.

Features

- Orthotopic transplantation
- Green Fluorescent Protein expression
- Red Fluorescent Protein expression

ORGAN SPECIFIC MODELS

- Bladder
- Brain
- Breast
- Colon
- Kidney
- Lung
- Liver
- Melanoma
- Ovarian
- Pancreas
- Prostate
- Stomach
- Bone Metastases

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EXHIBIT 9

EXHIBIT 9

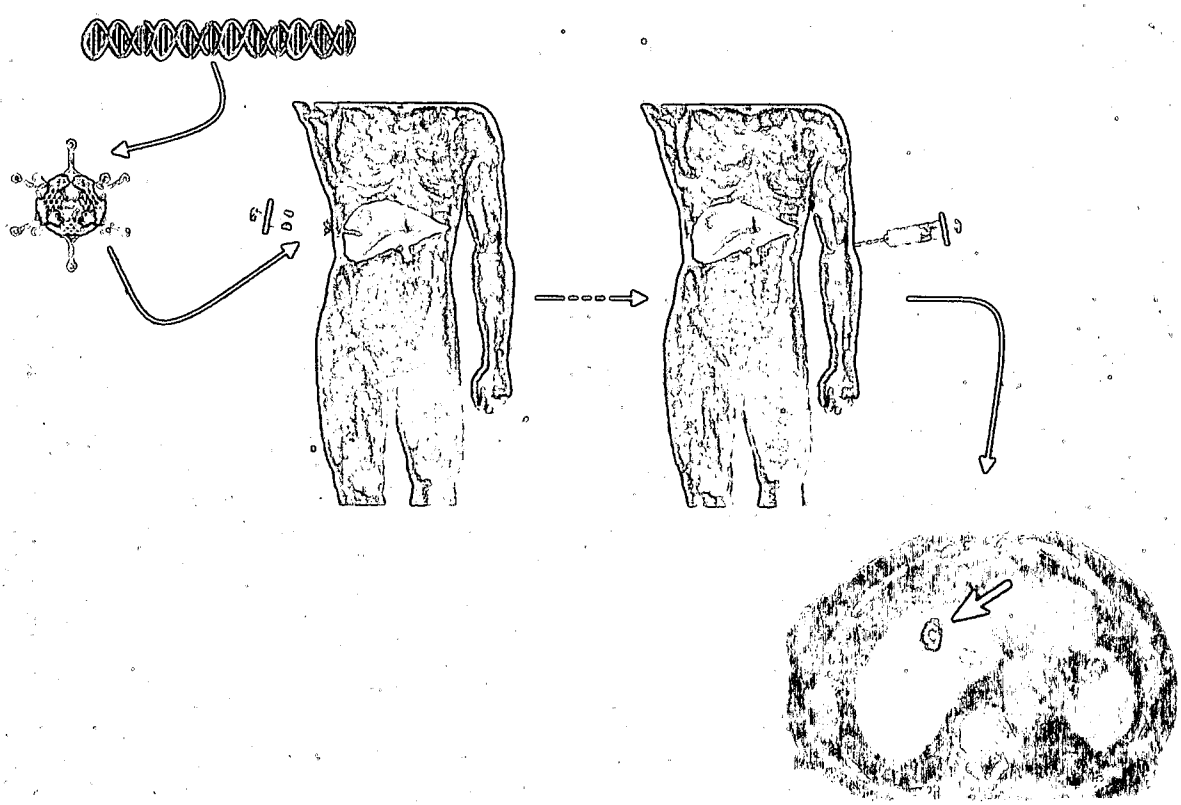
Cambridge Molecular Imaging

SERIES EDITORS: SIMON CHERRY, WOLFGANG A. WEBER, NICHOLAS VAN BRUGGEN

MOLECULAR IMAGING WITH REPORTER GENES

Edited by

Sanjiv Sam Gambhir • Shahriar S. Yaghoubi



MOLECULAR IMAGING

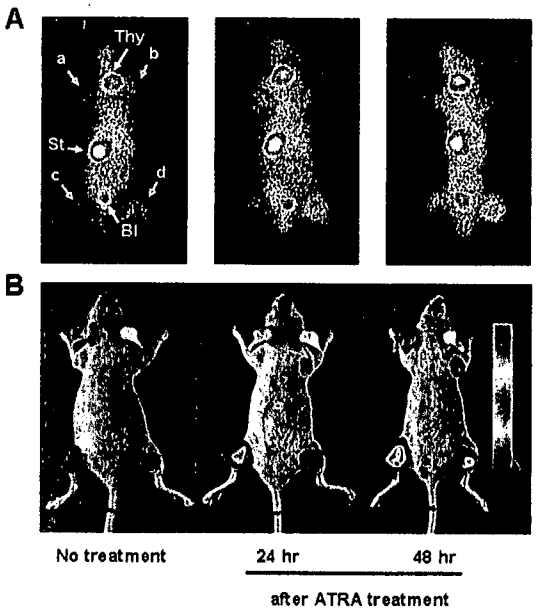


Figure 3.5. ^{99m}Tc scintigraphy (**A**) and bioluminescent (**B**) images of nude mice bearing SK-HEP1 and SK-HEP1/RARE/NIS-Luc tumors before and after all transretinoic acid (ATRA) treatment. Xenograft tumors derived from SK-HEP1 (a, 1×10^7 wild-type) and SK-HEP1/RARE/NIS-Luc cells (b, 1×10^6; c, 1×10^7; d, 1×10^8) were grown in nude mice. (**A**) Serial planar gamma camera images of the same mouse showing increased radioactivity after ATRA treatment in an SK-HEP1/RARE/NIS-Luc tumor, but not in an SK-HEP1 tumor (Thy = thyroid; St = stomach; Bl = bladder). (**B**) Serial optical images of the same mouse showing an increased optical signal after ATRA treatment in SK-HEP1/RARE/NIS-Luc tumors, but not in SK-HEP1 tumors.

two fusion proteins in the presence of doxycycline were grown subcutaneously in mice, and after treatment with doxycycline, PET imaging using F-18 FHBG detected and allowed the activation of reporter gene to be measured. Paulmurugan et al. [99] produced the splitted firefly luciferase as N-terminal and C-terminal halves that are fused into ID and MyoD, respectively (Nfluc-ID and MyoD-Cfluc). When Nfluc-ID and MyoD-Cfluc are simultaneously expressed, full luciferase activity could be recovered through MyoD and ID interaction. Therefore protein–protein interaction can be monitored using split reporter *in vivo* as well as *in vitro*.

Monitoring Tumor Mass

Noninvasive reporter gene imaging offers excellent opportunities to understand cancer progression, metastasis, and therapy in whole animals. Individual animals can be visually monitored for tumor burden at primary sites, differences in tumor progression rates can be distinguished, the possibility of metastases can be investigated, individual responses to alternative therapies can be repeatedly monitored, and therapies can be altered and the consequences of these alterations observed [100].

A large number of models of engrafted tumors have been marked with optical or nuclear medicine reporter genes and then used to monitor tumor burden and response to therapy. For example, we transfected hNIS and Fluc (firefly luciferase) genes under constitutive cytomegalovirus (CMV) promoter into human hepatoma cells. Using animal models, the effects of anticancer therapeutic regimens was easily monitored using a gamma camera system and radioiodine or Tc-99m. We found an excellent correlation ($R^2 = 0.99$) between accumulated radioiodine activity in cells and number of viable cancer cells [71]. In this study, reporter imaging using the NIS gene reflected viable cancer cell numbers and allowed changes in cell numbers to be detected after anticancer treatment. Furthermore, *in vivo* scintigraphic imaging using [^{99m}Tc] showed a moderate correlation ($R^2 = 0.87$) between tumor weight and radioactivity (Figure 3.6). However, it is likely that radioactivity reflects viable cancer cell numbers more accurately than tumor weight because tumor tissues contain immune cells, necrotic, and fibrous tissues as well as cancer cells. In addition, our group developed a lentiviral vector system carrying sodium iodide symporter (hNIS) gene under UbC promoter to establish stable and long-term gene expression *in vitro* and *in vivo* and subsequently demonstrated a marked therapeutic effect by radioiodine gene therapy using [^{131}I] in a tumor xenograft model [101].

Cell Therapy

Molecular imaging using imaging reporter genes can also be applied to the monitoring of *in vivo* distributions of target cells or therapy cells, such as immune and stem cells. The imaging of targeted T-cell trafficking using optical luciferase bioluminescence imaging has been demonstrated in several models of autoimmunity, including collagen-induced arthritis [102] and experimental autoimmune encephalomyelitis [103].

It would be of great value if the antigen-dependent activation states of T cells could be monitored noninvasively clinically and in animal models. Ponomarev et al. [104] transfected HSV1-tk/GFP fusion protein under the control of an NFAT-responsive promoter into a human T-cell line. In this study, cells were selected by flow cytometry for the NFAT induction of GFP expression in response to T-cell activation. Expression from NFAT promoter was measured by PET using I-124 FIAU as a noninvasive measure of T-cell receptor-dependent gene expression.

The transplantation of cells, such as stem cells or progenitor cells, into damaged tissues has tremendous potential for the treatment of a number of disorders. After stem cells have been administered systemically or locally, they may be able to migrate and repopulate pathologic sites. Reporter gene imaging techniques have

is difficult to convert an optical signal sensitivity into units of the molar concentration of and/or number of cells expressing the gene of interest. Finally, for FLI, due to strong autofluorescence background contamination from endogenous fluorochromes existing in tissue, the typical photon signal sensitivity is over an order of magnitude lower than for BLI [16].

Spatial resolution: The light photons that escape the body through a given area and direction from the surface of the animal are known as the surface *radiance*, typically expressed in units of watts/cm^2/steradians, where steradian is a unit of "solid" or volumetric angle. This surface radiance is used for noninvasive, *in vivo* optical imaging. Spatial resolution for *in vivo* optical imaging is mainly limited by the surface radiance spatial resolution (spot size), which is in turn a function of the emission source depth. The camera spatial resolution, which is a function of the camera lens magnification factor and the light sensor pixel size, is typically high (in the micron range), and so it contributes insignificantly to the overall measured surface radiance spot size compared to the emission source depth effect. Figure 7.1c plots the approximate surface radiance signal spot size (i.e., spatial resolution) versus emission source depth within tissue for red (~650 nm), orange (~590 nm), and yellow–green (~550 nm) light [16, 20]. Due to the highly diffusive nature of red and NIR light propagation, the measured spatial resolution is worse (surface radiance spot size is larger) for a light signal emitting in that portion of the spectrum. From Figure 7.1c we can induce the coarse rule of thumb that the measured spatial resolution (FWHM) of the surface radiance signal for a red light source is roughly equal to the depth below the surface in which it resides (e.g., ~1 cm FWHM for a 1 cm deep point source although somewhat less than 2 cm for a 2 cm deep 650 nm emission source) [16, 20]. Note that extrapolating from Figure 7.1c, if NIR wavelength emission sources are used, the FWHM resolution observed at the surface of the animal will be even greater than the source depth.

Thus, obtaining high resolution, quantitative optical detection, and imaging of gene expression *in vivo* and noninvasively is challenging due to the optical properties of intact biological tissues. Choosing excitation sources and reporters that emit in the red and NIR portions of the optical spectrum appear most promising with regard to penetration of tissues, with the drawback of producing and accepting a higher fraction of multiple-scattered photons into the detector that result in radiance resolution loss.

Fluorescence Imaging (FLI)

Overview

Optical fluorescence imaging (FLI) can image a variety of *in vivo* processes including gene expression occurring in cells located within tissues of live small laboratory animal subjects (mainly mice) by observing the body surface distribution of FL signal. Specific genes of interest can be linked with reporter genes in transgenic animals and their expression followed *in vivo* over the animal's lifetime. This approach has also been used in important *in vivo* applications such as monitoring therapeutic gene delivery strategies [21], tracking infectious diseases [22], and following the proliferation of cancer cells and their progeny in xenograft and transplant tumor models [23]. The FL proteins are isolated from living organisms and the gene that encodes for these proteins may be inserted into cells and used as a reporter gene [24]. GFP has been widely used in biological research for cell culture and *ex vivo* study of tissue sections [3, 4] as well as *in vivo* studies [25]. However, due to its low peak light absorption wavelength (~470–490 nm) a matched excitation source light cannot easily penetrate into tissue to excite GFP for *in vivo* FL imaging of live subjects. Similarly, due to the low peak emission wavelength (~510 nm) the GFP emission light cannot escape out of tissue. Furthermore, GFP's absorption and emission spectra also overlap with that of the background autofluorescence of surrounding tissue due to absorption by endogenous tissue fluorophores. This autofluroescence signal is not relevant for characterization of the gene expression level and must be subtracted from the overall detected signal. Thus, GFP is poorly suited for a wide variety of *in vivo* imaging studies. For *in vivo* FLI it is clear that a high light yield fluorophore with longer wavelength absorption and emission spectra within the optical window are desirable. These properties can be achieved by exploiting mutations in the naturally occurring gene encoding the FL protein (e.g., GFP and other FL proteins) [7]. It is also desired to use a high intensity, matching long wavelength excitation source and appropriate optical filters.

FLI Instrumentation

The distribution of the reporter gene can be localized within the animal subject by illuminating the animal with an external light source that excites the fluorophore within the FL protein [25]. The light source can be an intense laser (e.g., a red-emitting Ti:sapphire laser) that emits a well-defined frequency of light, ideally well matched to the absorption spectrum of the fluorophore to maximize signal intensity, minimize autoflourescence, and minimize any damage to tissue. The light source can also be a broadband source such as a fluorescent lamp equipped with a bandpass, in particular, a *low pass* filter that preferentially passes the lower frequency (longer wavelength) portion of the spectrum and that will have better penetration into the subject tissues for excitation of the reporter molecule. The filters on the emission light reaching the detector should pass the emitted light and block the excitation light that happens to propagate

EXHIBIT 10

EXHIBIT 10

Advances in Imaging Techniques Help Drive Stem Cell Research Forward

A 57-year-old man, suffering seizures and headaches, was diagnosed with a brain tumor in 2005. Doctors at the City of Hope cancer center in Duarte, California, removed the mass, but knew his cancer was of a type likely to return.

So they enrolled him in a cell therapy trial, and collected T cells from his blood. When the cancer did indeed recur, nine months later, they combined surgery with infusions of his own T cells, souped up with extra genes to make them seek and destroy the remaining cancer.

But once the new cells were in the patient, would they go to the right place? Would they function as expected? Would they even survive? "If they did not target the tumor, then the therapy would not work," said Shahriar Yaghoubi, a research radiologist at Stanford University in Palo Alto, California. "And if they went somewhere else, they might cause problems."

The answers to those questions will be necessary for cell-based therapies, including stem cell treatments, to make it through Food and Drug Administration review and into the clinic. "Right now, a lot of procedures are actually a black box," said Charles Lin, who is investigating cell tracking at Harvard Medical School in Boston, Massachusetts. "Imaging technology would allow us to peek inside the black box and see what's happening." Researchers are developing long-lasting tags to track cells, in vivo, with fluorescence microscopy, magnetic resonance imaging (MRI) and positron emission tomography (PET) (reviewed in Rodriguez-Porcel et al., 2009). "It is a very new technique right now," said Ali Arbab, a research radiologist at Henry Ford Hospital in Detroit, Michigan.

Doctors can inject cells directly into the target area, or rely on attractors such as cytokines to guide the new cells to the right place. But the majority of cells may fail to reach the target, Arbab said, and may not survive at all. The propensity for cells to find the right site is also likely to be cell type-specific, said Khalid Shah, a neurobiologist interested in cancer at Harvard Medical School and Massachusetts General Hospital in Boston.

Preclinical and Particle Tags

The first place to examine stem cell homing and function is in preclinical animal models, and the most straightforward technique is to mark cells with fluorescent proteins or luciferase. Optical imaging offers single-cell resolution. It is most commonly used with tissue samples once the animal has been sacrificed, but now there are ways to look at cells in a living organism with intravital microscopy. "There has been a real explosion of interest in this technique," Lin said, although few labs are using it for stem cell tracking yet. Lin had to build his own microscope, but top manufacturers are starting to develop commercial versions, he said.

The advantage of intravital microscopy is not only longitudinal studies of living animals, but also its ability to find rare cell types. Lin and colleagues have used the technique to image hematopoietic stem cells that migrated to the bone marrow inside a mouse skull (Lo Celso et al., 2009: PMID19052546). Only five to ten cells may get there, he said, and picking them out in a set of thin sections would be tedious. With confocal imaging of the entire skull, he can more easily find the cells of interest.

Shah has used fluorescent markers to track stem cells migrating toward mouse gliomas, and determine that neural stem cells move through brain tissue better than mesenchymal stem cells. (Shah et al., 2008: PMID18434519; Sasportas et al., 2009: PMID19264968). But fluorescence only travels through a few millimeters of tissue; Shah must cut a porthole in the skull to image the brain. This kind of microscopy would not be able to reach deep into the brain of someone with a tumor, for example. "The translation of optical imaging to clinics is limited," Shah said.

The clinical method that has been used most so far it is to force cells to take up super-paramagnetic iron oxide (SPIO) nanoparticles. The iron particles interfere with MRI, appearing as a shadow on the scan. However, the technique has limitations. "It only tells you about the location," Yaghoubi said. "If the cells multiply, you keep diluting your probe." And if the cells die, the nanoparticles may hang around for a while.

Four human SPIO trials (reviewed in Bulte, 2009: PMID19620426) have shown that it is feasible to detect cells this way. However, all four were done outside of the United.States. Stringent government regulations make it difficult to do human trials in the U.S., said Jeff Bulte, a radiologist at the Johns Hopkins University School of Medicine in Baltimore, Maryland. Another roadblock is that the FDA-approved SPIO particle Feridex has been off the market since 2008, and its producer, AMAG Pharmaceuticals of Lexington, Massachusetts, has no plans to make it again.

It may be worthwhile, then, to explore alternative tracers. Celsense, Inc., of Pittsburgh, Pennsylvania, is developing perfluorocarbon tags (reviewed in Janjic and Ahrens, 2009: PMID19920872). They work much like SPIO, in that cells take up the inert particles. Radiologists detect the marker with an MRI tuned to fluorine. Because fluorine is rare in the body, only tagged cells show up. Doctors can then overlay that image with standard MRI, showing organs and tissues, to see where the labeled cells are. Scientists are "on the verge" of trying the marker in human trials, said Celsense president Charlie O'Hanlon.

It's in the Genes

Both perfluorocarbon and SPIO signals may fade over time as cells divide or die, and neither will tell doctors what the cells are up to. "The next question is do they survive, and that's where you need another approach," said Raphael Guzman, a neurosurgeon at Stanford University. So the next big thing in the field is imaging techniques based on reporter genes that cells transcribe as long as they live.

This is the approach taken in the man (his identity is protected by privacy laws) from the City of Hope trial, which enrolled a handful of patients. When his doctors engineered his T cells, they added a gene for interleukin 13 zetakine, a receptor to home in on the cancer. In case something went wrong with the transplanted cells, they also added a failsafe, a suicide gene from herpes simplex virus 1 called thymidine kinase (HSV1-tk). In addition to phosphorylating nucleosides, its

natural role, HSV1-tk also phosphorylates the drug ganciclovir. Harmless on its own, ganciclovir becomes cytotoxic upon phosphorylation. If the new cells went rogue, ganciclovir treatment would destroy only the transplanted cells.

Conveniently, HSV1-tk also forms the basis for a PET imaging system. Yaghoubi and his colleagues, led by Sanjiv Gambhir of Stanford University, piggybacked onto the City of Hope study in 2006 to test-drive the concept. In addition to nucleosides and ganciclovir, HSV1-tk phosphorylates a probe called FHBG. FHBG can move in and out of cells, but once phosphorylated—as would only happen in the transplanted T cells or their descendants—it gains a negative charge and can no longer cross the cell membrane. "The only way the probe gets trapped inside the cells is if these cells are alive," Yaghoubi said. The researchers used FHBG containing radioactive fluorine so the labeled cells will light up on a PET scan. The approach is "a good way" to follow living cells, said Arbab, who was not involved in the trial.

This study (Yaghoubi et al., 2009: PMID19015650), the first to use reporter gene-based imaging of therapeutic cells in a person, enrolled the man with the brain tumor in 2006. The researchers were able to see the site of the returned tumor, but they also got a surprise: "We noticed there was another signal coming from another site in the brain," Yaghoubi said. The City of Hope doctors confirmed that there was a second tumor. "Had it not been for this imaging, they might not have detected that until later on," Yaghoubi said.

The man survived for fourteen months after the recurrence. The researchers have tried the same technique in a second patient, and intend to re-team with City of Hope in a larger cell therapy trial expected to begin enrollment in February, Yaghoubi said.

The More the Merrier

Gambhir founded a company, Cellsight Technologies in Sunnyvale, California, to pursue reporter-based imaging techniques. They will develop methods to image "anything about the status of the cell," said Yaghoubi, who serves part-time as chief scientific officer. Celsense is also working on a reporter system.

Reporters have their own drawbacks. "Transforming cells genetically adds a significant layer of complication" to safety studies, reducing the clinical appeal, O'Hanlon said. And HSV1-tk, being a viral gene, could cause an immune response. Researchers are working on developing other reporters, such as the human sodium iodide symporter, that might be safer (Terrovitis et al., 2008: PMID18992656). PET also does not give as detailed resolution as MRI.

"I do not think there is a single best method," Lin said. "It is going to be a combination of methods." Ultimately, doctors might select an MRI-based technique for the better resolution plus a reporter to confirm cell survival.

Will cell labels become standard procedure for all cell therapies? "The real clinical application of SPIO-based cell tracking will be to make sure the cells go to the right site when performing the injection procedure," Bulte said. Longer-term tracking may only be necessary at the clinical trial stage, Arbab said, to prove that cells go to the right place and do the right thing. But it is possible, Yaghoubi said, that different people will respond differently to cell infusions. "You may actually want to see what happens to the cell in each individual, so the imaging might actually become part of the therapy."

References

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Rodriguez-Porcel, M., Wu, J.C., and Gambhir, S.S. Molecular imaging of stem cells (July 30, 2009), StemBook, ed. The Stem Cell Research Community, StemBook, doi/10.3824/stembook.1.49.1, .

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Terrovitis, J., Kwok, K.F., Lautamäki, R., Engles, J.M., Barth, A.S., Kizana, E., Miake, J., Leppo, M.K., Fox, J., Seidel, J., et al. (2008). Ectopic expression of the sodium-iodide symporter enables imaging of transplanted cardiac stem cells in vivo by single-photon emission computed tomography or positron emission tomography. J. Am. Coll. Cardiol., 1652-1660.

Yaghoubi, S.S., Jensen, M.C., Satyamurthy, N., Budhiraja, S., Paik, D., Czernin, J., and Gambhir, S.S. (2009). Noninvasive detection of therapeutic cytolytic T cells with 18F-FHBG PET in a patient with glioma. Nat. Clin. Pract. Oncol., 53-58.

EXHIBIT 11

EXHIBIT 11

IRS.gov

Qualifying Therapeutic Discovery Project Grants for the State of California

The listings below represent the applicants that have been awarded a Qualifying Therapeutic Discovery Program Grant.

The project descriptions below were extracted from the applicant certification forms as submitted.

California $ 278,089,566.84

Applicant Name	Project Name	Grants Awarded for 2009	Grants Awarded for 2010
3-V BIOSCIENCES INC	BROAD SPECTRUM HOST FACTOR DIRECTED ANTIVIRAL THERAPEUTIC	$ 244,479.24	
5iSciences, Inc.	Application of continous negative external pressure (cNEP) to the upper airway	$ 22,834.35	$ 141,124.00
A.P. Pharma, Inc	APF530- Prevention of Chemotherapy-induced Nausea and Vomiting	$ 244,479.25	
Aaken Laboratories	Guided Electrical Stimulation	$ 49,026.40	$ 36,267.00
Aardvark Medical, Inc.	Nasal Irrigation/Aspiration System	$ 244,479.24	
Ablation Numerics Inc	Rapid and Simple Cure for Atrial Fibrillation	$ 500.00	$ 243,979.25
Acadia Pharmaceuticals Inc.	Pimavanserin	$ 244,479.25	
Acaduceus Pharmaceutics Inc	Developing Novel Platinum based Targeted-Nanoparticles for Anti-Cancer Drugs	$ 12,400.00	$ 132,500.00
Acaduceus Pharmeceutics Inc	Developing Targeted Probe for PET imaging of Primary and Metastatic Melanoma	$ 56,150.00	$ 180,000.00
Accelalox Inc.	Acceleration of Bone Fracture Healing	$ 54,062.50	$ 132,430.00
Access Scientific Inc	The Wand	$ 244,479.25	
Accumetrics, Inc	Gravitas: Gauging Responsiveness With a VerifyNow Assay-impact on Thrombosis & Safety	$ 244,479.25	
ACEA Biosciences Inc.	Development of and cancer treatment with Bis(4-fluorobenzyl)trisulfide (BFBTS)	$ 244,479.25	
AcelRx Pharmaceuticals, Inc	Sufentanil NanoTab ™ Patient Controlled Anagesia (PCA) System	$ 244,479.25	
AcelRx Pharmaceuticals, Inc.	Triazolam/Sufentanil NanoTab ™	$ 244,479.24	
Achaogen, Inc	ACHN-490 - A Novel Second-Generation Aminoglycoside Antibiotic for the Treatment of Resistant Bacterial Infections	$ 244,479.25	
Achaogen, Inc	Novel Expanded-Spectrum Aminoglycosides (ES-AGs) for the Treatment of Pan-Resistant Bacterial Infections Caused by Pseudomonas aeruginosa or Acinetobacter baumannii	$ 244,479.25	
Achaogen, Inc	Discovery and Development of a Novel Class of Antibacterials, LpxC Inhibitors for the Treatment of Multi-Drug Resistant Bacterial Infections Caused by Gram-Negative Bacteria	$ 244,479.25	
Achaogen, Inc.	Discovery & Development of a Novel Orally Bioavailable Beta-Lactam for the Treatment of MRSA and Other Multi-Drug resistant Bacterial Infections	$ 244,479.24	
Acologix Inc	AC 100	$ 88,245.46	
Acologix, Inc.	AC820	$ 244,479.25	
Acologix, Inc.	AC 200	$ 170,732.65	
Acorn Biomedical Inc	T-Fusion ™ Platform for oral Delivery of Recombinant Proteins	$ 125,005.10	$ 119,474.15
Acorn Biomedical Inc	Development of Adenosine A3 Receptor Antagoinst for the Treatment of Glaucoma.	$ 172,773.37	$ 71,705.88
Act Biotech , Inc	Telatinib is a new cancer drug that can readily combine with chemotherapy.	$ 244,479.25	

Catalyst Biosciences, Inc.	Improved recombinant human Factor VIIa	$ 244,479.24	
Catalyst Biosciences, Inc.	Anti-complement Alterase TM therapeutic program	$ 244,479.24	
Catalyst Biosciences, Inc.	Improved recombinant human Factor IX	$ 244,479.24	
CEBIX Incorporated	C-Peptide Replacement Therapy	$ 244,479.24	
Cell Biosciences, Inc	Nanoimmunoassay for Biomarker Detection	$ 244,479.24	
Cell Viable Corp	Small Molecule to treat Neurodegenerative Disease such as Alzheimers		$ 17,250.00
Celladon Corporation	MYDICAR (AAV1/SERCA2a) for treatment of NYHA Class III/IV symptoms of heart failure	$ 244,479.24	
CellScape Corporation	CellScape NIPD System	$ 244,479.24	
CellSight Technologies Inc	Imaging cell trafficking to advance clinical translation of cell and gene therapeutics		$ 202,209.00
Celprogen Inc.	Therapeutic agents for the treatment of Breast Cancer triple negative patients	$ 107,500.00	$ 136,979.24
Celula Inc	High-Capacity Single-Cell Molecular Diagnostics for Non-Invasive Prenatal Genetic Testing	$ 244,479.25	
Cenna Biosciences Inc	Peptide Drugs to Treat Alzheimer's Disease	$ 16,248.50	$ 228,230.75
Ceregene, Inc	Ceregene, Inc	$ 244,479.25	
Ceregene, Inc	Neurotrophic Factor Therapy (AAV-NGF) for the Treatment of Alzheimer's Disease (CERE-110)	$ 244,479.25	
Ceregene, Inc.	Delivery of NT-4 for the Treatment of Degenerative Ocular Diseases (CERE-140)	$ 244,479.25	
Cerimon Pharmaceuticals	Diclofenac sodium topical patch for treatment of acute musculoskeletal pain	$ 244,479.24	
Cerus Corporation	INTERCEPT Pathoogen Inactivation for Platelets	$ 244,479.25	
Cerus Corporation	INTERCEPT Pathogen Inactivation for Red Blood Cells	$ 244,479.25	
Charisela Technologies Inc	Charisela Technologies: Advanced Reagent Technologies		$ 213,581.03
CHEM DIV INC	NOVEL SMALL MOLECULE HEDGEHOG PATHWAY INHIBITORS AS ANTICANCER AGENTS	$ 73,690.00	$ 65,481.00
ChemGenex Pharmaceuticals, Inc.	Omacetaxine Mepesuccinate	$ 244,479.24	
ChemoCentryx Inc	Treating Chronic Hepatitis with C0334578	$ 244,479.25	
ChemoCentryx, Inc	Treating Glioblastoma Cancers with CCX662	$ 244,479.24	
ChemoCentryx, Inc	Treating Vasculitis with CCX168	$ 244,479.24	
ChemoCentryx, Inc	Treating Rheumatoid Arthritis with CCX354	$ 244,479.24	
ChemoCentryx, Inc.	Treating Inflammatory Bowel Disease (IBD) with CCX282	$ 244,479.25	
ChemoCentryx, Inc.	Treating Type 2 Diabetes with CCX140	$ 244,479.24	
ChemoCentryx, Inc.	Treating Atopic Dermatitis with C0333158	$ 125,808.00	$ 118,671.25
ChemoCentryx, Inc.	Treating Inflammatory Bowel Disease (IBD) with CCx025	$ 244,479.24	
ChemRegen, Inc.	New Stem Cell Therapies for Heart Disease		$ 244,479.25
CHF Technologies Inc	PliCath HF System	$ 244,479.24	
ChromoLogic LLC	Occular Radiation Dosimeter (OCDOS)		$ 244,479.25
ChromoLogic LLC	Rapid brain injury dosimeter (RAPIDOS)		$ 143,668.50

JS 44 (Rev. 12/07)

CIVIL COVER SHEET

The JS 44 civil cover sheet and the information contained herein neither replace nor supplement the filing and service of pleadings or other papers as required by law, except as provided by local rules of court. This form, approved by the Judicial Conference of the United States in September 1974, is required for the use of the Clerk of Court for the purpose of initiating the civil docket sheet. (SEE INSTRUCTIONS ON THE REVERSE OF THE FORM.)

I. (a) PLAINTIFFS

ANTICANCER, INC.

(b) County of Residence of First Listed Plaintiff San Diego
(EXCEPT IN U.S. PLAINTIFF CASES)

(c) Attorney's (Firm Name, Address, and Telephone Number)

Matt Valenti, AntiCancer, Inc., 7917 Ostrow Street, San Diego CA 92111

DEFENDANTS

CELLSIGHT TECHNOLOGIES, INC.

10 DEC -8 PM 2:32

County of Residence of First Listed Defendant San Francisco
(IN U.S. PLAINTIFF CASES ONLY)

NOTE: IN LAND CONDEMNATION CASES, USE THE LOCATION OF THE LAND INVOLVED.

Attorneys (If Known)

'10 CV 2515 MMA BLM

II. BASIS OF JURISDICTION (Place an "X" in One Box Only)

- ☐ 1 U.S. Government Plaintiff
- ☒ 3 Federal Question (U.S. Government Not a Party)
- ☐ 2 U.S. Government Defendant
- ☐ 4 Diversity (Indicate Citizenship of Parties in Item III)

III. CITIZENSHIP OF PRINCIPAL PARTIES (Place an "X" in One Box for Plaintiff and One Box for Defendant)
(For Diversity Cases Only)

	PTF	DEF		PTF	DEF
Citizen of This State	☐ 1	☐ 1	Incorporated *or* Principal Place of Business In This State	☐ 4	☐ 4
Citizen of Another State	☐ 2	☐ 2	Incorporated *and* Principal Place of Business In Another State	☐ 5	☐ 5
Citizen or Subject of a Foreign Country	☐ 3	☐ 3	Foreign Nation	☐ 6	☐ 6

IV. NATURE OF SUIT (Place an "X" in One Box Only)

CONTRACT
- ☐ 110 Insurance
- ☐ 120 Marine
- ☐ 130 Miller Act
- ☐ 140 Negotiable Instrument
- ☐ 150 Recovery of Overpayment & Enforcement of Judgment
- ☐ 151 Medicare Act
- ☐ 152 Recovery of Defaulted Student Loans (Excl. Veterans)
- ☐ 153 Recovery of Overpayment of Veteran's Benefits
- ☐ 160 Stockholders' Suits
- ☐ 190 Other Contract
- ☐ 195 Contract Product Liability
- ☐ 196 Franchise

REAL PROPERTY
- ☐ 210 Land Condemnation
- ☐ 220 Foreclosure
- ☐ 230 Rent Lease & Ejectment
- ☐ 240 Torts to Land
- ☐ 245 Tort Product Liability
- ☐ 290 All Other Real Property

TORTS

PERSONAL INJURY
- ☐ 310 Airplane
- ☐ 315 Airplane Product Liability
- ☐ 320 Assault, Libel & Slander
- ☐ 330 Federal Employers' Liability
- ☐ 340 Marine
- ☐ 345 Marine Product Liability
- ☐ 350 Motor Vehicle
- ☐ 355 Motor Vehicle Product Liability
- ☐ 360 Other Personal Injury

PERSONAL INJURY
- ☐ 362 Personal Injury - Med. Malpractice
- ☐ 365 Personal Injury - Product Liability
- ☐ 368 Asbestos Personal Injury Product Liability

PERSONAL PROPERTY
- ☐ 370 Other Fraud
- ☐ 371 Truth in Lending
- ☐ 380 Other Personal Property Damage
- ☐ 385 Property Damage Product Liability

CIVIL RIGHTS
- ☐ 441 Voting
- ☐ 442 Employment
- ☐ 443 Housing/ Accommodations
- ☐ 444 Welfare
- ☐ 445 Amer. w/Disabilities - Employment
- ☐ 446 Amer. w/Disabilities - Other
- ☐ 440 Other Civil Rights

PRISONER PETITIONS
- ☐ 510 Motions to Vacate Sentence

Habeas Corpus:
- ☐ 530 General
- ☐ 535 Death Penalty
- ☐ 540 Mandamus & Other
- ☐ 550 Civil Rights
- ☐ 555 Prison Condition

FORFEITURE/PENALTY
- ☐ 610 Agriculture
- ☐ 620 Other Food & Drug
- ☐ 625 Drug Related Seizure of Property 21 USC 881
- ☐ 630 Liquor Laws
- ☐ 640 R.R. & Truck
- ☐ 650 Airline Regs.
- ☐ 660 Occupational Safety/Health
- ☐ 690 Other

LABOR
- ☐ 710 Fair Labor Standards Act
- ☐ 720 Labor/Mgmt. Relations
- ☐ 730 Labor/Mgmt.Reporting & Disclosure Act
- ☐ 740 Railway Labor Act
- ☐ 790 Other Labor Litigation
- ☐ 791 Empl. Ret. Inc. Security Act

IMMIGRATION
- ☐ 462 Naturalization Application
- ☐ 463 Habeas Corpus - Alien Detainee
- ☐ 465 Other Immigration Actions

BANKRUPTCY
- ☐ 422 Appeal 28 USC 158
- ☐ 423 Withdrawal 28 USC 157

PROPERTY RIGHTS
- ☐ 820 Copyrights
- ☒ 830 Patent
- ☐ 840 Trademark

SOCIAL SECURITY
- ☐ 861 HIA (1395ff)
- ☐ 862 Black Lung (923)
- ☐ 863 DIWC/DIWW (405(g))
- ☐ 864 SSID Title XVI
- ☐ 865 RSI (405(g))

FEDERAL TAX SUITS
- ☐ 870 Taxes (U.S. Plaintiff or Defendant)
- ☐ 871 IRS—Third Party 26 USC 7609

OTHER STATUTES
- ☐ 400 State Reapportionment
- ☐ 410 Antitrust
- ☐ 430 Banks and Banking
- ☐ 450 Commerce
- ☐ 460 Deportation
- ☐ 470 Racketeer Influenced and Corrupt Organizations
- ☐ 480 Consumer Credit
- ☐ 490 Cable/Sat TV
- ☐ 810 Selective Service
- ☐ 850 Securities/Commodities/ Exchange
- ☐ 875 Customer Challenge 12 USC 3410
- ☐ 890 Other Statutory Actions
- ☐ 891 Agricultural Acts
- ☐ 892 Economic Stabilization Act
- ☐ 893 Environmental Matters
- ☐ 894 Energy Allocation Act
- ☐ 895 Freedom of Information Act
- ☐ 900Appeal of Fee Determination Under Equal Access to Justice
- ☐ 950 Constitutionality of State Statutes

V. ORIGIN (Place an "X" in One Box Only)

☒ 1 Original Proceeding ☐ 2 Removed from State Court ☐ 3 Remanded from Appellate Court ☐ 4 Reinstated or Reopened ☐ 5 Transferred from another district (specify) ☐ 6 Multidistrict Litigation ☐ 7 Appeal to District Judge from Magistrate Judgment

VI. CAUSE OF ACTION

Cite the U.S. Civil Statute under which you are filing (Do not cite jurisdictional statutes unless diversity): Titles 35, 17, and 15 of the United States Code and 28 U.S.C. §§ 1331 and 1338(a) 15:1126 YES

Brief description of cause: patent infringement, copyright infringement, trademark infringement, and unfair competition

VII. REQUESTED IN COMPLAINT:

☐ CHECK IF THIS IS A CLASS ACTION UNDER F.R.C.P. 23

DEMAND $ According to proof

CHECK YES only if demanded in complaint:
JURY DEMAND: ☒ Yes ☐ No

VIII. RELATED CASE(S) IF ANY (See instructions): JUDGE _____ DOCKET NUMBER _____

DATE 12/08/2010

SIGNATURE OF ATTORNEY OF RECORD

FOR OFFICE USE ONLY

RECEIPT # 20986 AMOUNT $350.00 APPLYING IFP (S) JUDGE _____ MAG. JUDGE _____

12-8-10

Court Name: USDC California Southern
Division: 3
Receipt Number: CAS020986
Cashier ID: nsiefken
Transaction Date: 12/08/2010
Payer Name: ANTICANSERINC

CIVIL FILING FEE
For: ANTICANCER INC V CELLSIGHT TEC
Case/Party: D-CAS-3-10-CV-002515-001
Amount: $350.00

CHECK
Check/Money Order Num: 20121
Amt Tendered: $350.00

Total Due: $350.00
Total Tendered: $350.00
Change Amt: $0.00

There will be a fee of $45.00
charged for any returned check.