



(51) International Patent Classification:
G01N 21/64 (2006.01)

(21) International Application Number:
PCT/US2020/015436

(22) International Filing Date:
28 January 2020 (28.01.2020)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/797,579 28 January 2019 (28.01.2019) US

(71) Applicant: **THE BOARD OF TRUSTEES OF THE LE-
LAND STANFORD JUNIOR UNIVERSITY** [US/US];
P.O. Box 20386, Office of the General Counsel, Build-
ing 170, 3rd Floor, Main Quad, Stanford, California
94305-2038 (US).

(72) Inventors: **ROSENTHAL, Eben Lloyd**; 975 Blake Wilbur
Drive, 2nd Floor, Palo Alto, California 94304 (US). **VAN
KEULEN, Stan**; 975 Blake Wilbur Drive, 2nd Floor, Palo
Alto, California 94304 (US).

(74) Agent: **BUCHBINDER, Jenny L.**; Bozicevic, Field &
Francis LLP, 201 Redwood Shores Pkwy., Suite 200, Red-
wood City, California 94065 (US).

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP,
KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,
SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR,
TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(54) Title: RAPID IDENTIFICATION OF CLOSE SURGICAL MARGINS ON SURGICAL SPECIMENS

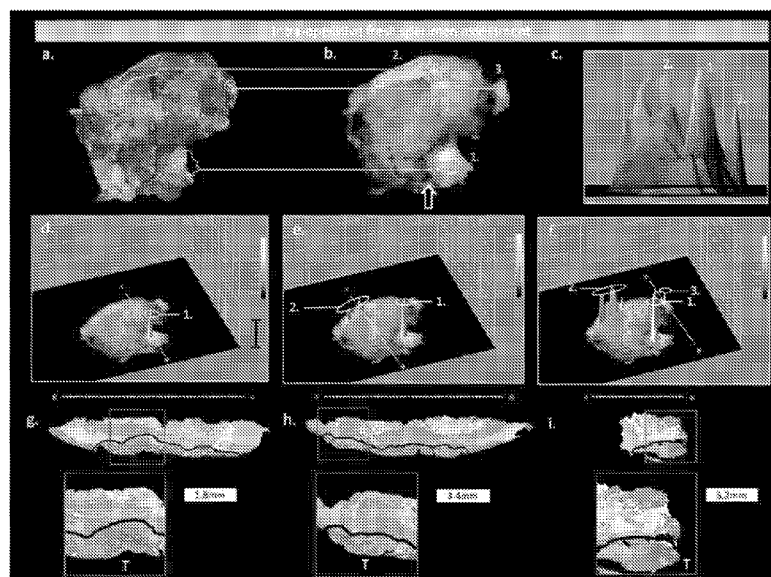


FIG. 1

(57) Abstract: Methods, kits, and systems for identifying suspicious areas in surgical specimens that potentially contain cancerous cells are provided. The methods utilize fluorescence imaging of a surgical specimen with a fluorescent contrast agent that selectively localizes to cancerous cells to identify fluorescence peak-intensity regions corresponding to locations in the specimen where the distance between the tumor and the margin border is the closest. The methods disclosed herein are useful in identifying sections of a tumor specimen that should be selected for further histopathological examination. Additionally, these methods aid in providing intraoperative guidance to allow a surgeon to take corrective action during surgical procedures.



(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

RAPID IDENTIFICATION OF CLOSE SURGICAL MARGINS ON SURGICAL SPECIMENS

FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0001] This invention was made with Government support under contract CA190306 awarded by the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND

[0002] Despite the central role of surgery in the management of cancer, the positive margin rates have not substantially changed over the past 30 years. Failure to develop new technologies to identify cancer in real-time during surgery has impeded surgical management of tumors despite significant advances in medical technology. Dependence on surgical experience, visual cues and manual palpation has resulted in a 15%-30 rate of close (< 5 mm) or positive (< 1 mm) surgical margins upon final pathological assessment (Woolgar et al. (2005) Oral Oncol. 41(10):1034-1043, McMahon et al. (2003) Br. J. Oral Maxillofac. Surg. 41(4):224-231, Ravasz et al. (1991) J. Cranio-Maxillo-fac Surg. Off. Publ. Eur. Assoc. Cranio-Maxillo-fac Surg. 19(7):314-318). Failure to obtain clear surgical margins is associated with locoregional recurrence and poor overall survival rates (Eldeeb et al. (2012) Cancer Biol Med. 9(1):29-33, Pawlik et al. (2005) Ann. Surg. 241(5):715-722, discussion 722-724, Hinni et al. (2013) Head Neck 35(9):1362-1370) and therefore requires additional therapy such as chemotherapy and/or radiotherapy or possibly second surgeries (Cooper et al. (2004) N. Engl. J. Med. 350(19):1937-1944, Bernier et al. (2004) N. Engl. J. Med. 350(19):1945-1952). Margins from the specimen are most difficult to assess at the deep margin because the tumor cannot be visualized. The epithelial margins of a resected specimen are easiest to assess because the surgeon can measure the distance throughout the removal. However, the surgeon remains blind to the deep surgical margin - the infiltrative tumor border and absence of landmarks within deeper tissue layers is exceptionally challenging to predict (Roh et al. (2012) Head Neck 34(11):1529-1536) and as a result deep margins are more at risk of being positive (Tirelli et al. (2019) Laryngoscope 129(8):1810-1815, Huang et al. (2007) Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 13(20):6099-106).

[0003] Although frozen section analysis (FSA) can successfully detect cancer - it can only sample a very small percent of the wound bed and surgeons struggle to identify which suspicious areas are appropriate for histopathological assessment (Tirelli et al., *supra*). Considering that FSA is time-consuming (15-20 minutes per section) and results in significant sampling errors (Black et al. (2006) Cancer 107(12):2792-2800), FSA is only as helpful as the ability to select suspicious areas for further assessment.

[0004] The need remains for methods that identify suspicious areas in surgical specimens (i.e., within 5 mm of a tumor) and reduce sampling error in assessment of specimens.

SUMMARY

[0005] Methods, kits, and systems for identifying suspicious areas in surgical specimens that potentially contain cancerous cells are provided. The methods utilize fluorescence imaging of a surgical specimen with a fluorescent contrast agent that selectively localizes to cancerous cells to identify fluorescence peak-intensity regions corresponding to locations in the specimen where the distance between the tumor and the margin border is the closest. The methods disclosed herein are useful in identifying areas of a tumor specimen that should be selected for further examination and provide intraoperative guidance to allow a surgeon to take corrective action during procedures.

[0006] In one aspect, a method of detecting a close or positive margin in a surgical tumor specimen is provided, the method comprising: a) administering a fluorescent contrast agent to a subject, wherein the fluorescent contrast agent selectively localizes to cancerous cells of a tumor in the subject; b) performing a resection of the tumor to produce the surgical tumor specimen; c) illuminating the surgical tumor specimen with light at a fluorescence excitation wavelength of the fluorescent contrast agent; d) performing fluorescence imaging on a tumor margin of the surgical tumor specimen by detecting fluorescent signals emitted from the fluorescent contrast agent localized to the cancerous cells in the tumor specimen; e) plotting a two-dimensional map or a three-dimensional map of the fluorescent signals; f) quantifying the fluorescent signals; g) identifying at least one fluorescence peak-intensity area in the two-dimensional map or the three-dimensional map, wherein the fluorescence peak-intensity area corresponds to a specimen location where the surgical tumor specimen has a close margin; and h) detecting cancerous cells if present at the specimen location where the surgical tumor specimen has a close margin to determine if the close margin is a positive margin. In some embodiments, the method further comprises collecting a tissue section of the surgical tumor specimen comprising tissue from the specimen location where the surgical tumor specimen has a close margin; and detecting cancerous cells if present in the tissue section to determine if the tissue section comprises a positive margin. In other embodiments, tissue at the specimen location where the surgical tumor specimen has a close margin is analyzed for cancerous cells without first sectioning the tumor specimen. In certain embodiments, cancerous cells are detected by histopathological examination or fluorescent examination of the tissue.

[0007] In certain embodiments, this method is performed intraoperatively or postoperatively to provide guidance to a surgeon for removal of additional cancerous tissue from the subject. In certain embodiments, the method further comprises surgically removing additional

cancerous tissue from the subject if cancerous cells are detected in the tissue section indicating that the tissue section comprises a positive margin, wherein the location of the positive margin in the tumor specimen is used to identify the location of the cancerous tissue in the subject in need of surgical removal.

[0008] In certain embodiments, the fluorescence peak-intensity area has the highest fluorescence intensity, second highest fluorescence intensity, third highest fluorescence intensity, or fourth highest fluorescence intensity of all detected fluorescence peak-intensity areas in the two-dimensional map or three-dimensional map.

[0009] In certain embodiments, the imaging is performed on a tumor margin of less than or equal to about 7 mm in length, such as a tumor margin of about 7, 6, 5, 4, 3, 2, or 1 mm, or any length in between. In some embodiments, the tumor margin is a close margin of less than or equal to about 5 mm in length, such as a tumor margin of about 5, 4, 3, 2, or 1 mm, or any length in between. In some embodiments, the tumor margin is a positive margin of less than or equal to about 2 mm in length, such as a tumor margin of about 2, 1.5, 1, 0.5, or 0 mm, or any length in between.

[0010] In certain embodiments, the method further comprises measuring a surgical margin thickness at the specimen location corresponding to the fluorescence peak-intensity area.

[0011] In certain embodiments, the method further comprises identifying the closest surgical margin corresponding to a fluorescence peak-intensity area in the two-dimensional map or three-dimensional map (e.g., surgical margin less than 5 mm from the tumor).

[0012] In certain embodiments, the fluorescent signals are quantified with or without scaling relative to fluorescent signals emitted from specimen regions known to have tumor or normal tissue (e.g., see Examples).

[0013] In certain embodiments, the fluorescent contrast agent comprises a fluorophore conjugated to a binding agent that specifically binds to a tumor marker or antigen in or on the cancerous cells of interest. Exemplary binding agents include antibodies, antibody fragments, antibody mimetics, ligands, and aptamers that specifically bind to the tumor marker or antigen.

In certain embodiments, the fluorescent contrast agent comprises a fluorescent dye (e.g., 5-aminolevulinic acid (5-ALA) and indocyanine green (ICG) dyes and derivatives) that selectively localizes to cancerous cells.

[0014] In certain embodiments, the fluorescent contrast agent comprises a fluorophore conjugated to a delocalized lipophilic cation (e.g., triphenylphosphonium derivatives and rhodamine derivatives) that selectively localizes to cancerous cells.

[0015] In certain embodiments, the fluorescent contrast agent comprises a fluorophore that emits visible, near-infrared, or near-infrared II fluorescent light. In one embodiment, the fluorescent contrast agent is a panitumumab-IRDye800 conjugate.

[0016] Fluorescence imaging may be performed using, for example, an open- or closed-field fluorescence imaging system. In some embodiments, fluorescence imaging is performed with a medical fluorescence imaging device. In some embodiments, a fluorescence image of the fluorescent signals from the contrast agent is recorded with a digital microscope, a charged coupled device (CCD) image sensor, a complementary metal-oxide-semiconductor (CMOS) image sensor, a digital camera, a fiber-optic imaging system, or a medical fluorescence imaging system.

[0017] In certain embodiments, the method further comprises superimposing a photographic image of the surgical tumor specimen and the fluorescence image of the surgical tumor specimen to determine the specimen location corresponding to the fluorescence peak-intensity area.

In another aspect, a method of detecting a close or positive margin in a surgical specimen is provided, the method comprising: a) obtaining the surgical specimen comprising at least a portion of a tumor; b) contacting the surgical specimen with a fluorescent contrast agent, wherein the fluorescent contrast agent selectively localizes to cancerous cells in the tumor specimen; c) illuminating the surgical tumor specimen with light at a fluorescence excitation wavelength of the fluorescent contrast agent; d) performing fluorescence imaging on a tumor margin of the surgical tumor specimen by detecting fluorescent signals emitted from the fluorescent contrast agent localized to the cancerous cells of the tumor specimen; e) plotting a two-dimensional map or a three-dimensional map of the fluorescent signals; f) quantifying the fluorescent signals; g)) identifying at least one fluorescence peak-intensity area in the two-dimensional map or the three-dimensional map, wherein the fluorescence peak-intensity area corresponds to a specimen location where the surgical specimen has a close margin; and h) detecting cancerous cells if present at the specimen location where the surgical tumor specimen has a close margin to determine if the close margin is a positive margin. In some embodiments, the method further comprises collecting a tissue section of the surgical tumor specimen comprising tissue from the specimen location where the surgical tumor specimen has a close margin; and detecting cancerous cells if present in the tissue section to determine if the tissue section comprises a positive margin. In other embodiments, tissue at the specimen location where the surgical tumor specimen has a close margin is analyzed for cancerous cells without first sectioning the tumor specimen. In certain embodiments, cancerous cells are detected by histopathological examination or fluorescent examination of the tissue.

[0018] In certain embodiments, this method is performed intraoperatively or postoperatively to provide guidance to a surgeon for removal of additional cancerous tissue from the subject.

[0019] In certain embodiments, the method further comprises surgically removing additional cancerous tissue from the subject if cancerous cells are detected in the tissue section indicating that the tissue section comprises a positive margin, wherein the location of the positive margin in the tumor specimen is used to identify the location of the cancerous tissue in the subject in need of surgical removal.

[0020] In another aspect, a computer implemented method for identifying a close or positive surgical margin in a tumor specimen is provided, the computer performing steps comprising: a) receiving inputted fluorescence imaging data of the tumor specimen, wherein the fluorescence imaging data comprises fluorescent signals from a fluorescent contrast agent localized to cancerous cells in the tumor specimen; b) plotting a two-dimensional map or a three-dimensional map of the fluorescent signals; c) quantifying the fluorescent signals; d) identifying a fluorescence peak-intensity area in the two-dimensional map or the three-dimensional map, wherein the fluorescence peak-intensity area corresponds to a specimen location where the surgical tumor specimen has a close margin; e) mapping at least one fluorescence peak-intensity area to a specimen location; f) marking a photographic image of the tumor specimen to identify the specimen location corresponding to the fluorescence peak-intensity area; and g) displaying the marked photographic image of the tumor specimen, wherein the specimen location corresponding to the fluorescence peak-intensity area is identified. For example, the specimen location corresponding to the fluorescence peak-intensity area can be marked to identify it in the photographic image of the tumor specimen by labeling, outlining, coloring, or highlighting it. In certain embodiments, the computer implemented method further comprises sending the marked photographic image to a surgeon or pathologist. In certain embodiments, the computer implemented method further comprises storing the marked photographic image of the tumor specimen in a database.

[0021] In another aspect, a non-transitory computer-readable medium is provided comprising program instructions that, when executed by a processor in a computer, causes the processor to perform a computer implemented method for identifying a close or positive surgical margin in a tumor specimen, as described herein.

[0022] In another aspect, a kit is provided comprising the non-transitory computer-readable medium described herein. The kit may further comprise instructions for identifying a close or positive surgical margin in a tumor specimen according to the methods described herein. In certain embodiments, the kit further comprises a fluorescent contrast agent. For example, the fluorescent contrast agent in the kit may be a fluorescently labeled antibody that specifically binds to a tumor antigen. In some embodiments, the fluorescent contrast agent emits visible, near-infrared, or near-infrared II fluorescence. In one embodiment, the kit comprises a panitumumab-IRDye800 conjugate.

[0023] In another aspect, a system for identifying a close or positive surgical margin in a tumor specimen using a computer implemented method, described herein, is provided, the system comprising: a) a storage component for storing data, wherein the storage component has instructions for identifying a close or positive surgical margin in a tumor specimen based on analysis of fluorescence imaging data of the tumor specimen stored therein; b) a computer processor for processing the fluorescence imaging data, wherein the computer processor is coupled to the storage component and configured to execute the instructions stored in the storage component in order to receive the inputted fluorescence imaging data and analyze the data according to the computer implemented method described herein; and c) a display component for displaying a marked photographic image of the tumor specimen, wherein the specimen location corresponding to the fluorescence peak-intensity area is identified.

[0024] In certain embodiments, the system further comprises a light source capable of illuminating a surgical specimen at a fluorescence excitation wavelength of the fluorescent contrast agent that is localized to cancerous cells of the surgical specimen.

[0025] In certain embodiments, the system further comprises a photodetector capable of detecting fluorescent light emitted by the fluorescent contrast agent. The photodetector may include without limitation a charged coupled device (CCD) image sensor, a complementary metal-oxide-semiconductor (CMOS) image sensor, a digital camera, a fiber-optic imaging system, or a medical fluorescence imaging system.

[0026] In another aspect, a kit is provided comprising a system for identifying a close or positive surgical margin in a tumor specimen, as described herein. The kit may further comprise instructions for identifying a close or positive surgical margin in a tumor specimen according to the methods described herein. In certain embodiments, the kit further comprises a fluorescent contrast agent. For example, the fluorescent contrast agent in the kit may be a fluorescently labeled antibody that specifically binds to a tumor antigen. In some embodiments, the fluorescent contrast agent emits visible, near-infrared, or near-infrared II fluorescence. In one embodiment, the kit comprises a panitumumab-IRDye800 conjugate.

BRIEF DESCRIPTION OF THE DRAWINGS

[0027] The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not to-scale. On the contrary, the dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures.

[0028] **FIG. 1** shows an overview of the workflow. Representative brightfield (a) and fluorescence image (b) of a specimen's deep margin. Numbers 1-3 allocate the fluorescence

peaks in order of first appearance throughout the whole figure. (c) Side view on the entire fluorescence surface map of deep margin (white arrow image (b) indicates angle). (d-f) Identification of highest fluorescence intensity-peaks on the deep surface (with color bar). The white dotted lines and asterisks (red/blue) indicate the orientation in which the hematoxylin & eosin (H&E) slides were cut. (g-i) H&E slides with delineated tumor (black line) on which the tumor depth was measured (white box). T=tumor.

[0029] **FIG. 2A** shows a graph of mean fluorescence intensity, showing the relative increase in MFI of all areas of no significance (ANS) to peak intensity areas (PIAs) per patient. **FIG. 2B** shows a graph illustrating the difference in tumor depth for ANS and PIAs per patient.

[0030] **FIG. 3A** shows the peak-intensity versus tumor depth. Representative brightfield (top) and fluorescence (middle) images of the deep margin are shown with identification of highest fluorescence intensity-peaks on the deep surface (bottom). Numbers 1-3 allocate the fluorescence peaks in order of first appearance. **FIG. 3B** shows a graph of the tumor depth at the 1st, 2nd and 3rd peak-intensity area per patient.

[0031] **FIG. 4** shows a schematic of the workflow. PIA: peak-intensity area; SCC: squamous cell carcinoma; H&E: hematoxylin & eosin.

[0032] **FIG. 5** shows annotation of a suspicious area by suture. Bright field (a) and corresponding fluorescence (b) images of the specimen's deep margin after maxillectomy are shown. The white solid line indicates peak intensity area in the bright field (a) and fluorescence (b) images. A white dotted line delineates the infiltrated left concha inferior (nasal cavity). A hematoxylin & eosin slide of the suture marked area (c, black solid line delineates tumor from healthy tissue) and an isolated fluorescence peak-intensity area where the suture was placed (d) are also shown.

DETAILED DESCRIPTION OF EMBODIMENTS

[0033] Methods, kits, and systems for identifying suspicious areas in surgical specimens that potentially contain cancerous cells are provided. The methods utilize fluorescence imaging of a surgical specimen with a fluorescent contrast agent that selectively localizes to cancerous cells to identify fluorescence peak-intensity regions corresponding to locations in the specimen where the distance between the tumor and the margin border is the closest. The methods disclosed herein are useful in identifying suspicious sections of a tumor specimen, potentially containing cancerous cells, which should be selected for further examination. Additionally, these methods aid in providing intraoperative guidance to allow a surgeon to take corrective action during surgical procedures.

[0034] Before the present treatment methods are described, it is to be understood that this invention is not limited to a particular method or composition described, as such may, of

course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0035] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0036] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, some potential and preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. It is understood that the present disclosure supersedes any disclosure of an incorporated publication to the extent there is a contradiction.

[0037] As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present invention. Any recited method can be carried out in the order of events recited or in any other order which is logically possible.

[0038] It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a contrast agent" includes a plurality of such contrast agents and reference to "the contrast agent" includes reference to one or more contrast agents and equivalents thereof, such as fluorescently labeled tumor binding agents (e.g., fluorescently labeled antibodies specific for tumor antigens), known to those skilled in the art, and so forth.

[0039] The term "fluorescence characteristics" means an ability to emit fluorescence by irradiation of excitation light. Examples of parameters of fluorescence characteristics include fluorescence intensity, excitation wavelength, fluorescence wavelength, and pH sensitivity.

Fluorescent emissions may include fluorescent light, for example, at wavelengths in the visible, near-infrared, or near-infrared II regions of the light spectrum.

[0040] The term "margin" or "tumor margin" refers to tissue surrounding a tumor in a surgical specimen, including tissue between the specimen edge and the tumor. By "close margin" is meant, when referring to a surgical specimen, that the distance between the specimen edge and tumorous tissue is less than or equal to 5 mm. By "positive margin" is meant, when referring to a surgical specimen, that the tissue between the specimen edge and the tumor comprises cancerous cells.

[0041] By "isolated" is meant, when referring to a polypeptide, that the indicated molecule is separate and discrete from the whole organism with which the molecule is found in nature or is present in the substantial absence of other biological macro molecules of the same type. The term "isolated" with respect to a polynucleotide is a nucleic acid molecule devoid, in whole or part, of sequences normally associated with it in nature; or a sequence, as it exists in nature, but having heterologous sequences in association therewith; or a molecule disassociated from the chromosome.

[0042] An "antibody" or "antibody molecule" is any immunoglobulin, including antibodies and fragments thereof, that binds to a specific antigen. The term includes polyclonal, monoclonal, chimeric, single domain (Dab) and bispecific antibodies. As used herein, antibody or antibody molecule contemplates recombinantly generated intact immunoglobulin molecules and immunologically active portions of an immunoglobulin molecule such as, without limitation: Fab, Fab', F(ab')₂, F(v), scFv, scFv₂, scFv-Fc, minibody, diabody, tetrabody, single variable domain (e.g., variable heavy domain, variable light domain), bispecific, Affibody molecules (Affibody, Bromma, Sweden), and peptabodies (Terskikh et al. (1997) PNAS 94:1663-1668). Dabs can be composed of a single variable light or heavy chain domain. In a certain embodiment, the variable light domain and/or variable heavy domain specific for MISIR are inserted into the backbone of the above-mentioned antibody constructs. Methods for recombinantly producing antibodies are well known in the art. For example, commercial vectors comprising constant genes to make IgGs from scFvs are provided by Lonza Biologics (Slough, United Kingdom). The phrase "Affibody molecule" may also refer to "Affibody.RTM. molecule." Affibody is a trademark owned by Affibody AB. Affibody is a trademark registered in Sweden, Europe, and the United States and under trademark application in Japan.

[0043] "Fv" is an antibody fragment which contains an antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable

domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although often at a lower affinity than the entire binding site.

[0044] "Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see, for example, Pluckthun, A. in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

[0045] The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) on the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Holliger et al., (1993) *Proc. Natl. Acad. Sci. USA*, 90: 6444-6448.

[0046] With respect to antibodies, the terms "specifically binds" and "selectively binds" are used interchangeably and refer to binding agents (e.g., antibodies, aptamers) that bind to one or more epitopes of a protein (e.g., tumor antigen) or compound of interest, but which do not substantially recognize and bind other molecules in a sample containing a mixed population of antigenic biological molecules. Typically in assays, specific or selective binding will be at least twice background signal or noise and more typically more than 10 to 100 times background.

[0047] The term "affibody molecule" refers to a molecule that consists of three alpha helices with 58 amino acids and has a molar mass of about 6 kDa. A monoclonal antibody, for comparison, is 150 kDa, and a single-domain antibody, the smallest type of antigen-binding antibody fragment, 12-15 kDa. See, for exemplary details of affibody structures and uses, Orlova, A; Magnusson, M; Eriksson, T L; Nilsson, M; Larsson, B; Hoiden-Guthenberg, I; Widstrom, C; Carlsson, J et al. (2006). "Tumor imaging using a picomolar affinity HER2 binding affibody molecule", *Cancer Res.* 66 (8): 4339-48. Exemplary Affibody. Molecules are commercially available from Abcam Corp. Cambridge Mass.

[0048] The term "conjugated" refers to the joining by covalent or noncovalent means of two compounds or agents (e.g., binding agent specific for a tumor antigen conjugated to a fluorophore).

[0049] The terms "treatment", "treating", "treat" and the like are used herein to generally refer to obtaining a desired pharmacologic and/or physiologic effect. The effect can be prophylactic in terms of completely or partially preventing a disease or symptom(s) thereof and/or may be

therapeutic in terms of a partial or complete stabilization or cure for a disease and/or adverse effect attributable to the disease. The term "treatment" encompasses any treatment of a disease in a mammal, particularly a human, and includes: (a) preventing the disease and/or symptom(s) from occurring in a subject who may be predisposed to the disease or symptom but has not yet been diagnosed as having it; (b) inhibiting the disease and/or symptom(s), i.e., arresting their development; or (c) relieving the disease symptom(s), i.e., causing regression of the disease and/or symptom(s). Those in need of treatment include those already inflicted (e.g., those with cancer) as well as those in which prevention is desired (e.g., those with increased susceptibility to cancer, those suspected of having cancer, those with a risk of recurrence, etc.).

[0050] The terms "tumor," "cancer" and "neoplasia" are used interchangeably and refer to a cell or population of cells whose growth, proliferation or survival is greater than growth, proliferation or survival of a normal counterpart cell, e.g., a cell proliferative, hyperproliferative or differentiative disorder. Typically, the growth is uncontrolled. The term "malignancy" refers to invasion of nearby tissue. The term "metastasis" or a secondary, recurring or recurrent tumor, cancer or neoplasia refers to spread or dissemination of a tumor, cancer or neoplasia to other sites, locations or regions within the subject, in which the sites, locations or regions are distinct from the primary tumor or cancer. Neoplasia, tumors and cancers include benign, malignant, metastatic and non-metastatic types, and include any stage (I, II, III, IV or V) or grade (G1, G2, G3, etc.) of neoplasia, tumor, or cancer, or a neoplasia, tumor, cancer or metastasis that is progressing, worsening, stabilized or in remission. In particular, the terms "tumor," "cancer" and "neoplasia" include carcinomas, such as squamous cell carcinoma, adenocarcinoma, adenosquamous carcinoma, anaplastic carcinoma, large cell carcinoma, and small cell carcinoma, and include cancers such as, but are not limited to, head and neck cancer, skin cancer, breast cancer, ovarian cancer, melanoma, pancreatic cancer, peripheral neuroma, glioblastoma, adrenocortical carcinoma, AIDS-related lymphoma, anal cancer, bladder cancer, meningioma, glioma, astrocytoma, cervical cancer, chronic myeloproliferative disorders, colon cancer, endometrial cancer, ependymoma, esophageal cancer, Ewing's sarcoma, extracranial germ cell tumors, extrahepatic bile duct cancer, gallbladder cancer, gastric cancer, gastrointestinal carcinoid tumors, gestational trophoblastic tumors, hairy cell leukemia, Hodgkin lymphoma, non-Hodgkin lymphoma, hypopharyngeal cancer, islet cell carcinoma, Kaposi sarcoma, laryngeal cancer, leukemia, lip cancer, oral cavity cancer, liver cancer, male breast cancer, malignant mesothelioma, medulloblastoma, Merkel cell carcinoma, metastatic squamous neck cell carcinoma, multiple myeloma and other plasma cell neoplasms, mycosis fungoides and the Sezary syndrome, myelodysplastic syndromes, nasopharyngeal cancer, neuroblastoma, non-small cell lung cancer, small cell lung cancer,

oropharyngeal cancer, bone cancers, including osteosarcoma and malignant fibrous histiocytoma of bone, paranasal sinus cancer, parathyroid cancer, penile cancer, pheochromocytoma, pituitary tumors, prostate cancer, rectal cancer, renal cell cancer, retinoblastoma, rhabdomyosarcoma, salivary gland cancer, small intestine cancer, soft tissue sarcoma, supratentorial primitive neuroectodermal tumors, pineoblastoma, testicular cancer, thymoma, thymic carcinoma, thyroid cancer, transitional cell cancer of the renal pelvis and ureter, urethral cancer, uterine sarcoma, vaginal cancer, vulvar cancer, and Wilm's tumor and other childhood kidney tumors.

[0051] "Substantially purified" generally refers to isolation of a substance (compound, drug, polynucleotide, protein, polypeptide) such that the substance comprises the majority percent of the sample in which it resides. Typically in a sample, a substantially purified component comprises 50%, preferably 80%-85%, more preferably 90-95% of the sample. Techniques for purifying substances of interest are well-known in the art and include, for example, ion-exchange chromatography, affinity chromatography and sedimentation according to density.

[0052] The terms "recipient", "individual", "subject", "host", and "patient", are used interchangeably herein and refer to any mammalian subject for whom diagnosis, treatment, or therapy is desired, particularly humans. "Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, sheep, goats, pigs, etc. Preferably, the mammal is human.

[0053] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

Methods

[0054] Methods for identifying suspicious areas in surgical specimens that potentially contain cancerous cells are provided. The methods utilize fluorescence imaging of a surgical specimen with a fluorescent contrast agent that selectively localizes to cancerous cells to identify fluorescence peak-intensity regions corresponding to locations in the specimen where the distance between the tumor and the margin border is the closest. In particular, the methods disclosed herein are useful in identifying suspicious sections of a tumor specimen containing close or positive tumor margins that should be selected for further examination.

Fluorescent Contrast Agents

[0055] As explained above, the methods of the present invention utilize fluorescence imaging of tumor specimens with fluorescent contrast agents that selectively localize to cancerous cells. The fluorescent contrast agent may comprise a fluorophore having fluorescent light emissions at wavelengths, for example, in the visible, near-infrared, or near-infrared II regions of the light spectrum.

[0056] In certain embodiments, the fluorophore has a fluorescence emission in the visible region of the light spectrum, which ranges from about 400 nm to about 700 nm. Exemplary fluorophores that emit fluorescent light in the visible region of the light spectrum include, but are not limited to, CAL Fluor dyes such as CAL Fluor Gold 540, CAL Fluor Orange 560, CAL Fluor Red 590, CAL Fluor Red 610, and CAL Fluor Red 635; Quasar dyes such as Quasar 570, Quasar 670, and Quasar 705; Alexa Fluor dyes such as Alexa Fluor 350, Alexa Fluor 488, Alexa Fluor 546, Alexa Fluor 555, Alexa Fluor 594, Alexa 633, and Alexa Fluor 647; cyanine dyes such as Cy 3, Cy3.5, Cy5, Cy5.5, and Cy7, fluorescein and derivatives such as 2', 4', 5', 7'-tetrachloro-4-7-dichlorofluorescein (TET), carboxyfluorescein (FAM), 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE), and hexachlorofluorescein (HEX); rhodamine and derivatives such as carboxy-X-rhodamine (ROX), tetramethylrhodamine (TMR), tetramethylrhodamine-isothiocyanate (TRITC), carboxytetramethylrhodamine (TAMRA), sulforhodamine 101, and Texas red; DyLight dyes such as DyLight 350, DyLight 405, DyLight 488, DyLight 550 DyLight 594, and DyLight 633; and HiLyte dyes such as HiLyte Fluor 405, HiLyte Fluor 488, HiLyte Fluor 532, HiLyte Fluor 555, and HiLyte 594.

[0057] In certain embodiments, the fluorophore has a fluorescence emission in the near-infrared (NIR) region of the light spectrum, which ranges from about 700 nm to about 1700 nm. The use of NIR fluorophores is advantageous in minimizing interference from tissue autofluorescence and enhancing tissue penetration compared to other fluorophores. Preferably, the fluorescent emission wavelength is in a region of the light spectrum where blood and tissue absorb minimally, and tissue penetration is maximal, such as in the range from 700 nm to 1000 nm. Any NIR fluorophore with an emission in the NIR region of the light spectrum may be used, including, but not limited to, fluorophores with fluorescence emissions at about 700, 720, 740, 760, 780, 800, 820, 840, 860, 880, 900, 920, 940, 960, 980, or 1000 nm, or any wavelength in between. Exemplary NIR fluorophores include IRDye dyes (e.g., IRDye 800CW, IRDye 680RD, IRDye 750, and IRDye 800RS), CF dyes (e.g., CF680, CF680R, CF750, CF770, and CF790), Tracy dyes (e.g., Tracy 645 and Tracy 652), cyanine dyes (e.g., Cy7 and Cy7.5), thienothiadiazole dyes, phthalocyanine dyes, squaraine dyes, rhodamine dyes and analogues (e.g., Si-pyrone, Si-rhodamine, Te-rhodamine, and Changsha), borondipyrromethane (BODIPY) dyes, seminaphthofluorone xanthene dyes,

benzo[c]heterocycle dyes (e.g., isobenzofuran dyes), and quantum dots. For a review of NIR fluorophores and their use in fluorescence imaging, see, e.g., Escobedo et al. (2010) *Curr. Opin. Chem. Biol.* 14(1):64-70, Hilderbrand et al. (2010) *Curr. Opin. Chem. Biol.* 14(1):71-79, Yuan et al. (2013) *Chem. Soc. Rev.* 42(2):622-661, Vats et al. (2017) *Int. J. Mol. Sci.* 18(5), Zhang et al. (2017) *Nat. Rev. Clin. Oncol.* 14(6):347-364, Gao et al. (2010) *Curr. Top. Med. Chem.* 10(12):1147-1157, Zhao et al. (2018) *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* 10(3):e1483, and Haque et al. (2017) *Bioorg. Med. Chem.* 25(7):2017-2034; herein incorporated by reference in their entireties.

[0058] In certain embodiments, the fluorophore has an emission in the NIR II region of the light spectrum (i.e., about 1000 nm to about 1700 nm), such as at about 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1450, 1500, 1550, 1600, 1650, or 1700 nm, or any wavelength in between. Exemplary NIR II fluorophores include benzobisthiadiazole (BBTD) derivatives, ECX, FD-1080, CH1055, SCH1100, CQS1000, H2a-4T, polymethine dyes, cyanine dyes, quantum dots, rare-earth (RE)-doped nanoparticles (RENPs), and single-walled carbon nanotubes (SWCNTs). Off-peak emissions of NIR-I dyes such as indocyanine green (ICG), IRDye800CW, and IR-12N3 may also be used. See, e.g., Sun et al. (2017) *Chem. Sci.* 8(5):3489-3493, Antaris et al. (2016) *Nat. Mater.* 15(2):235-242, Yang et al. (2017) *Adv. Mater.* 29(12), Zhu et al. (2018) *Theranostics* 8(15):4141-4151, Ding et al. (2018) *Chem. Sci.* 9(19):4370-4380, Miao et al. (2018) *ChemBiochem.* 19(24):2522-2541, Zhu et al. (2018) *Adv. Mater.* e1802546, U.S. Patent Application Publication No. 2017/0266323 A1, U.S. Patent Application Publication No. 2015/0056142 A1, and U.S. Patent No. 10,124,111; herein incorporated by reference in their entireties.

[0059] In certain embodiments, the contrast agent comprises a dye that selectively localizes to cancerous cells. For example, 5-aminolevulinic acid (5-ALA) and indocyanine green (ICG) dyes and derivatives thereof selectively accumulate in tumors. ICG (sodium 4-[2-[(1E,3E,5E,7Z)-7-[1,1-dimethyl-3-(4-sulfonatobutyl)benzo[e]indol-2-ylidene]hepta-1,3,5-trienyl]-1,1-dimethylbenzo[e]indol-3-ium-3-yl]butane-1-sulfonate) is a NIR cyanine dye, which selectively accumulates in tumors, in part, because of the increased permeability of tumor vessels and neovascularization around tumors. In addition, ICG binds to serum proteins such as albumin to form complexes that are accumulated and retained in tumors (see, e.g., Intes et al. (2003) *Med Phys.* 30:1039-1047, Licha et al. (2000) *Photochem. Photobiol.* 72:392-398). 5-ALA uptake by tumor cells results in biosynthesis and accumulation of fluorescent protoporphyrin IX (PpIX). 5-ALA selectively accumulates in tumors, in part, because the metabolism of ALA to PpIX in mitochondria is impaired in cancerous cells. To enhance uptake into cancerous cells, such dyes may be conjugated to tumor targeting agents or binding

agents, such as antibodies, antibody mimetics, peptide ligands, aptamers, and the like, as discussed further below.

[0060] In other embodiments, the fluorescent contrast agent comprises a fluorophore conjugated to a delocalized lipophilic cation that selectively localizes to cancerous cells. Delocalized lipophilic cations (DLCs) accumulate in mitochondria of cancerous cell because of the higher plasma and/or mitochondrial membrane potentials of cancerous cells compared to normal cells. Delocalized lipophilic cations include, but are not limited to, alkyltriphenylphosphonium (e.g., triphenylphosphonium) derivatives and rhodamine derivatives (see, e.g., Zielonka et al. (2017) *Chem Rev.* 117(15):10043-10120, Yang et al. (2017) *Cell Death Dis.* 8(2):e2587, Modica-Napolitano et al. (2001) *Adv. Drug Deliv. Rev.* 49(1-2):63-70, and Lu et al. (2016) *J. Control Release.* 240:38-35; herein incorporated by reference in their entireties).

[0061] In other embodiments, the fluorescent contrast agent comprises a fluorophore conjugated to a binding agent that specifically binds to a tumor marker or antigen of interest (i.e., target antigen on or in the tumor that is resected). In some embodiments, the binding agent binds to a tumor antigen of interest with high affinity.

[0062] Examples of binding agents include, without limitation, antibodies, antibody fragments, antibody mimetics, ligands, and aptamers. The conjugates of the subject methods include at least one fluorophore attached to the binding agent. Fluorescent dyes with different excitation and emission wavelengths can be conjugated to different binding agents that specifically bind to different tumor markers or antigens. It is expected that simultaneous binding to multiple tumor markers or antigens will increase the sensitivity and specificity of detecting cancerous cells by fluorescence imaging.

[0063] Such binding agents may be designed to selectively bind to cell surface molecules or receptors that are highly expressed in tumor cells and/or the tumor environment to increase localization of the contrast agent to the tumor. Binding of targeted fluorophores to cell surface receptors may result in cellular internalization in tumor cells (Becker et al. (2001) *Nat. Biotechnol.* 19:327-331. Various cell surface molecules, including, but not limited to, epidermal growth factor receptor (EGFR), Her-2/Neu, α V β 3 integrin, urokinase plasminogen activator receptor, mucin 1, carcinoembryonic antigen, and the folate receptor can be targeted by binding agents (Moon et al. (2003) *Bioconjug. Chem.* 14:539-545, Ke et al. (2003) *Cancer Res.* 63:78700-78705, Wang et al. (2004) *Mol. Imaging* 3:343-351, Sampath et al. (2007) *J Nucl. Med.* 48:1501-10, Cheng et al. (2005) *Bioconjug. Chem.* 16:1433-1441, Lisy et al. (2008) *Radiology* 247:779-787, Kelly et al. (2004) *Cancer Res.* 64:6247-6251, Yang et al. (2009) *Small* 5:235-43).

[0064] In certain embodiments, the binding agent comprises an antibody that specifically binds to the tumor antigen of interest. Any type of antibody may be used, including polyclonal and monoclonal antibodies, hybrid antibodies, altered antibodies, chimeric antibodies and, humanized antibodies, as well as: hybrid (chimeric) antibody molecules (see, for example, Winter et al. (1991) *Nature* 349:293-299; and U.S. Pat. No. 4,816,567); F(ab')₂ and F(ab) fragments; F_v molecules (noncovalent heterodimers, see, for example, Inbar et al. (1972) *Proc. Natl. Acad. Sci. USA* 69:2659-2662; and Ehrlich et al. (1980) *Biochem.* 19:4091-4096); single-chain Fv molecules (sFv) (see, e.g., Huston et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883); nanobodies or single-domain antibodies (sdAb) (see, e.g., Wang et al. (2016) *Int. J. Nanomedicine* 11:3287-3303, Vincke et al. (2012) *Methods Mol. Biol.* 911:15-26; dimeric and trimeric antibody fragment constructs; minibodies (see, e.g., Pack et al. (1992) *Biochem* 31:1579-1584; Cumber et al. (1992) *J Immunology* 149B:120-126); humanized antibody molecules (see, e.g., Riechmann et al. (1988) *Nature* 332:323-327; Verhoeyan et al. (1988) *Science* 239:1534-1536; and U.K. Patent Publication No. GB 2,276,169, published 21 Sep. 1994); and, any functional fragments obtained from such molecules, wherein such fragments retain specific-binding properties of the parent antibody molecule (i.e., specifically binds to a target tumor antigen).

[0065] In other embodiments, the binding agent comprises an aptamer that specifically binds to the tumor antigen of interest. Any type of aptamer may be used, including a DNA, RNA, xeno-nucleic acid (XNA), or peptide aptamer that specifically binds to the tumor antigen. Such aptamers can be identified, for example, by screening a combinatorial library. Nucleic acid aptamers (e.g., DNA or RNA aptamers) that bind selectively to a target tumor antigen can be produced by carrying out repeated rounds of *in vitro* selection or systematic evolution of ligands by exponential enrichment (SELEX). Peptide aptamers that bind to a target tumor antigen may be isolated from a combinatorial library and improved by directed mutation or repeated rounds of mutagenesis and selection. For a description of methods of producing aptamers, see, e.g., *Aptamers: Tools for Nanotherapy and Molecular Imaging* (R.N. Veedu ed., Pan Stanford, 2016), *Nucleic Acid and Peptide Aptamers: Methods and Protocols* (Methods in Molecular Biology, G. Mayer ed., Humana Press, 2009), *Nucleic Acid Aptamers: Selection, Characterization, and Application* (Methods in Molecular Biology, G. Mayer ed., Humana Press, 2016), *Aptamers Selected by Cell-SELEX for Theranostics* (W. Tan, X. Fang eds., Springer, 2015), Cox et al. (2001) *Bioorg. Med. Chem.* 9(10):2525-2531; Cox et al. (2002) *Nucleic Acids Res.* 30(20): e108, Kenan et al. (1999) *Methods Mol. Biol.* 118:217-231; Platella et al. (2016) *Biochim. Biophys. Acta Nov* 16 pii: S0304-4165(16)30447-0, and Lyu et al. (2016) *Theranostics* 6(9):1440-1452; herein incorporated by reference in their entireties.

[0066] In yet other embodiments, the binding agent comprises an antibody mimetic. Any type of antibody mimetic may be used, including, but not limited to, affibody molecules (Nygren (2008) FEBS J. 275 (11):2668-2676), affilins (Ebersbach et al. (2007) J. Mol. Biol. 372 (1):172-185), affimers (Johnson et al. (2012) Anal. Chem. 84 (15):6553-6560), affitins (Krehenbrink et al. (2008) J. Mol. Biol. 383 (5):1058-1068), alphabodies (Desmet et al. (2014) Nature Communications 5:5237), anticalins (Skerra (2008) FEBS J. 275 (11):2677-2683), avimers (Silverman et al. (2005) Nat. Biotechnol. 23 (12):1556-1561), darpins (Stumpp et al. (2008) Drug Discov. Today 13 (15-16):695-701), fynomers (Grabulovski et al. (2007) J. Biol. Chem. 282 (5):3196-3204), and monobodies (Koide et al. (2007) Methods Mol. Biol. 352:95-109).

[0067] Fluorophores may be conjugated to binding agents by any suitable method. In some instances, the fluorophore and binding agent may be directly linked, e.g., via a single bond, or indirectly linked e.g., through the use of a suitable linker, e.g., a polymer linker, a chemical linker, or one or more linking molecules or moieties. In some instances, attachment of the fluorophore and binding agent may be by way of one or more covalent interactions. In some instances, the fluorophore or binding agent may be functionalized, e.g., by addition or creation of a reactive functional group. Functionalized fluorophores or binding agents may be modified to contain any convenient reactive functional group for conjugation such as an amine functional group, a carboxylic functional group, a sulfhydryl group, a thiol functional group, and the like.

[0068] Any convenient method of bioconjugation may be used including, but not limited to, glutaraldehyde crosslinking, carbodiimide crosslinking, succinimide ester crosslinking, imidoester, crosslinking, maleimide crosslinking, iodoacetamide crosslinking, benzidine crosslinking, periodate crosslinking, isothiocyanate crosslinking, and the like. Such conjugation methods may optionally use a reactive sidechain group of an amino acid residue of the binding agent (e.g., a reactive side-chain group of a Lys, Cys, Ser, Thr, Tyr, His or Arg amino acid residue of the protein, i.e., a polypeptide linking group may be amino-reactive, thiol-reactive, hydroxyl-reactive, imidazolyl-reactive or guanidinyll-reactive). In some cases, a chemoselective reactive functional group may be utilized. Other conjugation reagents that can be used include, but are not limited to, e.g., homobifunctional conjugation reagents (e.g., (bis(2-[succinimidooxycarbonyloxy]ethyl) sulfone, 1,4-Di-(3'-[2'pyridyldithio]-propionamido) butane, disuccinimidyl suberate, disuccinimidyl tartrate, sulfodisuccinimidyl tartrate, dithiobis(succinimidyl propionate), 3,3'-dithiobis(sulfosuccinimidyl propionate), ethylene glycol bis(succinimidyl succinate), and the like), heterobifunctional conjugation reagents (e.g., m-maleimidobenzoyl-N-hydroxysuccinimide ester, m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester, N-γ-maleimidobutyryloxysuccinimide ester, N-γ-maleimidobutyryloxysulfosuccinimide ester, N-(8-maleimidocaproic acid) hydrazide, N-(ε-

maleimidocaproyloxy) succinimide ester, N-(8-maleimidocaproyloxy) sulfo succinimide ester, N-(p-maleimidophenyl) isocyanate, N-succinimidyl(4-iodoacetyl)aminobenzoate, succinimidyl 4-(N-maleimidomethyl) cyclohexane-l-carboxylate, succinimidyl 4-(p-maleimidophenyl) butyrate, N-sulfosuccinimidyl(4-iodoacetyl)aminobenzoate, sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-l-carboxylate, sulfo succinimidyl 4-(p-maleimidophenyl) butyrate, l-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, l-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, maleimide PEG N-hydroxysuccinimide ester, and the like), photoreactive conjugation reagents (e.g., p-azidobenzoyl hydrazide, N-5-azido-2-nitrobenzyloxysuccinimide, p-azidophenyl glyoxal monohydrate, N-(4-[p-azidosalicylamido]butyl)-3'-(2'-pyridyl)dithio) propionamide, bis(P-[4-azidosalicylamido]-ethyl) disulfide, N-hydroxysuccinimideyl-4-azidosalicyclic acid, N-hydroxysulfosuccinimidyl-4-azidobenzoate, sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamide)ethyl-l,3-dithiopropionate, sulfosuccinimidyl 2-(m-azido-o-nitrobenzamido)-ethyl-1,3 '-propionate, sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate, sulfosuccinimidyl (4-azidophenyl dithio)propionate, sulfosuccinimidyl-2-(p-azidosalicylamido)ethyl-l,3-dithiopropionate, and the like).

[0069] In some instances, attachment of a fluorophore to a binding agent of interest is mediated by one or more functional linkers. A functional linker, as used herein, refers to any suitable linker that has one or more functional groups for the attachment of one molecule to another. For example, in some instances the functional linker comprises an amino functional group, a thiol functional group, a hydroxyl functional group, an imidazolyl functional group, a guanidinyl functional group, an alkyne functional group, an azide functional group, or a strained alkyne functional group. Further exemplary functional groups and methods of crosslinking and conjugation are described in, e.g., Hermanson *Bioconjugate Techniques* (Academic Press, 3rd edition, 2013), herein incorporated by reference in its entirety.

[0070] In certain embodiments, the tumor antigen targeted by a binding agent is the epidermal growth factor receptor (EGFR). A number of anti-EGFR antibodies are available including panitumumab, cetuximab, zalutumumab, nimotuzumab, and matuzumab, which can be conjugated to suitable fluorophores for use as fluorescent contrast agents, according to the methods described herein, for fluorescence imaging of cancerous cells expressing EGFR in a tumor specimen, including those tumor specimens comprising head and neck cancer, colorectal cancer, lung cancer, ovarian cancer, breast cancer, endometrial cancer, cervical cancer, bladder cancer, gastric cancer, and esophageal cancer.

[0071] In other embodiments, the tumor antigen targeted by a binding agent is HER2. A number of anti-HER2 antibodies are also available including trastuzumab, pertuzumab, and margetuximab, which can be conjugated to suitable fluorophores for use as fluorescent

contrast agents, according to the methods described herein, for fluorescence imaging of cancerous cells expressing HER2 in a tumor specimen, including those tumor specimens comprising breast cancer, ovarian cancer, stomach cancer, lung cancer, uterine cancer, gastric cancer, colon cancer, head and neck cancer, and salivary duct carcinoma.

[0072] In other embodiments, the tumor antigen targeted by a binding agent is the epithelial cell adhesion molecule (EpCAM) 17-1A. A number of anti-EpCAM 17-1A antibodies are also available including edrecolomab, catumaxomab, and nofetumomab, which can be conjugated to suitable fluorophores for use as fluorescent contrast agents, according to the methods described herein, for fluorescence imaging of cancerous cells expressing EpCAM 17-1A in tumor specimens, including those tumor specimens comprising epithelial-derived neoplasms and various carcinomas, such as lung cancer, gastrointestinal cancer, breast cancer, ovarian cancer, pancreatic cancer, renal cancer, cervical cancer, colorectal cancer, and bladder cancer.

[0073] In other embodiments, the tumor antigen targeted by a binding agent is CD20. A number of anti-CD20 antibodies are also available including rituximab, tositumomab, ocrelizumab, obinutuzumab, ocaratuzumab, ofatumumab, ibritumomab tiuxetan, ublituximab, and veltuzumab, which can be conjugated to suitable fluorophores for use as fluorescent contrast agents, according to the methods described herein, for fluorescence imaging of cancerous cells expressing CD20 in tumor specimens, including those tumor specimens comprising lymphoma such as, but not limited to, marginal zone lymphoma, Hodgkins lymphoma, non-Hodgkins lymphoma; leukemia such as, but not limited to, chronic lymphocytic leukemia, acute lymphoblastic leukemia, myelogenous leukemia, and chemotherapy-resistant hairy cell leukemia; and thyroid cancer.

[0074] In other embodiments, the tumor antigen targeted by a binding agent is CD52. A number of anti-CD52 antibodies are also available including alemtuzumab, which can be conjugated to suitable fluorophores for use as fluorescent contrast agents, according to the methods described herein, for fluorescence imaging of cancerous cells expressing CD52 in tumor specimens, including those tumor specimens comprising lymphoma such as, but not limited to, cutaneous T-cell lymphoma (CTCL) and T-cell lymphoma and chronic lymphocytic leukemia (CLL).

[0075] In other embodiments, the tumor antigen targeted by a binding agent is CD22. A number of anti-CD22 antibodies are also available including inotuzumab, which can be conjugated to suitable fluorophores for use as fluorescent contrast agents, according to the methods described herein, for fluorescence imaging of cancerous cells expressing CD22 in tumor specimens, including those tumor specimens comprising leukemia such as, but not limited to, lymphoblastic leukemia and hairy cell leukemia; lymphoma, and lung cancer.

[0076] In other embodiments, the tumor antigen targeted by a binding agent is CD19. A number of anti-CD19 antibodies are also available including blinatumomab, MEDI-551 and MOR-208, which can be conjugated to suitable fluorophores for use as fluorescent contrast agents, according to the methods described herein, for fluorescence imaging of cancerous cells expressing CD19 in tumor specimens, including those tumor specimens comprising B-cell neoplasms, non-Hodgkin lymphoma (NHL), chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), and multiple myeloma (MM).

Fluorescence Imaging

[0077] Tissue containing a tumor or cancerous cells can be contacted with a fluorescent contrast agent, such that the fluorescent contrast agent selectively localizes to the tumor or cancerous cells. In some embodiments, a detectably effective amount of a fluorescent contrast agent is administered to a subject before surgical resection of a tumor; that is, an amount that is sufficient to yield an acceptable fluorescence image using the fluorescence imaging equipment that is available for clinical use. A detectably effective amount of the fluorescent contrast agent may be administered in more than one injection if needed. The detectably effective amount of the fluorescent contrast agent needed for an individual may vary according to factors such as the degree of susceptibility to uptake into the target area of interest, the age, sex, and weight of the individual, and the particular fluorescence imaging device used. Optimization of such factors is within the level of skill in the art. In other embodiments, the surgical specimen is contacted with a detectably effective amount of a fluorescent contrast agent after removal from the subject.

[0078] The tumor specimen is illuminated with light at a fluorescence excitation wavelength of the fluorescent contrast agent. Any suitable light source may be used for fluorescence excitation. Exemplary light sources include halogen lamps, light-emitting diodes (LEDs), and laser diodes. In addition, band-pass filters can be used to produce multiple excitation channels from a light source and narrow the band of excitation.

[0079] A fluorescence image of the tumor or cancerous cells in a surgical specimen is recorded using a fluorescence imaging device or photodetector capable of detecting the fluorescence emitted by the fluorescent contrast agent. Fluorescence images may be recorded by any suitable method. For example, a CCD image sensor (e.g., intensified CCD (ICCD) or electron multiplying CCD (EMCCD)), a CMOS image sensor, or a digital camera may be used to capture images. The image may be a still photo or a video in any format (e.g., bitmap, Graphics Interchange Format, JPEG file interchange format, TIFF, or mpeg). Alternatively, images may be captured by an analog camera and converted into an electronic form. In addition, fluorescence can be detected by a fluorescence microscope, a closed-field

fluorescence imaging system, a fiber-optic fluorescence imaging system, or a medical fluorescence imaging device (e.g., a handheld fluorescence microscope, a laparoscope, an endoscope, or a microendoscope).

[0080] In one embodiment, fluorescence imaging is performed with near-infrared (near-IR) light, which has the advantage that it can penetrate several millimeters to centimeters into living tissues and be used to visualize cancerous tissue and cells below the surface. Because tissue exhibits almost no autofluorescence in the near-IR spectrum, interfering background fluorescence is minimal.

[0081] An imaging system that can simultaneously detect fluorescence at multiple wavelengths can be used for detection of fluorescence from multiple fluorescent contrast agents that emit fluorescence at different wavelengths. For example, two or more fluorescent contrast agents targeting different tumor antigens can be used. In some devices, the excitation light source and photodetector are integrated into the fluorescence imaging device. In other devices, the excitation light source and/or photodetector reside apart and are used with remote delivery of excitation light. For a review of fluorescence imaging devices, see, e.g., Gray et al. (2012) Biomed. Opt. Express. 3(8):1880-1890; Flusberg et al. (2005) Nat. Methods 2(12):941-950; Choyke et al. (2012) IEEE J. Sel. Top. Quantum. Electron. 18(3):1140-1146; Gray et al. (2012) Proc. SPIE February 3: 8207; Vahrmeijer et al. (2013) Nat. Rev. Clin. Oncol. 10:507-518; herein incorporated by reference in their entireties.

System and Computer implemented Methods for Identifying a Close or Positive Surgical Margin in a Tumor Specimen

[0082] In a further aspect, the invention includes a computer implemented method for identifying a close or positive surgical margin in a tumor specimen. The computer performs steps comprising: a) receiving inputted fluorescence imaging data of a tumor surgical specimen, wherein the fluorescence imaging data comprises fluorescent signals from a fluorescent contrast agent localized to cancerous cells in the surgical specimen; b) plotting a two-dimensional map or a three-dimensional map of the fluorescent signals; c) quantifying the fluorescent signals; d) identifying a fluorescence peak-intensity area in the two-dimensional map or the three-dimensional map, wherein the fluorescence peak-intensity area corresponds to a specimen location where the surgical tumor specimen has a close margin; e) mapping the fluorescence peak-intensity area to a specimen location; f) marking a photographic image of the tumor surgical specimen to identify the specimen location corresponding to the fluorescence peak-intensity area; and g) displaying the marked photographic image of the tumor surgical specimen, wherein the specimen location corresponding to the fluorescence peak-intensity area is identified. In certain embodiments, the fluorescence peak-intensity area

having the highest fluorescence intensity, second highest fluorescence intensity, third highest fluorescence intensity, or fourth highest fluorescence intensity of all detected fluorescence peak-intensity areas in the two-dimensional map or the three-dimensional map is mapped to a location in the tumor surgical specimen, and a photographic image of the tumor surgical specimen is marked to identify the specimen location corresponding to that fluorescence peak-intensity area. In certain embodiments, multiple fluorescence peak-intensity areas are mapped to locations in the tumor surgical specimen and marked on a photographic image of the tumor surgical specimen to identify the specimen locations corresponding to the fluorescence peak-intensity areas. For example, the fluorescence peak-intensity area having the highest fluorescence intensity, second highest fluorescence intensity, third highest fluorescence intensity, and fourth highest fluorescence intensity of all detected fluorescence peak-intensity areas in the two-dimensional map or the three-dimensional map can be mapped to locations in the tumor surgical specimen, and a photographic image of the tumor surgical specimen can be marked to identify the specimen locations corresponding to those fluorescence peak-intensity areas. In certain embodiments, the fluorescent signals are quantified with or without scaling relative to fluorescent signals emitted from specimen regions known to have tumor or normal tissue (e.g., see Examples). In certain embodiments, the computer implemented method further comprises calculating a surgical margin thickness at a specimen location corresponding to a fluorescence peak-intensity area. In certain embodiments, the computer implemented method further comprises identifying the closest surgical margin corresponding to a fluorescence peak-intensity area (e.g., surgical margin less than 5 mm from the tumor).

[0083] In certain embodiments, the computer implemented method further comprises storing a marked photographic image of the tumor specimen (i.e., having at least one specimen location corresponding to a fluorescence peak-intensity area identified) in a database. In certain embodiments, the computer implemented method further comprises sending a marked photographic image to a surgeon or pathologist.

[0084] The method can be implemented in digital electronic circuitry, or in computer software, firmware, or hardware. The disclosed and other embodiments can be implemented as one or more computer program products, i.e., one or more modules of computer program instructions encoded on a computer readable medium for execution by, or to control the operation of, a data processing apparatus. The computer readable medium can be a machine-readable storage device, a machine-readable storage substrate, a memory device, a composition of matter effecting a machine-readable propagated signal, or any combination thereof.

[0085] A computer program (also known as a program, software, software application, script, or code) can be written in any form of programming language, including compiled or interpreted languages, and it can be deployed in any form, including as a stand-alone program

or as a module, component, subroutine, or other unit suitable for use in a computing environment. A computer program does not necessarily correspond to a file in a file system. A program can be stored in a portion of a file that holds other programs or data (e.g., one or more scripts stored in a markup language document), in a single file dedicated to the program in question, or in multiple coordinated files (e.g., files that store one or more modules, sub programs, or portions of code). A computer program can be deployed to be executed on one computer or on multiple computers that are located at one site or distributed across multiple sites and interconnected by a communication network.

[0086] In a further aspect, a system for performing the computer implemented method, as described, is provided. Such a system includes a computer containing a processor, a storage component (i.e., memory), a display component, and other components typically present in general purpose computers. The storage component stores information accessible by the processor, including instructions that may be executed by the processor and data that may be retrieved, manipulated or stored by the processor.

[0087] The storage component includes instructions for analyzing fluorescence imaging data of a surgical specimen to identify suspicious regions (e.g., a close or positive surgical margin in need of further assessment to determine if cancerous cells are present). For example, the storage component includes instructions for identifying a close or positive surgical margin in a tumor specimen based on analysis of fluorescence imaging data stored therein. The computer processor is coupled to the storage component and configured to execute the instructions stored in the storage component in order to receive fluorescence imaging data of a surgical specimen and analyze the data according to one or more algorithms as described herein. The display component displays a marked photographic image of the tumor specimen, wherein the specimen location corresponding to the fluorescence peak-intensity area is identified. For example, the specimen location corresponding to the fluorescence peak-intensity area can be marked to identify it in the photographic image of the tumor specimen by labeling, outlining, coloring, or highlighting it.

[0088] The storage component may be of any type capable of storing information accessible by the processor, such as a hard-drive, memory card, ROM, RAM, DVD, CD-ROM, USB Flash drive, write-capable, and read-only memories. The processor may be any well-known processor, such as processors from Intel Corporation. Alternatively, the processor may be a dedicated controller such as an ASIC.

[0089] The instructions may be any set of instructions to be executed directly (such as machine code) or indirectly (such as scripts) by the processor. In that regard, the terms "instructions," "steps" and "programs" may be used interchangeably herein. The instructions may be stored in object code form for direct processing by the processor, or in any other

computer language including scripts or collections of independent source code modules that are interpreted on demand or compiled in advance.

[0090] Data may be retrieved, stored or modified by the processor in accordance with the instructions. For instance, although the system is not limited by any particular data structure, the data may be stored in computer registers, in a relational database as a table having a plurality of different fields and records, XML documents, or flat files. The data may also be formatted in any computer-readable format such as, but not limited to, binary values, ASCII or Unicode. Moreover, the data may comprise any information sufficient to identify the relevant information, such as numbers, descriptive text, proprietary codes, pointers, references to data stored in other memories (including other network locations) or information which is used by a function to calculate the relevant data.

[0091] In certain embodiments, the processor and storage component may comprise multiple processors and storage components that may or may not be stored within the same physical housing. For example, some of the instructions and data may be stored on removable CD-ROM and others within a read-only computer chip. Some or all of the instructions and data may be stored in a location physically remote from, yet still accessible by, the processor. Similarly, the processor may comprise a collection of processors which may or may not operate in parallel.

[0092] In certain embodiments, the system further comprises a light source capable of illuminating a surgical specimen at a fluorescence excitation wavelength of the fluorescent contrast agent used in fluorescently labeling cancerous cells of the surgical specimen and a switch configured to control the switching on and off of the light source. Exemplary light sources that can be included in the system include halogen lamps, LEDs, and laser diodes.

[0093] In certain embodiments, the system further comprises a photodetector and a controller configured to automate recording of photographic or fluorescent images of a tumor specimen. Exemplary photodetectors that may be used in the system include a charged coupled device (CCD) image sensor, a complementary metal-oxide-semiconductor (CMOS) image sensor, a digital camera, a fiber-optic imaging system, and a medical fluorescence imaging system.

Kits

[0094] Kits are also provided for carrying out the methods described herein. In some embodiments, the kit comprises software for carrying out the computer implemented methods for identifying a close or positive surgical margin in a tumor specimen using fluorescence imaging data, as described herein. In some embodiments, the kit comprises a system for identifying a close or positive surgical margin in a tumor specimen as described herein.

[0095] In certain embodiments, the kit further comprises at least one fluorescent contrast agent that selectively localizes to cancerous cells. Compositions can be in liquid form or can be lyophilized and contained in one or more containers. Suitable containers for the compositions include, for example, bottles, vials, syringes, and test tubes. Containers can be formed from a variety of materials, including glass or plastic. A container may have a sterile access port (for example, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle).

[0096] The kit can further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution, or dextrose solution. It can also contain other materials useful to the end-user, including other pharmaceutically acceptable formulating solutions such as buffers, diluents, filters, needles, and syringes or other delivery devices. The delivery device may be pre-filled with the compositions.

[0097] The kit can also comprise a package insert containing written instructions for methods of using the compositions comprising fluorescent contrast agents for fluorescence imaging of a surgical specimen according to the methods described herein. The package insert can be an unapproved draft package insert or can be a package insert approved by the Food and Drug Administration (FDA) or other regulatory body.

[0098] In certain embodiments, the kit further comprises a light source capable of illuminating a surgical specimen at a fluorescence excitation wavelength of the fluorescent contrast agent used in fluorescently labeling cancerous cells of the surgical specimen.

[0099] In certain embodiments, the kit further comprises a photodetector capable of recording photographic or fluorescent images of a tumor specimen.

Utility

[00100] The methods described herein are useful for accurately determining surgical margins for tumor resection (i.e., the distance from a tumor border to healthy tissue). Surgeons typically remove a tumor along with a 5 mm margin that is presumed to be cancer-free healthy tissue surrounding the tumor. However, this margin is sometimes insufficient and failure to remove all cancerous cells results in locoregional recurrence and poor overall survival rates of patients. Tumor borders within deeper tissue layers are especially difficult to determine, and deep margins are consequently more prone to develop local recurrence. Although frozen section analysis (FSA) can detect cancer, typically only a very small percent of the wound bed is sampled, and surgeons struggle to identify which suspicious areas are appropriate for histopathological assessment. Thus, the usefulness of frozen section analysis is limited by the ability to select the appropriate areas of a tumor specimen for sampling.

[00101] The methods of the present invention address the need for better ways of assessing tumor margins for cancer surgery. The methods disclosed herein consistently detect the smallest margin of "normal" tissue surrounding a tumor in surgical specimens and provide strategical guidance in identifying potentially positive or close margins that need further assessment by a surgeon or pathologist. In particular, the methods of the invention can be performed intraoperatively to provide guidance on whether or not additional surgery is required to remove additional cancerous tissue from a subject. The methods can further direct a surgeon to the appropriate tissue location in a patient wherein additional cancerous tissue needs removal. That is, a surgeon can surgically remove additional cancerous tissue from a subject if cancerous cells are detected in a tissue section of a tumor specimen indicating that the tissue section comprises a positive margin, wherein the location of the positive margin in the tumor specimen is used to identify the location of the cancerous tissue remaining in the subject.

[00102] The methods disclosed herein can be used to evaluate margins in surgical tumor specimens for a wide variety of cancers, including, but not limited to, head and neck cancer, skin cancer, breast cancer, ovarian cancer, melanoma, pancreatic cancer, peripheral neuroma, glioblastoma, adrenocortical carcinoma, AIDS-related lymphoma, anal cancer, bladder cancer, meningioma, glioma, astrocytoma, cervical cancer, chronic myeloproliferative disorders, colon cancer, endometrial cancer, ependymoma, esophageal cancer, Ewing's sarcoma, extracranial germ cell tumors, extrahepatic bile duct cancer, gallbladder cancer, gastric cancer, gastrointestinal carcinoid tumors, gestational trophoblastic tumors, hairy cell leukemia, Hodgkin lymphoma, non-Hodgkin lymphoma, hypopharyngeal cancer, islet cell carcinoma, Kaposi sarcoma, laryngeal cancer, leukemia, lip cancer, oral cavity cancer, liver cancer, male breast cancer, malignant mesothelioma, medulloblastoma, Merkel cell carcinoma, metastatic squamous neck cell carcinoma, multiple myeloma and other plasma cell neoplasms, mycosis fungoides and the Sezary syndrome, myelodysplastic syndromes, nasopharyngeal cancer, neuroblastoma, non-small cell lung cancer, small cell lung cancer, oropharyngeal cancer, bone cancers, including osteosarcoma and malignant fibrous histiocytoma of bone, paranasal sinus cancer, parathyroid cancer, penile cancer, pheochromocytoma, pituitary tumors, prostate cancer, rectal cancer, renal cell cancer, retinoblastoma, rhabdomyosarcoma, salivary gland cancer, small intestine cancer, soft tissue sarcoma, supratentorial primitive neuroectodermal tumors, pineoblastoma, testicular cancer, thymoma, thymic carcinoma, thyroid cancer, transitional cell cancer of the renal pelvis and ureter, urethral cancer, uterine sarcoma, vaginal cancer, vulvar cancer, and Wilm's tumor and other childhood kidney tumors.

[00103] It will be apparent to one of ordinary skill in the art that various changes and modifications can be made without departing from the spirit or scope of the invention.

EXPERIMENTAL

[00104] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (*e.g.* amounts, temperature, *etc.*) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

[00105] All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

[00106] The present invention has been described in terms of particular embodiments found or proposed by the present inventor to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. For example, due to codon redundancy, changes can be made in the underlying DNA sequence without affecting the protein sequence. Moreover, due to biological functional equivalency considerations, changes can be made in protein structure without affecting the biological action in kind or amount. All such modifications are intended to be included within the scope of the appended claims.

Example 1

Utilizing Relative Fluorescence Peak-intensity for Intraoperative Surgical Margins Assessment

Introduction

[00107] Close (1-5 mm) and positive surgical margins (< 1 mm) are directly correlated with poor survival. Efforts to mitigate positive margin rates are critical to improve outcomes for the vast majority of patients undergoing curative intent treatment. Here we describe a novel methodology to select areas most suspicious for a close or positive margin within minutes of resection to allow the surgeon to take corrective action during the procedure (13). To address

the challenges concerning deep margin status, we believe that the near-infrared fluorescence imaging could provide a new approach in surgical oncology. This study investigates intraoperative margin assessment using relative fluorescence peak-intensity isolation of the resected specimens' deep surface in order to identify suspicious margins (tumor < 5 mm).

Materials and Methods

Study design

[00108] The Phase I study evaluating panitumumab-IRDye800CW was approved by the Stanford Institutional Review Board (IRB-35064; NCT02415881). More details on the phase I study can be found in Gao et al. (14). Briefly, descending patients (n=18) were infused 1-4 days prior to surgery with panitumumab-IRDye800CW (excitation/emission max: 774/789nm; half-life: 24 hours (14)). After resection, the tumor specimens were imaged on the deep margin in a closed-field fluorescence imaging device (PEARL, LI-COR biosciences) before being sent to pathology for standard of care histological assessment. There, the specimen was formalin-fixed and cut in 5mm tissue sections. The specimen was then reconstructed from the 5mm sections and re-imaged. Thereafter, the 5mm tissue sections were processed and paraffin-embedded. From each 5mm section, a representative 5µm section was cut for routine hematoxylin and eosin (H&E) staining for diagnosis. On the acquired H&E slides, areas with invasive or *in situ* SCC were outlined by a board-certified pathologist. The slides were then digitized and analyzed for our study.

Fluorescence Peak-intensity Isolation

[00109] ImageJ (version 1.50i, National Institute of Health, Washington D.C., Maryland, USA) was used to quantify the fluorescent signal on the deep surface of the resected specimen after the entire surface was plotted using an interactive 3D signal-mapping program (ImageJ plugin, interactive 3D surface plot, Internationale Medieninformatik, Berlin, Germany). Utilizing this 3D signal-mapping tool, we were able to isolate high intensity areas, further described as peak-intensity areas (PIAs), from back ground signal (FIG. 1 WORKFLOW). By scaling up the threshold of the fluorescent signal in the 3D plot, multiple PIAs could be isolated up to a level where all signal reached above the surface (c). The PIAs were numbered in order of appearance ranging from 1st to 4th (d-g). Further isolation than the 3rd or 4th PIA was deemed unnecessary given the objective to find the closest tumor margin on the deep surface. In addition, because some specimen had no clear 4th PIA or presented with a simultaneous 3rd and 4th peak, we choose to average the values (i.e. fluorescence intensity and tumor depth) of these peaks. Subsequently, for each PIA, an opposing area of no significance (ANS) was

randomly assigned 10-15 mm from the PIA as reference. To quantify the fluorescence signal of both PIAs and ANSs, mean fluorescence intensities (MFIs) were subtracted from the deep margin by drawing circular regions of interests (ROIs) with a diameter of 5 mm. For MFI comparison between PIAs and ANSs, the average signal intensity of all spots of both PIAs and ANSs was used.

Correlation of Fluorescence Signal with Tumor Depth

[00110] First, the specimens were reconstructed in order to locate the H&E slides back to their original position in the specimen. Then, to measure the tumor depth (i.e. distance from tumor tissue to deep surface) at the PIAs and ANSs, ImageJ was used on the outlined H&E slides. Each tumor depth distance was measured five times and averaged. Statistical analysis was performed by comparing tumor depth at PIAs to tumor depth of corresponding ANSs using an unpaired, two-tailed t-test (Version 8.0c, GraphPad Software, La Jolla, California, USA). For within specimen PIA (and ANS) comparison an unpaired Mann-Whitney test was used. P-values of 0.05 or less were considered statistically significant.

Results

[00111] A total of 18 patients underwent evaluation of their primary specimen after systemic infusion of panitumumab-IRDye800 that we have shown to be sensitive and specific for squamous cell cancer (14,15). Primary surgical specimens underwent closed-field imaging of the deep margin immediately after surgical removal. Patient characteristics are summarized in Table 1. All 18 patients could undergo successful imaging and peak intensity analysis, but six of the patients were excluded because pathological reconstruction of the specimen could not be performed in a manner where the individual histological sections could be correlated precisely (< 1 mm) to the corresponding PIA on the fluorescence imaging of the primary specimen. For the remaining 12 patients, a total of 80 PIAs and corresponding ANSs were isolated whereby a total of 400 tumor depth measurements were performed.

Correlation of PIA and corresponding ANS

[00112] To determine the deep margin region where the leading edge of the tumor was closest to the deep margin of the specimen. The peak intensity areas and areas of negative fluorescence on the surgical specimen surface immediately after removal could be accurately mapped back to the histologic section of the pathologic reconstruction. Using this method, tumor depth was measured at areas of high and low fluorescence intensity (measured as mean fluorescence intensity, MFI) as shown in FIG. 2. The average tumor depth was found at $4.5 \pm 2.3\text{mm}$ (n=80) at areas of peak fluorescence, compared to $10.4 \pm 4.2\text{mm}$ (n=80) at areas

of relatively low fluorescence intensity. With the difference between averaged distances being $5.8 \pm 1.4\text{mm}$, a significant difference was found ($p < 0.0001$, $n = 160$) between tumor depth identified at areas of peak fluorescence compared to areas of low fluorescence intensity. The invasive front of the tumor was significantly closer to the deep margin in areas assessed by peak fluorescence intensity compared to tumor distance in corresponding low fluorescence intensity areas suggesting the closest margins on the specimen was always identified at the area of peak fluorescence intensity on the specimen.

Margin distance correlates with the fluorescence intensity

[00113] The margin distance (the measurement in mm between the invasive tumor edge and the cut surface of the specimen) correlates directly with the peak fluorescence region. In all specimens, tumor was always closer to the deep margin in the first region of peak intensity on the specimen compared to the second which harbored tumor closer in 83% compared to the averaged third/fourth fluorescence peak. FIG. 3 shows decreasing peak fluorescence intensity corresponds with increasing tumor depth on histological section for each patient. The first and second intensity peaks are consistently distinguishable in tumor depth shown by an average increase of 2.1mm. This suggests that in all 12 cases the first PIA identified harbored tumor significantly closer to the deep surface than all the subsequent PIAs ($p < 0.05$, $n = 36$). The average increase of tumor depth between the second and the third/fourth PIA was 1.6mm. In only two (2/12) cases was the tumor depth that correlated to the second PIA was found to be larger than that of the third/fourth PIA tumor distance. However, in both of these cases the second PIA and third/fourth PIA were not substantially different: the PIAs were found to correlate with a tumor thickness that was within the margin of error in measurement ($5.8 \pm 0.1\text{mm}$ versus $5.6 \pm 0.4\text{mm}$; and $11.4 \pm 0.6\text{mm}$ versus $10.2 \pm 0.6\text{mm}$). Failure to correlate more precisely as the fluorescence intensity decreases might be due to the tumor heterogeneity and undulating tumor boarder and inconsistent delivery of the contrast agent to the tumor, it might also be explained by the optical properties of the tissue, such that the limited penetration depth of the dye. Importantly, areas of low fluorescence intensity, located 10-15mm from the isolated peak fluorescence areas, showed no significant difference in tumor depth ($p > 0.05$, $n = 36$), indicating that only regions of peak fluorescence intensity were of value for image guidance.

Patient exclusion

[00114] Although the proper pathological correlation could only be made in 12 patients, the other 6 underwent imaging, but the specimens were too large or complex to successfully reconstruct for appropriate pathologic reconstruction since they contained bone, such as

maxillectomy. In one such case, a marking suture was leveraged to correlate the pathological findings back to fluorescence imaging results. As shown in a maxillectomy specimen (FIG. 5) peak fluorescence imaging of the specimen could be correlated with the margin for specific locations by correlating the PIA on the deep surface to a marked suture.

Discussion

[00115] Reduction in positive margin rates on final pathology have not significantly improved in decades despite dramatic improvement in surgical instrumentation (16,17). We enrolled patients undergoing curative intent surgery to receive a systemically administered fluorescent contrast agent (panitumumab-IRDye800) and showed that optical imaging could identify areas on the surgical specimen most at risk for a close or positive margin. We show that the peak areas of fluorescence should be chosen for frozen section analysis since that is most likely to be the closest surgical margin. While standard of care relies on frozen section analysis, the method is limited by the sampling error and the small amount of tissue that can be sampled (18). Frozen section itself is considered highly accurate (>90%;), but its application in head and neck surgery results in 10-20 frozen sections per case and as mentioned, has not impacted success rates despite wide availability (12,19). The current study has shown that identifying fluorescence peak-intensity can be performed which could direct intraoperative margin sampling for frozen section analysis. Retrospective data analysis on patient specimens suggests that intraoperative *ex vivo* fluorescence imaging of the resected specimen can direct the surgeon to the closest involved margin on the deep surface.

[00116] The success of this *ex vivo* specimen mapping method relies on several key optical principles. First, the NIR fluorescent signal will not travel much further than 6mm through tissue. This allows the signal to become relatively constant after the margin exceeds 5mm, an important number clinically. Detection of a truly positive number is not as common as a close margin (1-5mm) which can be successfully identified using this technique. Other surgical imaging methods dependent on cancer on the surface of the specimen are less helpful. Second, the optical signal will intensify as the thickness of the surgical margin decreases. The region with the peak fluorescence intensity may or may not be positive, however it is most likely the spot where tumor tissue is closest to the surface. It objectively delineates the most likely suspicious region and therefore transcends the need to identify positive margins. The corollary is that if all the fluorescence is the same, the tumor surface is either all positive or all negative and requires a frozen section assessment for confirmation. The difficulty in accounting for tumor thickness, tumor heterogeneity and size of the tumor is clear, but retrospective analysis was not affected by these results. Thus, using this technique, if infiltrative tumor tissue is found closer than 5 mm to the margin using FSA, the surgeon has

the immediate possibility to resect additional tissue from the wound cavity using the fluorescence intensity peaks as guidance of where to resect. The proposed intraoperative workflow is shown in FIG. 4.

[00117] In a previous study we showed that fluorescent surface mapping is highly sensitive for detection of cancer within 5 mm of the surgical margin (13) using an absolute fluorescence value. We add to these findings using the relative intensity where a patient serves as its own control, difficulties concerning interpatient variabilities such as dose differences, drug uptake, signal intensity and tumor location encountered in previous work have therefore been eliminated (13,15,20). Unfortunately, large intratumor heterogeneity is more difficult to account for in the model, although the results were not affected in this study, it might suggest intratumoral factors. Moreover, our method of relative MFI peak-intensity isolation is presumed to be compatible with a variety of fluorescent probes as well as different imaging systems as it avoids the need for absolute quantitative analysis of the imaging data but rather observes relative differences in MFI peak-intensities to guide intraoperative decision making.

[00118] The relatively small sample size limits the conclusions that can be drawn from the results, and the motivation for exclusion of the six patients offers valuable information. In order to face challenges such as bone involvement, tumor size and location we believe that our current reconstruction method necessitates a different strategy. Future clinical trials should be designed to gain knowledge about the clinical value of this technique for complex cases (i.e. bone involvement, perineural invasion and extracapsular invasion). Currently, we are investigating a prospective intraoperative research setting, where we mark the 1st and 2nd we can simultaneously guide the surgeon and pathologist to areas that might need extra attention and specifically investigate complex situations like bone involvement, perineural invasion and extracapsular invasion. Another constraint may be the manner in which margins are being sampled for FSA. Although the majority of surgeons nowadays prefer to obtain frozen sections from the specimen, there are some who obtain sections from the wound cavity to test margin status (21). Since our technique relies not only on specimen driven sampling but also on obtaining perpendicular margins to measure tumor distance, it might necessitate change in current practice. Nevertheless, it is worth noting that the literature suggest that perpendicular margins have a significant advantage over en-face sampling (6). Although en-face sampling may assess a greater surface area, lack of information concerning tumor depth makes perpendicular sampling superior.

[00119] Our proposed non-invasive imaging of the tumor specimen allows use of MFI peak-isolation that may have clinical value in expediting analysis of the entire specimen surface with a single frozen section. Most importantly, this strategy of margin assessment can be performed on all patients undergoing specimen resection with an antibody-dye bioconjugate.

Consequently, this technique could significantly decrease our current poor margin control and locoregional recurrence rate to increase 5-year overall survival. Furthermore, we believe that this technique might have implication in other fields besides head and neck where wide local excision is required (e.g. melanoma, colon cancer and vulva cancer; (22-24)).

Conclusion

[00120] We can successfully identify regions on the cut surface where a tumor is closest to the border. This technique changes intraoperative decision-making, and the data presented in this study supports the use of peak-intensity isolation.

References

- [00121] 1. Woolgar JA, Triantafyllou A. A histopathological appraisal of surgical margins in oral and oropharyngeal cancer resection specimens. *Oral Oncol.* 2005 Nov;41(10):1034-43.
- [00122] 2. McMahon J, O'Brien CJ, Pathak I, Hamill R, McNeil E, Hammersley N, et al. Influence of condition of surgical margins on local recurrence and disease-specific survival in oral and oropharyngeal cancer. *Br J Oral Maxillofac Surg.* 2003 Aug;41(4):224-31.
- [00123] 3. Ravasz LA, Slootweg PJ, Hordijk GJ, Smit F, van der Tweel I. The status of the resection margin as a prognostic factor in the treatment of head and neck carcinoma. *J Cranio-Maxillo-fac Surg Off Publ Eur Assoc Cranio-Maxillo-fac Surg.* 1991 Oct;19(7):314-8.
- [00124] 4. Eldeeb H, Macmillan C, Elwell C, Hammod A. The effect of the surgical margins on the outcome of patients with head and neck squamous cell carcinoma: single institution experience. *Cancer Biol Med.* 2012 Mar;9(1):29-33.
- [00125] 5. Pawlik TM, Scoggins CR, Zorzi D, Abdalla EK, Andres A, Eng C, et al. Effect of surgical margin status on survival and site of recurrence after hepatic resection for colorectal metastases. *Ann Surg.* 2005 May;241(5):715-22, discussion 722-724.
- [00126] 6. Hinni ML, Ferlito A, Brandwein-Gensler MS, Takes RP, Silver CE, Westra WH, et al. Surgical margins in head and neck cancer: a contemporary review. *Head Neck.* 2013 Sep;35(9):1362-70.
- [00127] 7. Cooper JS, Pajak TF, Forastiere AA, Jacobs J, Campbell BH, Saxman SB, et al. Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck. *N Engl J Med.* 2004 May 6;350(19):1937-44.
- [00128] 8. Bernier J, Domette C, Ozsahin M, Matuszewska K, Lefèbvre J-L, Greiner RH, et al. Postoperative Irradiation with or without Concomitant Chemotherapy for Locally Advanced Head and Neck Cancer. *N Engl J Med.* 2004 May 6;350(19):1945-52.

- [00129] 9. Roh J-L, Westra WH, Califano JA, Sidransky D, Koch WM. Tissue Imprint for Molecular Mapping of Deep Surgical Margins in Patients with Head and Neck Squamous Cell Carcinoma. *Head Neck*. 2012 Nov;34(11):1529-36.
- [00130] 10. Tirelli G, Nata FB, Gatto A, Bussani R, Spinato G, Zacchigna S, et al. Intraoperative Margin Control in Transoral Approach for Oral and Oropharyngeal Cancer. *The Laryngoscope* [Internet]. [cited 2018 Nov 9];0(0).
- [00131] 11. Huang X, Pateromichelakis S, Hills A, Sherriff M, Lyons A, Langdon J, et al. p53 mutations in deep tissues are more strongly associated with recurrence than mutation-positive mucosal margins. *Clin Cancer Res Off J Am Assoc Cancer Res*. 2007 Oct 15;13(20):6099-106.
- [00132] 12. Black C, Marotti J, Zarovnaya E, Paydarfar J. Critical evaluation of frozen section margins in head and neck cancer resections. *Cancer*. 2006 Dec 15;107(12):2792-800.
- [00133] 13. van Keulen S, van den Berg NS, Nishio N, Birkeland A, Zhou Q, Lu G, et al. Rapid, non-invasive fluorescence margin assessment: Optical specimen mapping in oral squamous cell carcinoma. *Oral Oncol*. 2019 Jan 1;88:58-65.
- [00134] 14. Gao RW, Teraphongphom N, de Boer E, van den Berg NS, Divi V, Kaplan MJ, et al. Safety of panitumumab-IRDye800CW and cetuximab-IRDye800CW for fluorescence-guided surgical navigation in head and neck cancers. *Theranostics*. 2018;8(9):2488-95.
- [00135] 15. Gao RW, Teraphongphom NT, van den Berg NS, Martin BA, Oberhelman NJ, Divi V, et al. Determination of Tumor Margins with Surgical Specimen Mapping Using Near-Infrared Fluorescence. *Cancer Res*. 2018 Jul 2;canres.0878.2018.
- [00136] 16. Ramirez PT, Frumovitz M, Pareja R, Lopez A, Vieira M, Ribeiro R, et al. Minimally Invasive versus Abdominal Radical Hysterectomy for Cervical Cancer. *N Engl J Med*. 2018 15;379(20):1895-904.
- [00137] 17. Poon H, Li C, Gao W, Ren H, Lim CM. Evolution of robotic systems for transoral head and neck surgery. *Oral Oncol*. 2018 Dec;87:82-8.
- [00138] 18. Olson SM, Hussaini M, Lewis Jr JS. Frozen section analysis of margins for head and neck tumor resections: reduction of sampling errors with a third histologic level. *Mod Pathol*. 2011 May;24(5):665-70.
- [00139] 19. Ord RA, Aisner S. Accuracy of frozen sections in assessing margins in oral cancer resection. *J Oral Maxillofac Surg Off J Am Assoc Oral Maxillofac Surg*. 1997 Jul;55(7):663-9; discussion 669-671.
- [00140] 20. Moore LS, Rosenthal EL, Chung TK, de Boer E, Patel N, Prince AC, et al. Characterizing the Utility and Limitations of Repurposing an Open-Field Optical Imaging Device for Fluorescence-Guided Surgery in Head and Neck Cancer Patients. *J Nucl Med*. 2017 Feb;58(2):246-51.

- [00141] 21. Jaafar H. Intra-operative frozen section consultation: concepts, applications and limitations. *Malays J Med Sci MJMS*. 2006 Jan;13(1):4-12.
- [00142] 22. Sladden MJ, Nieweg OE, Howle J, Coventry BJ, Thompson JF. Updated evidence-based clinical practice guidelines for the diagnosis and management of melanoma: definitive excision margins for primary cutaneous melanoma. *Med J Aust*. 2018 Feb 19;208(3):137-42.
- [00143] 23. Kuvshinoff B, Maghfoor I, Miedema B, Bryer M, Westgate S, Wilkes J, et al. Distal margin requirements after preoperative chemoradiotherapy for distal rectal carcinomas: are < or = 1 cm distal margins sufficient? *Ann Surg Oncol*. 2001 Mar;8(2):163-9.
- [00144] 24. Nooij LS, van der Slot MA, Dekkers OM, Stijnen T, Gaarenstroom KN, Creutzberg CL, et al. Tumour-free margins in vulvar squamous cell carcinoma: Does distance really matter? *Eur J Cancer Oxf Engl 1990*. 2016;65:139-49.

Table 1: Patient Characteristics

Age	Gender	Tumor Location	pTNM
71	M	Lateral tongue	T1NxMx
52	M	Floor of mouth	T2N2bMx
73	M	Lateral tongue	T3N2Mx
69	F	Buccal mucosa	T1N0Mx
51	F	Lateral tongue	T3N1Mx
48	M	Lateral tongue*	T3N2cMx
58	F	Retromolar trigone*	T3N0Mx
65	F	Buccal mucosa	T2N2bMx
70	F	Buccal mucosa	T3N0Mx
63	F	Alveolar ridge*	T2N0Mx
71	F	Lateral tongue	T2N2bMx
68	F	Lateral tongue	T3N2bMx
71	F	Lateral tongue	T1N1Mx
47	F	Retromolar trigone*	T4bN3bMx
62	M	Buccal mucosa	T2N0Mx
56	M	Floor of mouth	T4aN1Mx
69	M	Maxillary Sinus*	T4aN0Mx
57	M	Anterior tongue*	T4aN3b

*Excluded patients

WHAT IS CLAIMED IS:

1. A method of detecting a close or positive margin in a surgical tumor specimen, the method comprising:
 - a) administering a fluorescent contrast agent to a subject, wherein the fluorescent contrast agent selectively localizes to cancerous cells of a tumor in the subject;
 - b) performing a resection of the tumor to produce the surgical tumor specimen;
 - c) illuminating the surgical tumor specimen with light at a fluorescence excitation wavelength of the fluorescent contrast agent;
 - d) performing fluorescence imaging on a tumor margin of the surgical tumor specimen by detecting fluorescent signals emitted from the fluorescent contrast agent localized to the cancerous cells in the tumor specimen;
 - e) plotting a two-dimensional map or a three-dimensional map of the fluorescent signals emitted from the fluorescent contrast agent localized to the cancerous cells in the tumor specimen;
 - f) quantifying the fluorescent signals;
 - g) identifying at least one fluorescence peak-intensity area in the two-dimensional map or the three-dimensional map, wherein the fluorescence peak-intensity area corresponds to a specimen location where the surgical tumor specimen has a close margin; and
 - h) detecting cancerous cells, if present, at the specimen location where the surgical tumor specimen has a close margin to determine if the close margin is a positive margin.
2. The method of claim 1, further comprising collecting a tissue section of the surgical tumor specimen comprising tissue from the specimen location corresponding to the fluorescence peak-intensity area; and detecting cancerous cells, if present, in the tissue section to determine if the tissue section comprises a positive margin.
3. The method of claim 1 or 2, wherein the fluorescence peak-intensity area has the highest fluorescence intensity, second highest fluorescence intensity, third highest fluorescence intensity, or fourth highest fluorescence intensity of all detected fluorescence peak-intensity areas in the three-dimensional map.
4. The method of any one of claims 1 to 3, further comprising measuring a surgical margin thickness at the specimen location corresponding to the fluorescence peak-intensity area.

5. The method of any one of claims 1 to 4, further comprising identifying the closest surgical margin corresponding to a fluorescence peak-intensity area in the three-dimensional map.
6. The method of claim 5, wherein the surgical margin is less than 5 mm from the tumor.
7. The method of any one of claims 1 to 6, wherein said detecting cancerous cells comprises performing histopathological examination or fluorescent examination of the tissue section.
8. The method of any one of claims 1 to 7, wherein the fluorescent contrast agent comprises a fluorophore conjugated to a binding agent that specifically binds to a tumor marker or antigen in or on the cancerous cells.
9. The method of claim 8, wherein the binding agent comprises an antibody, an antibody fragment, an antibody mimetic, a ligand, or an aptamer that specifically binds to the tumor antigen.
10. The method of claim 9, wherein the fluorescent contrast agent is a panitumumab-IRDye800 conjugate.
11. The method of any one of claims 1 to 9, wherein the fluorescent contrast agent comprises a fluorescent dye that selectively localizes to the cancerous cells.
12. The method of claim 11, wherein the fluorescent dye is 5-aminolevulinic acid (5-ALA) or indocyanine green (ICG).
13. The method of any one of claims 1 to 11, wherein the fluorescent contrast agent comprises a fluorophore conjugated to a delocalized lipophilic cation.
14. The method of claim 13, wherein the delocalized lipophilic cation is triphenylphosphonium or rhodamine cation.
15. The method of any one of claims 1 to 14, wherein the fluorescent contrast agent emits visible, near-infrared, or near-infrared II fluorescent light.

16. The method of any one of claims 1 to 15, wherein the tumor is a head and neck cancer tumor.
17. The method of any one of claims 1 to 16, wherein the tumor margin is less than 7 mm.
18. The method of any one of claims 1 to 17, wherein the fluorescence imaging is performed using an open-field or closed-field fluorescence imaging system.
19. The method of any one of claims 1 to 18, wherein the fluorescence imaging is performed with a medical fluorescence imaging device.
20. The method of any one of claims 1 to 19, the fluorescence imaging comprises recording a fluorescence image of the fluorescent signals with a digital microscope, a charged coupled device (CCD) image sensor, a complementary metal-oxide-semiconductor (CMOS) image sensor, a digital camera, a fiber-optic imaging system, or a medical fluorescence imaging system.
21. The method of any one of claims 1 to 20, further comprising superimposing a photographic image of the surgical tumor specimen and the fluorescence image of the surgical tumor specimen to determine the specimen location corresponding to the fluorescence peak-intensity area.
22. The method of any one of claims 1 to 21, wherein the method is performed intraoperatively or postoperatively to provide guidance to a surgeon for removal of additional cancerous tissue from the subject.
23. The method of claim 22, further comprising surgically removing additional cancerous tissue from the subject if cancerous cells are detected in the tissue section indicating that the tissue section comprises a positive margin, wherein the location of the positive margin in the tumor specimen is used to identify the location of the cancerous tissue in the subject in need of surgical removal.
24. The method of any one of claims 1 to 23, further comprising scaling the fluorescent signal relative to fluorescent signals emitted from specimen regions known to have tumor or normal tissue prior to said quantifying the fluorescent signals.

25. A method of detecting a close or positive margin in a surgical specimen, the method comprising:

- a) obtaining the surgical specimen comprising at least a portion of a tumor;
- b) contacting the surgical specimen with a fluorescent contrast agent, wherein the fluorescent contrast agent selectively localizes to cancerous cells in the tumor specimen;
- c) illuminating the surgical tumor specimen with light at a fluorescence excitation wavelength of the fluorescent contrast agent;
- d) performing fluorescence imaging on a tumor margin of the surgical tumor specimen by detecting fluorescent signals emitted from the fluorescent contrast agent localized to the cancerous cells in the tumor specimen;
- e) plotting a two-dimensional map or a three-dimensional map of the fluorescent signals;
- f) quantifying the fluorescent signals;
- g) identifying at least one fluorescence peak-intensity area in the two-dimensional map or the three-dimensional map, wherein the fluorescence peak-intensity area corresponds to a specimen location where the surgical specimen has a close margin; and
- h) detecting cancerous cells if present at the specimen location where the surgical tumor specimen has a close margin to determine if the close margin is a positive margin.

26. The method of claim 25, further comprising collecting a tissue section of the surgical tumor specimen comprising tissue from the specimen location corresponding to the fluorescence peak-intensity area; and detecting cancerous cells, if present, in the tissue section to determine if the tissue section comprises a positive margin.

27. The method of claim 25 or 26, wherein the method is performed intraoperatively or postoperatively to provide guidance to a surgeon for removal of additional cancerous tissue from the subject.

28. The method of any one of claims 25 to 27, further comprising surgically removing additional cancerous tissue from the subject if cancerous cells are detected in the tissue section indicating that the tissue section comprises a positive margin, wherein the location of

the positive margin in the tumor specimen is used to identify the location of the cancerous tissue in the subject in need of surgical removal.

29. A computer implemented method for identifying a close or positive surgical margin in a tumor specimen, the computer performing steps comprising:

- a) receiving inputted fluorescence imaging data of the tumor specimen, wherein the fluorescence imaging data comprises fluorescent signals from a fluorescent contrast agent bound to cancerous cells in the tumor specimen;
- b) plotting a two-dimensional map or a three-dimensional map of the fluorescent signals;
- c) quantifying the fluorescent signals;
- d) identifying a fluorescence peak-intensity area in the two-dimensional map or the three-dimensional map, wherein the fluorescence peak-intensity area corresponds to a specimen location where the surgical tumor specimen has a close margin;
- e) mapping the fluorescence peak-intensity area to the specimen location;
- f) marking a photographic image of the tumor specimen to identify the specimen location corresponding to the fluorescence peak-intensity area; and
- g) displaying the marked photographic image of the tumor specimen, wherein the specimen location corresponding to the fluorescence peak-intensity area is identified.

30. The computer implemented method of claim 29, further comprising sending the marked photographic image to a surgeon or pathologist.

31. The computer implemented method of claim 29 or 30, wherein said marking the photographic image of the tumor specimen comprises labeling, outlining, coloring, or highlighting the specimen location corresponding to the fluorescence peak-intensity area.

32. The computer implemented method of any one of claims 29 to 31, further comprising storing the marked photographic image of the tumor specimen in a database.

33. The computer implemented method of any one of claims 29 to 32, further comprising scaling the fluorescent signal relative to fluorescent signals emitted from specimen regions known to have tumor or normal tissue prior to said quantifying the fluorescent signals.

34. A system for identifying a close or positive surgical margin in a tumor specimen using the computer implemented method of any one of claims 29 to 33, the system comprising:

- a) a storage component for storing data, wherein the storage component has instructions for identifying a close or positive surgical margin in a tumor specimen based on analysis of the fluorescence imaging data stored therein;
- b) a computer processor for processing the fluorescence imaging data, wherein the computer processor is coupled to the storage component and configured to execute the instructions stored in the storage component in order to receive the inputted fluorescence imaging data and analyze the data according to the computer implemented method of any one of claims 29 to 33; and
- c) a display component for displaying the marked photographic image of the tumor specimen, wherein the specimen location corresponding to the fluorescence peak-intensity area is identified.

35. The system of claim 34, further comprising a light source capable of illuminating a surgical specimen at a fluorescence excitation wavelength of the fluorescent contrast agent.

36. The system of claim 34 or 35, further comprising a photodetector.

37. The system of claim 36, wherein the photodetector is a charged coupled device (CCD) image sensor, a complementary metal-oxide-semiconductor (CMOS) image sensor, a digital camera, a fiber-optic imaging system, or a medical fluorescence imaging system.

38. A non-transitory computer-readable medium comprising program instructions that, when executed by a processor in a computer, causes the processor to perform the method of any one of claims 29 to 33.

39. A kit comprising the non-transitory computer-readable medium of claim 38 and instructions for identifying a close or positive surgical margin in a tumor specimen.

40. The kit of claim 39, further comprising a fluorescent contrast agent.

41. The kit of claim 40, wherein the fluorescent contrast agent comprises a fluorophore conjugated to a binding agent that specifically binds to a tumor marker or antigen.

42. The kit of claim 41, wherein the binding agent comprises an antibody, an antibody fragment, an antibody mimetic, a ligand, or an aptamer that specifically binds to the tumor antigen.

43. The kit of claim 40 or 41, wherein the fluorophore emits visible, near-infrared, or near-infrared II fluorescent light.
44. The kit of claim 42 or 43, wherein the fluorescent contrast agent is a panitumumab-IRDye800 conjugate.
45. A kit comprising the system of any one of claims 34 to 37.

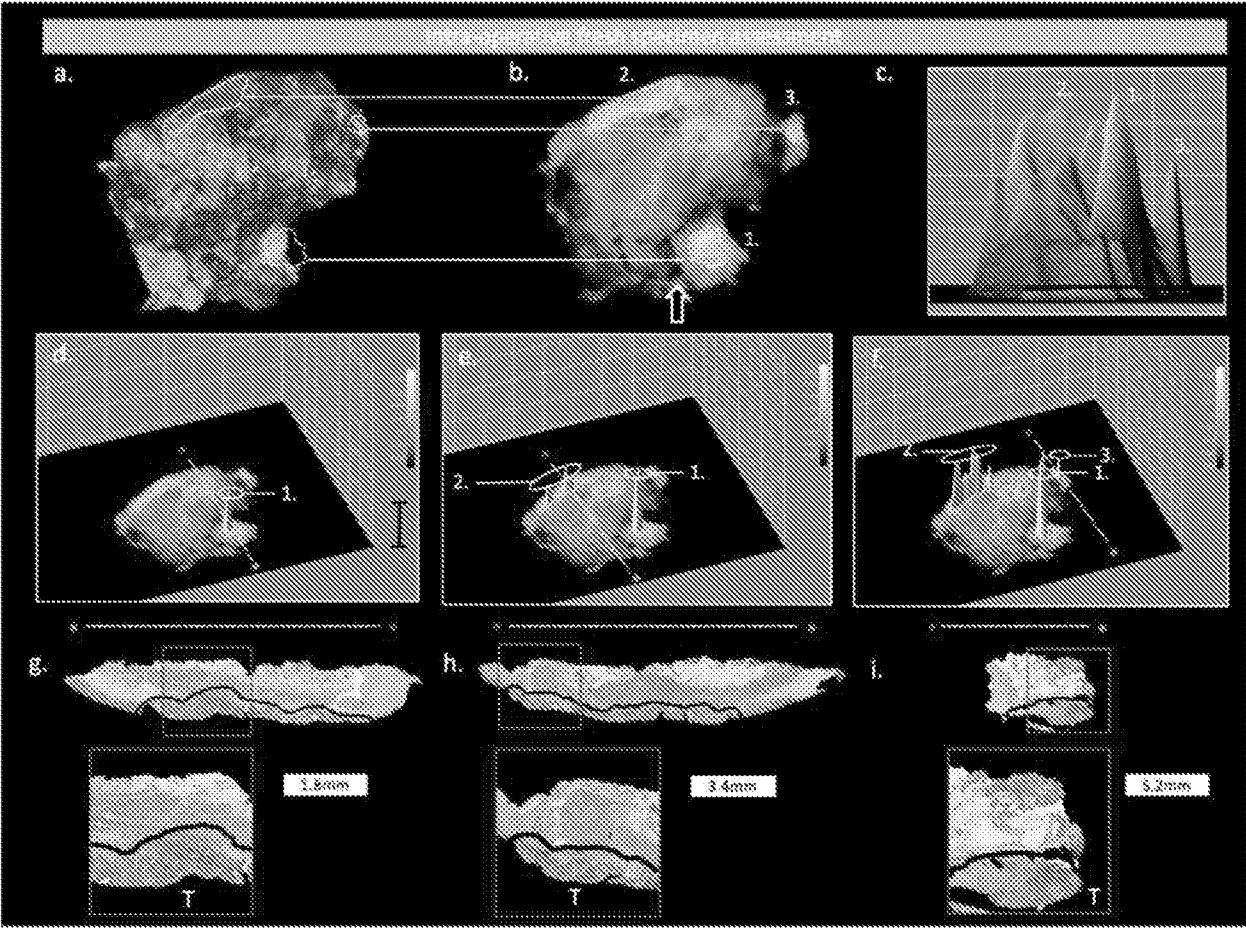


FIG. 1

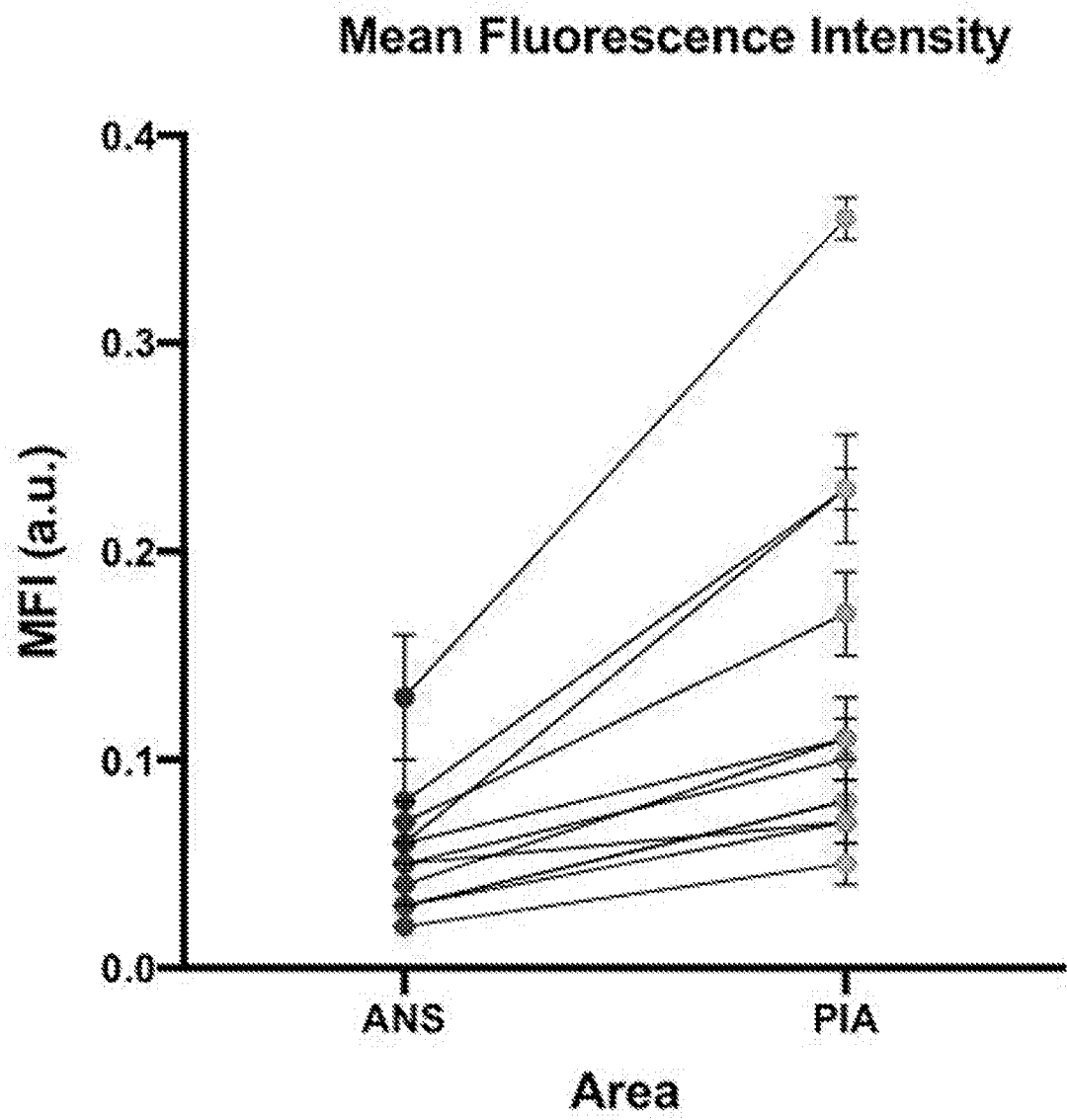


FIG. 2A

3/7

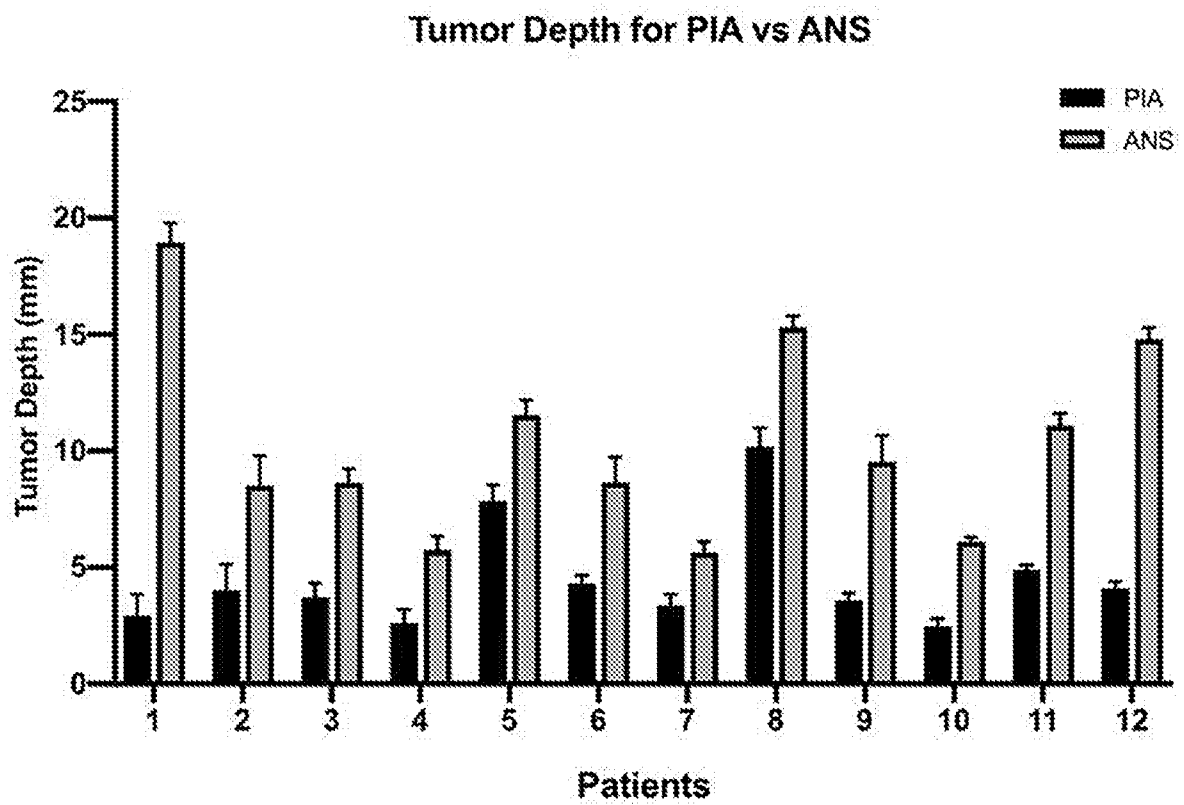


FIG. 2B

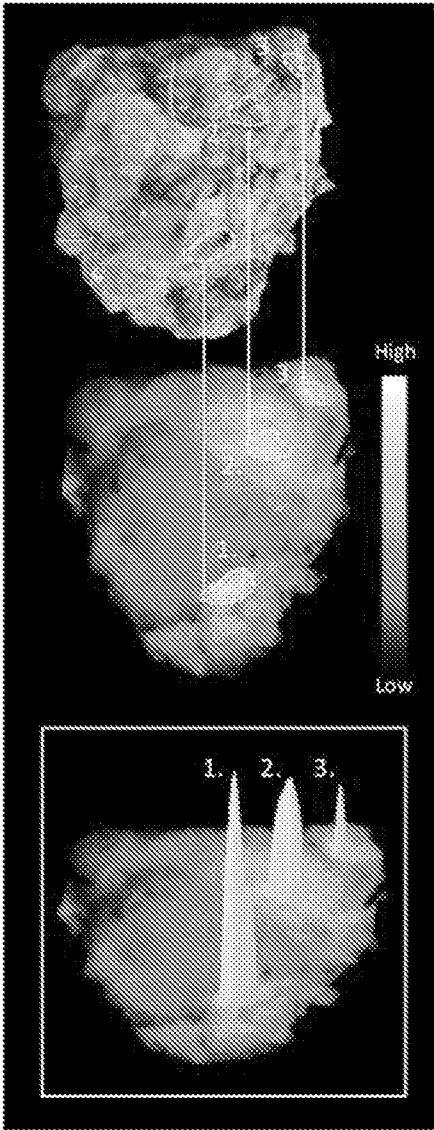


FIG. 3A

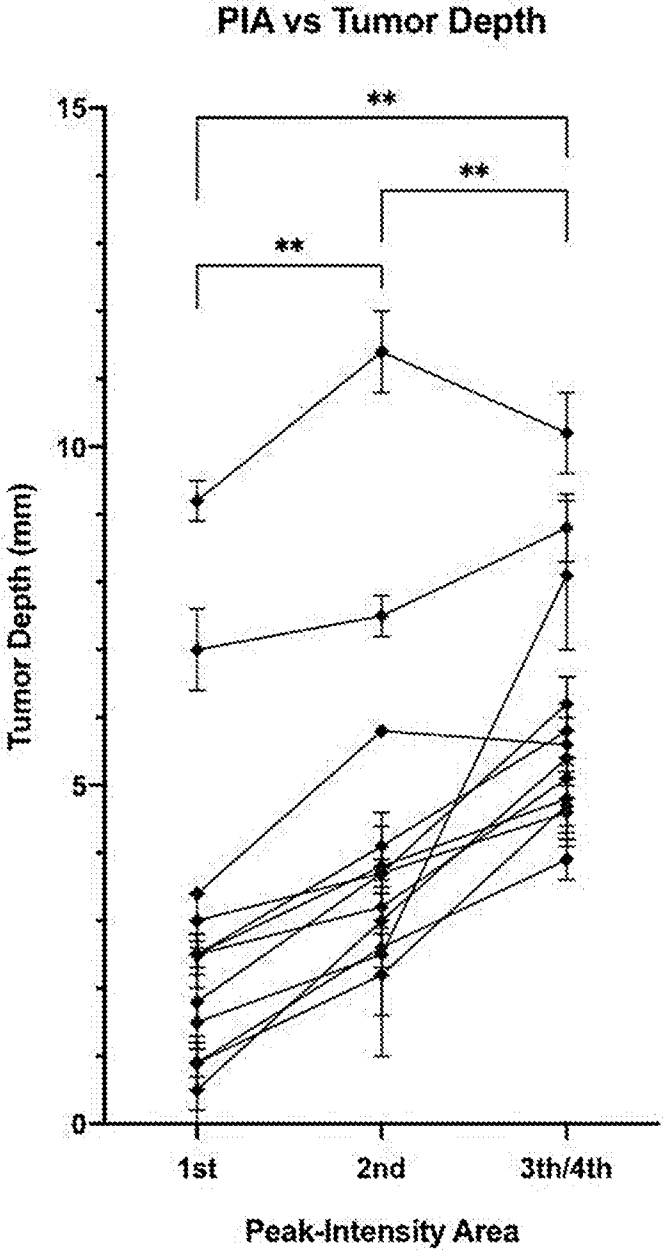


FIG. 3B

6/7

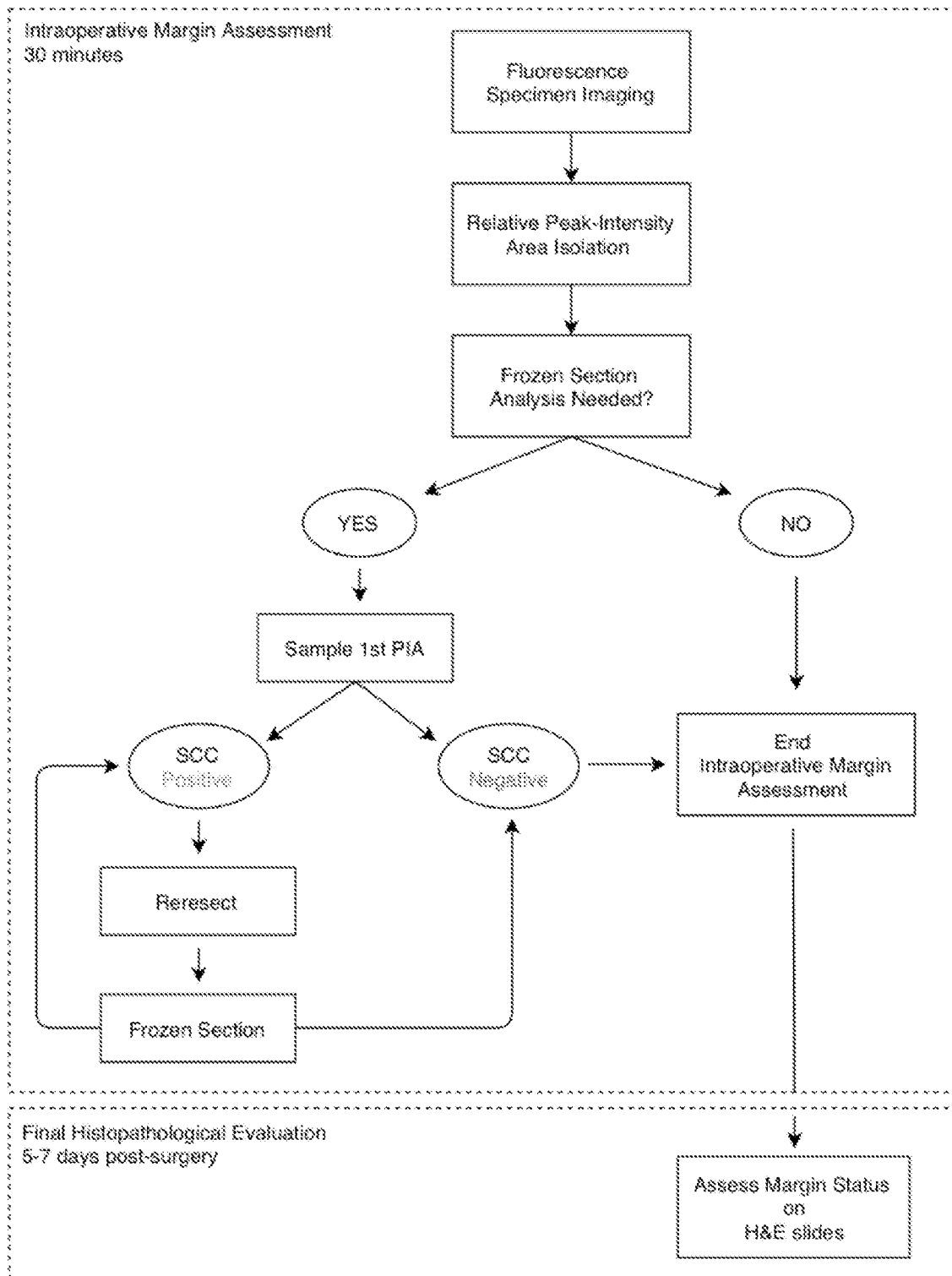


FIG. 4

7/7

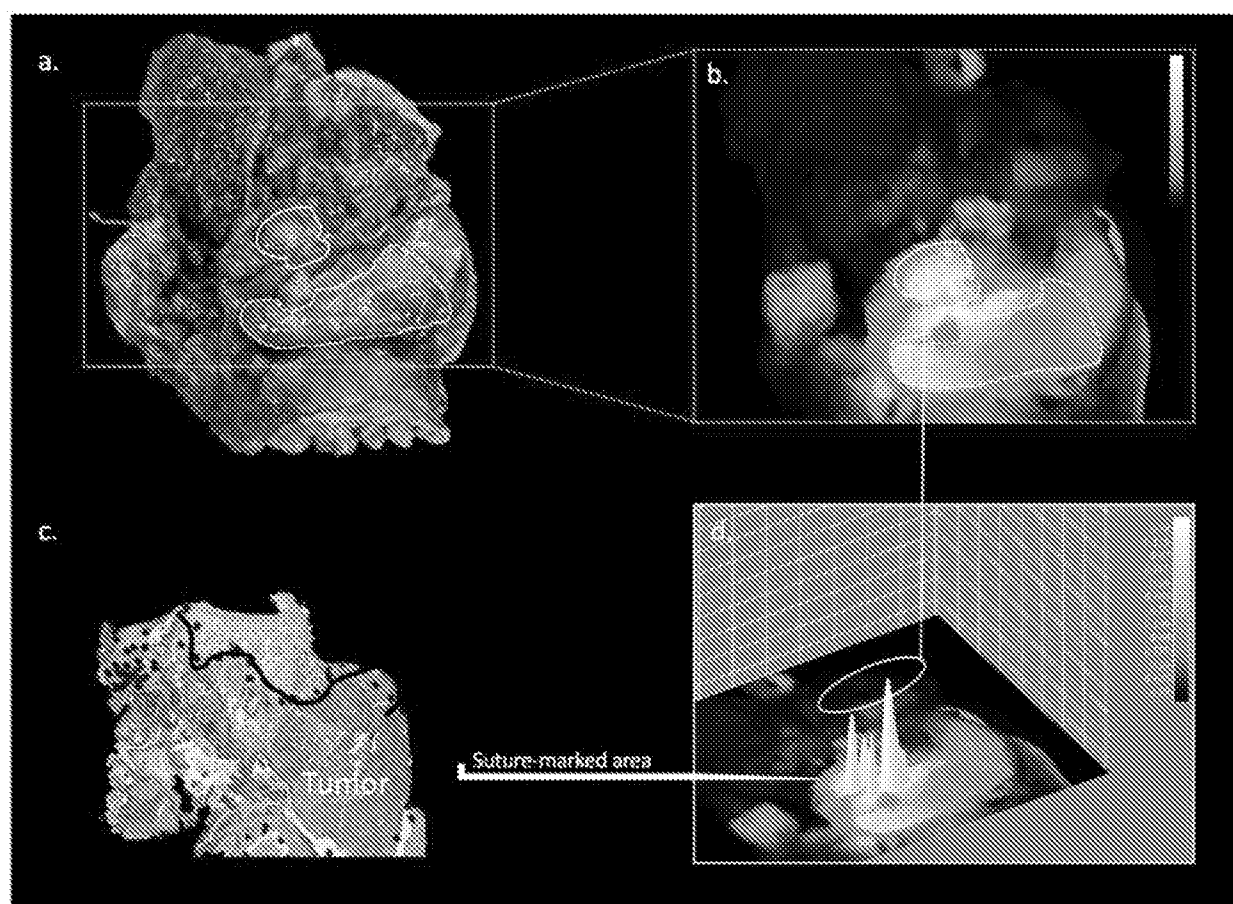


FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US20/15436

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-24, 28, 32-45
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

- Remark on Protest**
- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
 - ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
 - ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US20/15436

A. CLASSIFICATION OF SUBJECT MATTER

IPC - G01N 21/64 (2020.01)

CPC - A61B 5/0071, A61B 5/0075; G01N 21/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2017/0340755 A1 (BRACCO IMAGING S.P.A.) 30 November 2017; paragraphs [0044]-[0045], [0082], [0267]-[0268]; claim 1	1-3, 25-27, 29-31
Y	US 2008/0015448 A1 (KEELY, PJ et al.) 17 January 2008; figure 21; paragraphs [0038], [0041], [0068], [0106], [0109]	1-3, 25-27, 29-31
A	US 2015/0030542 A1 (SINGHAL, S) 29 January 2015; entire document	1-3, 25-27, 29-31
A	US 2013/0066199 A1 (RAMANUJAN, VK) 14 March 2013; entire document	1-3, 25-27, 29-31
A	US 2017/0059487 A1 (LI-COR, INC.) 02 March 2017; entire document	1-3, 25-27, 29-31

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

23 March 2020 (23.03.2020)

Date of mailing of the international search report

23 APR 2020

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

Telephone No. PCT Helpdesk: 571-272-4300