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(57) Abstract: The invention relates to a highly purified population of protoporphyrin IX (PpIX)-positive extracellular vesicles (EVs). The invention also relates to a method of identifying a neoplasm in a subject, the method comprising the steps of a) administering a 5-aminolevulinic acid to the subject; and b) detecting, in a biofluid of the subject, PpIX-positive EVs by imaging flow cytometry, wherein an increase in the amount of PpIX-positive EVs compared to a control sample is taken as an indication that the subject has the neoplasm.



EXTRACELLULAR VESICLES AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims benefit of U.S. Provisional Application No. 62/839,038, filed April 26, 2019, the contents of which are incorporated herein by reference in their entirety.

STATEMENT AS TO FEDERALLY FUNDED RESEARCH

10 This invention was made with government support through a contract from the National Institutes of Health (U01 CA230697, UH3 TR000931, P01 CA069246, K12CA090354, U19 CA179563). The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

15 The invention generally provides a minimally invasive method of detecting tumor specific extracellular vesicles (EVs) and sorting or separating highly purified populations of tumor specific extracellular vesicles for diagnosing malignant neoplasm in a patient.

 Conventionally, tumor tissue biopsy is used for histological and molecular analysis. Brain biopsy is highly limited by sampling frequency and incompletely representative of intra-tumor heterogeneity. Hence, liquid biopsy can provide a minimally invasive strategy for diagnosis and monitoring of brain
20 tumors. EVs are small membrane bound vesicles released by all cells including cancer cells into biofluids. EVs contain tumor specific genetic material which can provide a window into not only the existence of a malignancy in a patient but also insight into the genetic status of the tumor producing such EVs. Detection of tumor specific EVs and their cargo can provide a minimally invasive tool to diagnose and monitor brain tumors. This technology can be central in the era of personalized medicine to diagnose the tumor at a
25 genomic and transcriptomic level, monitor the growth and recurrence, and to assess a patient's response to therapy using a simple blood test.

 However, it is challenging to detect, sort and characterize tumor specific EVs amidst a vast background of EVs derived from all the cells of the body. Accordingly, there is a need for advanced methods to detect, sort and analyze tumor specific EVs with high sensitivity and specificity.

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SUMMARY OF THE INVENTION

 In one aspect, the invention, in general, features a highly purified population of protoporphyrin IX (PpIX)-positive extracellular vesicles (EVs). In some embodiments, the EVs are stored in a container impervious to light. In other embodiments, the EVs are obtained directly from virtually any biofluid (e.g.,
35 the plasma) of a subject (e.g., a human). In still other embodiments, the EVs are produced from a neoplasm or cancer found in a subject.

 In another aspect, the invention features a method of identifying a neoplasm in a subject, the method including the steps of: a) administering a 5-aminolevulinic acid (5-ALA) to the subject; and b) detecting, in a biofluid of the subject, PpIX-positive EVs by imaging flow cytometry, wherein an increase

in the amount of PpIX-positive EVs compared to a control sample is taken as an indication that the subject has the neoplasm.

In some embodiments, the neoplasm, following administration of 5-ALA, releases PpIX-positive EVs. In some embodiments, the biofluid is collected about 2 hours (or about 3 hours or about 4 hours or more) after administration of the 5-ALA. In some embodiments, the biofluid (as a control) is collected before administration of the 5-ALA to the subject. For example, plasma is obtained from a patient prior to being administered 5-ALA hydrochloride. In still other embodiments, the method further includes the step of sorting the PpIX-positive EVs. In another embodiment, the sorting includes laser-based sorting EVs of interest. In some embodiments, one or more fluorescent markers is detected on the sorted EVs.

In some embodiments, the biofluid a liquid biopsy, plasma, blood, cerebrospinal fluid or urine or any other fluid which may be obtained from the subject.

In some embodiments, the neoplasm is a cancer. In yet other embodiments, the neoplasm is an aggressive tumor. Such tumors may be a high grade malignant glioma. In yet other embodiments, the cancer is a low grade glioma, melanoma, a meningioma, non-small cell lung cancer, pancreatic cancer, or bladder cancer.

In some embodiments, the 5-ALA is 5-ALA hydrochloride.

In some embodiments, the subject (e.g., a human) has undergone surgery (e.g., fluorescence guided surgery (FGS)).

In still other embodiments, the PpIX-positive EVs are detected by multidimensional analysis. In some embodiments, the multidimensional analysis includes identifying EVs using an EV-specific marker. In some embodiments, the EV-specific marker is a stain (e.g., carboxyfluorescein diacetate succinimidyl ester stain (CFDA-SE)). In other embodiments, the EV-specific marker is an antibody (e.g., a fluorescent-labeled antibody that specifically binds to CD81+ or CD63+ tetraspanins or a fluorescent-labeled antibody that specifically binds to EGFR, EGFRvIII, or Tenascin C).

In other embodiments, the method distinguishes pseudoprogression from true progressive disease. In some embodiments, the method monitors a subject's cancer. In other embodiments, the method monitors a response of a subject's cancer treatment.

In still other embodiments, the subject is suspected of having a cancer.

In yet other embodiments, the PpIX-positive EVs are fluorescent.

In another aspect, the invention features methods of detecting EVs. Accordingly, the invention features a method of detecting EVs in a subject, the method including the steps of: a) administering a 5-ALA to the subject; and b) detecting, in a biofluid of the subject, PpIX-positive EVs by imaging flow cytometry.

In some embodiments, the method includes detecting PpIX-positive EVs from a neoplasm in the subject, following administration of 5-ALA.

In some embodiments, the biofluid is collected about 2 hours after administration of the 5-ALA.

In still other embodiments, the method further includes sorting the PpIX-positive EVs. In some embodiments, the sorting includes laser-based sorting of PpIX-positive EVs. In some embodiments, one or more fluorescent markers is detected on the sorted PpIX-positive EVs.

In yet other embodiments, the biofluid is a liquid biopsy, plasma, blood, cerebrospinal fluid or urine or any other fluid which may be obtained from the subject.

In some embodiments, the 5-ALA is 5-ALA hydrochloride.

In some embodiments, the subject is a mammal (e.g., a human).

In yet other embodiments, PpIX-positive EVs are detected by multidimensional analysis. In some embodiments, the multidimensional analysis includes identifying EVs using an EV-specific marker. In some embodiments, the EV-specific marker is a stain (e.g., carboxyfluorescein diacetate succinimidyl ester stain (CFDA-SE)) or is an antibody (e.g. a fluorescent-labeled antibody that specifically binds to CD81+ or CD63+ tetraspanins or a fluorescent-labeled antibody that specifically binds to EGFR, EGFRvIII, or Tenascin C).

In another embodiment, the PpIX-positive EVs are fluorescent.

In yet another aspect, the invention features methods of purifying PpIX-positive EVs. For example, the invention features a method of purifying EVs from a subject, the method including the steps of administering a 5-ALA to the subject; and isolating, from a biofluid of the subject, protoporphyrin IX (PpIX)-positive EVs by imaging flow cytometry, thereby purifying EVs from the subject.

In some embodiments, the method further includes detecting the presence or absence of an EV marker.

In some embodiments, the EV marker is a nucleic acid (e.g., an RNA or a DNA). Such markers may be a tumor specific marker.

In other embodiments, the marker may be a protein or a lipid.

In other embodiments, the method involves purifying EVs from a neoplasm that, following administration of 5-ALA to a subject, release PpIX-positive EVs. In some embodiments, the biofluid is collected about 2 hours (or 3 hours or 4 hours or more) after administration of the 5-ALA. In some embodiments, the method further includes sorting the PpIX-positive EVs. Such sorting may include a laser-based sorting of the EVs.

In yet other embodiments, one or more fluorescent markers is detected on the sorted EVs.

In still other embodiments, the biofluid is a liquid biopsy, plasma, blood, cerebrospinal fluid or urine.

In some embodiments, the neoplasm is a cancer. In yet other embodiments, the neoplasm is an aggressive tumor (e.g., a high grade malignant glioma). In other embodiments, the cancer is a low grade glioma, a meningioma, melanoma, non-small cell lung cancer, pancreatic cancer, or bladder cancer.

In some embodiments, the 5-ALA is 5-ALA hydrochloride.

In yet another aspect, the invention features a method of counting EVs from a subject, the method including the steps of administering a 5-ALA to the subject; isolating, from a biofluid of the subject, PpIX-positive EVs by imaging flow cytometry, and counting the EVs in the biofluid.

The term "purified population," relative to neoplastic-derived vesicles, as used herein refers to plurality of neoplastic-derived extracellular vesicles that have undergone one or more processes of selection for the enrichment or isolation of the desired exosome population relative to some or all of some other components with which neoplastic-derived vesicles are normally found in a biofluid of a subject. Alternatively, "purified" can refer to the removal or reduction of residual undesired components found in the biofluid (e.g., cell debris, soluble proteins, etc.).

A "highly purified population" as used herein, refers to a population which is at least 30% pure (e.g., at least 40% pure, at least 50% pure, at least 60% pure, at least 70% pure, at least 80% pure, at

least 90% pure, at least 99% pure, or even 100% pure) relative to the one or more contaminants or undesired components from the source material (e.g., a biofluid such as plasma) as measured according to the methods described herein. Preferably, at least 1% of contaminants or undesired components from a source material (e.g., a biofluid such as plasma) are removed (e.g., at least 2%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 45%, 50%, 55%, 60%, 70%, 80%, 90%, 95%, 96%, 98%, 99%, or even 100%) from the source material.

A highly purified population includes 5 PpIX-positive EVs, 5-10 PpIX-positive EVs, 10-50 PpIX-positive EVs, 50-60 PpIX-positive EVs, 60-70 PpIX-positive EVs, 70-80 PpIX-positive EVs, or even 90-100 PpIX-positive EVs to the exclusion of other components in a biofluid (such as plasma). In some embodiments, a highly purified population of PpIX-positive EVs includes 200, 300, 400 or 500 PpIX-positive EVs, 600 PpIX-positive EVs, 700 PpIX-positive EVs, 800 PpIX-positive EVs, 900 PpIX-positive EVs, 1000-2000 PpIX-positive EVs, 2000-3000 PpIX-positive EVs, 3000-4,000 PpIX-positive EVs, 4000-5,000 PpIX-positive EVs, 5000-6000 PpIX-positive EVs, 6000-7000 PpIX-positive EVs, 7000-8000 PpIX-positive EVs, 8,000-9,000 PpIX-positive EVs, or even 9,000 or greater PpIX-positive EVs. In still other embodiments, the highly purified population of PpIX-positive EVs includes 10,000 PpIX-positive EVs, 20,000 PpIX-positive EVs, 30,000 PpIX-positive EVs, 40,000 PpIX-positive EVs, 50,000 PpIX-positive EVs, 60,000 PpIX-positive EVs, 70,000 PpIX-positive EVs, 80,000 PpIX-positive EVs, 90,000 PpIX-positive EVs, or greater than 100,000 PpIX-positive EVs.

By "impervious to light" refers to a container which is, in general, opaque or inhibits light of a wavelength that promotes photo-initiated degradation of a compound (e.g., PpIX). For example, containers may be metalized (e.g., aluminized) or combined or coated with carbonaceous materials or other dye(s).

The invention provides several advantages. For example, the methods and compositions described herein are useful as a noninvasive means to detect aggressive tumors. A subject with a suspicious mass, for example, orally takes the 5-ALA for a FGS, and about 2-3 hours later plasma samples would be collected and EVs analyzed by imaging flow cytometry. A post-5-ALA plasma sample is then, for example, compared to the pre-5-ALA samples and an increase fold over, for example, a >1 fold change may be taken as an indication that a subject is positive for an aggressive tumor.

Analysis of PpIX-positive EVs also provide a useful means for noninvasively tracking tumor progression. Furthermore, since glioma tumors typically regress as high grade gliomas, dosing patients with 5-ALA provides a fast, noninvasive means to determine tumor recurrences and distinguish for pseudoprogression. The methods are also useful of monitoring the course of a neoplasm in a patient (e.g., during treatment for a cancer). Furthermore, the methods described herein provide a means for detecting high grade gliomas which would lead subsequently to more aggressive tumor treatment.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows an example for determining optimal 5-ALA dose for maximum fluorescence and viability of glioma cells. (a) Confocal images of PpIX fluorescence in Gli36 cells treated with 0.8 mM 5-ALA dose for 24 h. Brightfield image (left), fluorescence image (center), overlay image (right). (b) Dot-plot

showing PpIX fluorescence (mean intensity in relative light units, RLU) of Gli36 cells at different 5-ALA doses measured using confocal microscopy. (c) Histogram showing cell viability (%) of Gli36 cells at different 5-ALA doses measured using Countess II FL Automated Cell Counter. Data are mean 95% confidence interval ($n = 3$ biological replicates). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, unpaired two-tailed Student's t -test.

Fig. 2 shows an example of an ISX analysis for glioma cells dosed with 5-ALA. Visual interrogation and gate setting to demonstrate PpIX fluorescence: (a) ISX scatter plot of Gli36 cells by aspect ratio (x-axis, shape and measure of circularity) vs. area (y-axis, size). Gate R1 (green): Single cells as detected by aspect ratio ~ 1.0 and uniform area. Gate R2 (gray): Cell debris as detected by varying aspect ratio and low area. Gate R3 (pink): Doublet cells as detected by an aspect ratio ~ 0.5 and high area. Gate R4 (blue): Triplet cells as detected by an aspect ratio ~ 1.0 and high area. Ch 01 brightfield images of cells (R1, green), cell debris (R2, gray), doublet cells (R3, pink), triplet cells (R4, blue). (b) The representative histogram of gradient RMS (Ch 01) vs. normalized frequency to identify cells in focus based on the sharpness of the image. Gate R5 (red): Gating of single cells in focus with PpIX fluorescence (Ch 11: λ 660–745 nm). R5-gated brightfield images (Ch 01) and corresponding fluorescence images (Ch 11) demonstrating PpIX fluorescence. (c) Scatter plot of fluorescence intensity (Ch 11) vs. side scatter (Ch 06) showing a homogeneous clustered population of focused, single cells demonstrating PpIX fluorescence (Ch 11). (d) Dot-plot showing PpIX fluorescence (mean intensity in relative light units, RLU) of Gli36 cells at different 5-ALA doses showing maximal mean intensity at 0.8 mM. Data are mean 95% confidence interval ($n = 4$ biological replicates). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, unpaired two-tailed Student's t -test.

Fig. 3 shows an example of Confocal and ISX imaging assessment of optimal 5-ALA dose for maximum fluorescence of glioma cells. (a) Confocal images of PpIX fluorescence in Gli36 cells dosed with Mock, 0.8 mM, 8 mM, 16 mM, 32 mM and 64 mM of 5-ALA for 24 hours. Brightfield images (left), fluorescence images (center), overlay images (right); magnification scale 20 μ m. (b) ISX scatter plots (left) of PpIX fluorescence intensity (Ch 11) vs. side scatter (Ch 06) of Gli36 cells dosed with Mock, 0.8 mM, 8 mM, 16 mM, 32 mM and 64 mM of 5-ALA for 24 hours. Brightfield images (Ch 01, center) and fluorescence images (Ch 11, right) of representative Gli36 single cells.

Fig. 4 shows an example of ISX analysis and characterization of nanoparticles using 100 nm Alexa Fluor 647 (650/665) liposomes. (a) ISX scatter plot of time vs. flow speed; Gate R1 (green): selection of a population where the ISX flow speed is stable, excluding artifactual events from unstable flow speed (gray box) (b) ISX scatter-plot of aspect ratio vs. area of brightfield images (Ch 01); Gate R2 (gray): gated population for fluorescent liposome analysis, excluding doublets, triplets, debris and large aggregates (Gate R4, light blue). Representative images of R4 events visualized on brightfield (Ch 01, BF), side scatter (Ch 06, SSC), and fluorescence spectrum (Ch 11). (c) ISX scatter plot of fluorescence intensity (Ch 11) vs. side scatter (Ch 06) with R3 gate (red) representing 100 nm sized Alexa Fluor 647 liposomes with low side scatter and fluorescence in Ch 11 and are not captured on brightfield images. Gate R6 (royal blue) represents non-fluorescent events, which are not captured on brightfield images and have low side scatter. Gate R7 (gray) represents speed beads, which are captured on brightfield images, have high side scatter, and are non-fluorescent. Gate R8 (purple) represents fluorescent aggregates, captured on brightfield images, and have high side scatter. (d) ISX scatter plot of fluorescence intensity

(Ch 11) vs. side scatter (Ch 06) with PBS buffer (control, left), and Alexa Fluor 647 liposomes of sizes 100 nm, 200 nm, and in combination. The fluorescent events were not detected after detergent lysis.

Fig. 5 shows an example of an ISX calibration with nanoparticle-sized calibration beads and liposomes. (a) ISX scatter plot of fluorescence intensity (Ch 02, λ 480-560 nm) vs. side scatter (Ch 06) with PBS buffer (control, left) and ApogeeMix calibration beads (110 and 500 nm, green, right). (b) ISX scatter plot of fluorescence intensity (Ch 07, λ 420-505 nm) vs. side scatter (Ch 06) with PBS buffer (control, left), and Alexa Fluor 405 liposomes of sizes 100 nm, 200 nm, and in combination. The fluorescent events were not detected after detergent lysis (far right). (c) ISX scatter plot of fluorescence intensity (Ch 03, λ 560-595 nm) vs. side scatter (Ch 06) with PBS buffer (control, left), and Alexa Fluor 555 liposomes of sizes 100 nm, and 200 nm, and in combination. The fluorescent events were not detected after detergent lysis (far right).

Fig. 6 shows an example of INSPIRE data acquisition software settings for EV analysis. ISX software calibration is initiated upon every startup. (a) Sample is loaded, (b) After specifying the counting gate and the collection gate are specified, the acquisition file is mapped to the desired location. (c) Illumination controls: All excitation lasers used, are set to maximum power to ensure maximal sensitivity. Ch 06 is selected for side scatter channel and is set to 1.64 mW power. Ch 01 and 09 are set to brightfield. (d) Magnification controls: 60X magnification is selected. (e) Fluidics controls: High Sensitivity and Low Running Speed is selected (f) Advanced controls: under Flow Speed, the Core diameter is 7 μ m and Bead Percentage of 15% is selected by default depending on the Magnification and the Flow Speed. (g) The Flow Speed was stably maintained at around 44 and the Flow Speed Error CV was maintained under 0.2. The sample was allowed to run for 1-2 minutes prior to acquisition, to get a stable Flow Speed and a low Flow Speed Error CV. The sample was primed when the Flow Speed and the Flow Speed Error CV became unstable. (h) Acquisition: Prior to each acquisition, Unchecking of Remove beads option was performed in the 'Advanced Controls' menu. (i) Image gallery allows real-time viewing of images of events being collected, and the selected detection channels including BF and Side scatter (j,k,l) and the 12 spectral image detection channels, Ch 01-Ch 12. (m) Work area permits real-time graphical gating using scatter plots showing (n) R0 collection gate. The acquire tab is clicked once all the desired instrument controls are specified. For more details, see methods.

Fig. 7 shows an example of an ISX characterization of conditioned media and EVs derived from 5-ALA dosed Gli36 cells and HBMVEC. (a) Control samples: ISX scatter plots of PBS buffer only (left), buffer with 5-ALA (center), and elution buffer following processing with ExoEasy Kit (right). (b) ISX scatter plot of media from 5-ALA dosed (left) and mock dosed (right) Gli36 cells. (c) ISX scatter plot of media from 5-ALA dosed (left) and mock dosed (right) HBMVEC. (d) ISX scatter plot of EVs isolated from 5-ALA dosed (left) and mock dosed (right) Gli36 cells. (e) ISX scatter plot of EVs isolated from 5-ALA dosed (left) and mock dosed (right) HBMVEC.

Fig. 8 shows an example of an ISX characterization of conditioned media and EVs derived from 5-ALA treated Gli36 cells and HBMVEC following 0.1% Triton™ X-100 detergent lysis. (a) ISX scatter plot of media from 5-ALA dosed (left) and mock dosed (right) Gli36 cells after lysis. (b) ISX scatter plot media from 5-ALA dosed (left) and mock dosed (right) HBMVEC after lysis. (c) ISX scatter plot of EVs isolated from 5-ALA dosed (left) and mock dosed (right) Gli36 cells after lysis. (d) ISX scatter plot of EVs isolated from 5-ALA dosed (left) and mock dosed (right) HBMVEC after lysis.

Fig. 9 shows an example of an ISX characterization of Gli36 EVs following white light exposure. ISX scatter plot of EVs from conditioned media of Gli36 cells dosed with 5-ALA for 24 h, isolated using a commercial kit, before (left) and after (right) 20 min of exposure to white light.

Fig. 10 shows an example of an ISX characterization of plasma-derived PpIX-positive EVs from control and glioma-bearing mice dosed with 5-ALA. (a) Overview of the experiment (b) Line plot showing pre-5-ALA and post-5-ALA PpIX-positive EV levels for the control (gray) and glioma (red) mice. The dotted line represents the threshold (t) of background fluorescent signal prior to 5-ALA dosing based on this population. (c) ISX scatter plot of representative control mouse plasma before 5-ALA (left) and 3 h post- 5-ALA (right). (d) ISX scatter plot of representative glioma-bearing mouse plasma before 5-ALA (left) and 3 h post-5-ALA (right).

Fig. 11 shows an ISX characterization of plasma-derived PpIX-positive EVs from remaining control and glioma-bearing mice dosed with 5-ALA. (a) ISX scatter plot of control mice plasma before 5-ALA (left) and 3 hours post-5-ALA (right) (b) ISX scatter plot of glioma-bearing mouse plasma before 5-ALA (left) and 3 hours post-5-ALA dosing (right).

Fig. 12 shows an ISX characterization of plasma-derived PpIX-positive EVs from patients with malignant glioma following 5-ALA administration for FGS with both avidly fluorescent and minimally fluorescent tumors. (a) Avidly fluorescent tumor: The T1-post-gadolinium-contrast axial MRI (left) shows the posterior left temporal tumor which demonstrated avid intraoperative fluorescence under the blue light of the microscope (right); ISX scatter plot of representative patient plasma before 5-ALA (left) and following 5-ALA (right). (b) Minimally fluorescent tumor: The T1-post-gadolinium-contrast axial MRI shows the left occipital tumor (left) which demonstrated minimal intraoperative fluorescence under the blue light of the microscope (right); ISX scatter plot of representative patient plasma before 5-ALA (left) and following 5-ALA (right). (c) Line-plot of pre-5-ALA and post-5-ALA levels for patients with avidly fluorescent tumors (red) and minimally fluorescent tumors (gray). The dotted line represents the threshold (t) of background fluorescent signal prior to 5-ALA dosing based on this population. (d) Bar graph indicating the fold change (post-5-ALA/pre-5-ALA levels) of PpIX-positive EV signal for patients with avidly fluorescent tumors (red) and patients with minimally fluorescent tumors (gray). (e) Pearson correlation of enhancing tumor volumes and fold change of PpIX-fluorescent EV signal from the 4 patients with avidly fluorescent tumors (f) ISX scatter plots of Patient 2 plasma: pre-5-ALA, post-5-ALA (intraoperative, 3.5 h post-dosing), 2 weeks post-FGS, and 6 weeks post-FGS; wks = weeks.

Fig. 13 shows an example of the size and concentrations of EVs from mice and patient plasma-derived samples using NTA. Comparison of EV sizes and concentrations from the plasma samples of (a) Control and (b) Glioma-bearing mice; (c) Avidly fluorescent tumor and (d) Minimally fluorescent tumor patients. Data are seen $\pm$ s.d. (n+3 biological replicates). * $p \leq 0.05$, ** $\leq p 0.01$, *** ≤ 0.001 , unpaired Student's t-test.

Fig. 14 shows an example of an ISX characterization of plasma-derived PpIX EVs from patients with malignant glioma following 5-ALA administration for FGS with both avidly fluorescent and minimally fluorescent tumors. (a) T1-post-contrast axial MRI demonstrating the respective patient's tumor (left) and corresponding ISX scatter plot of patients plasma with avidly fluorescent tumors before 5-ALA (middle) and following 5-ALA treatment (right) (b) T1-post-contrast axial MRI demonstrating the respective

patient's tumor (left) and corresponding ISX scatter plot of patient plasma with minimally fluorescent tumor before 5-ALA (middle) and following 5-ALA treatment (right).

Fig. 15 shows secondary labeling EGFRvIII glioma cell derived EVs.

Fig. 16 shows detection of PpIX positive EVs in cerebrospinal fluid and urine of a glioblastoma patient.

Fig. 17 shows relative fluorescence in 5-ALA dosed cancer cells.

Fig. 18 shows PpIX EVs derived from 5-ALA dosed cancer cells.

Fig. 19 shows high purity 5-ALA EV sorting from glioma cell lines.

Fig. 20 shows high purity 5-ALA EV sorting from glioblastoma patient plasma.

Fig. 21 shows differential expression analysis of post-ALA plasma versus ALA positive EVs.

DETAILED DESCRIPTION OF THE INVENTION

Below we describe methods for detecting PpIX positive EVs in the plasma of subjects suspected of having a malignant neoplasm (such as a glioblastoma). We show that PpIX positive EVs increase significantly after 5-ALA dosing and they remain below background in cases where tumors do not fluoresce.

EXAMPLE 1: Characterization of cell line and plasma EVs following administration of 5-ALA

We have shown that glioma tumors that fluoresce upon oral administration of 5-ALA also release fluorescent extracellular vesicles (EVs) in the systemic circulation, while glioma patients whose tumor does not fluoresce have below-background levels of PpIX positive EVs. Furthermore, fluorescent EVs are also undetectable immediately following tumor resection suggesting a direct correlation with the tumor presence as well as level of fluorescence.

We have accordingly characterized EVs isolated from glioma cell lines treated with 5-ALA for 24 hours. We also have evaluated plasma-derived EVs from glioma patients following preoperative oral administration of 5-ALA. We used a fluorescence-based analysis system known as the Amnis ImageStream IsX MKII imaging flow cytometer to measure fluorescent signals from individual nanoparticles with the added value of being able to individually visualize particles being measured.

The Amnis ImageStream IsX MKII has the ability to analyse fluorescent nanoparticles, including EVs. The advantage of this instrument lies in its ability to measure scatter and multiple fluorescent markers in a high throughput manner and validate it using a corresponding image. This technology, with charge couple device (CCD) cameras, allows for a larger dynamic range, lower noise and larger quantum efficiency along with time delay integration to read out pixel intensities and coupled with slower flow rates gives maximal sensitivity. This allows for characterization of tumor specific fluorescent PpIX-positive EVs.

We first determined the optimal concentration of 5-ALA using glioma cells Gli36. We used a range of concentrations and based on two criteria: (i) brightest fluorescence signal on ImageStream and (ii) lowest cell death due to 5-ALA toxicity we determined 0.8 mM as the optimal concentration for all our downstream experiments. We reasoned that this provides the optimal ability to determine fluorescent EVs while reducing the risk of cell death and therefore studying released apoptotic bodies with the

confounding factor of autofluorescence. Control experiments in parallel included dosing normal host cells with 5-ALA and testing their presence for PpIX positive events.

We also then compared the EVs released from glioma cells and healthy human brain microvascular endothelial cells (HBMVEC) treated with 5-ALA and determined that there is a 21-fold higher PpIX positive EVs from glioma cells treated with 5-ALA compared to mock treated glioma cells, but 5-ALA treated HBMVEC cells only released 6-fold higher events compared to mock treated HBMVEC cells.

We have performed a direct analysis of conditioned media derived from 5-ALA treated glioma cells and HBMVEC, and we detected a 212-fold higher PpIX positive EVs from glioma cells treated with 5-ALA compared to mock treated glioma cells, while conditioned media derived from HBMVEC had only 6-fold higher PpIX positive EVs compared to mock treated HBMVEC.

We similarly compared the direct analysis of conditioned media to that of EVs purified by a commercial kit and determined that the extra exposure to light of EVs during the isolation using a commercial kit leads to a significant loss of PpIX positive EV signal. To confirm our findings, we exposed 5-ALA treated glioma cells derived EVs to white light for 20 minutes and compared the number of fluorescent events before and after exposure to light and determined an 8.4-fold reduction in PpIX positive EVs detected.

Finally, a comparison of the plasma samples from glioma patients collected upon oral administration of 5-ALA revealed that we can reliably detect fluorescent EVs in the plasma of these patients when the primary tumor fluoresces, while these events were undetectable in the cases where the primary tumor did not fluoresce. Furthermore, these events were undetectable upon tumor resection. Indeed, a comparison of the plasma samples collected from malignant glioma patients after administration of 5-ALA revealed reliable detection of PpIX EVs in the plasma of these patients when the primary tumor fluoresces intraoperatively, with these events were undetectable in the cases where the primary tumor did not fluoresce.

EXAMPLE 2: Characterization of protoporphyrin-IX-positive extracellular vesicles following 5-ALA use in patients with malignant glioma

MATERIALS AND METHODS

Cell line

The human Gli36 glioma cell line (RRID:CVCL_RL88) was generated at Massachusetts General Hospital with approved IRB protocol and cultured in high glucose Dulbecco's modified essential medium (DMEM; Gibco, Invitrogen Cell culture, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Life Technologies Corporation, Carlsbad, CA) and 1% Penicillin/Streptomycin (Penicillin Streptomycin Solution; Life Technologies Corporation, Carlsbad, CA). HBMVEC were kindly provided by Xandra O. Breakefield and cultured using endothelial basal medium (EGM-2 MV Microvascular Endothelial Cell Growth Medium-2 BulletKit, Lonza, Allendale, NJ). All *in vitro* experiments were performed with a cell confluency of 50–70% to minimize cell death. All the cell lines are periodically verified for mycoplasma

contamination using commercial mycoplasma PCR (PCR Mycoplasma Detection Kit, Applied Biological Materials Incorporated, Richmond, British Columbia).

Dosing with 5-ALA

5 5-ALA (Sigma-Aldrich; Saint Louis, MO) was dissolved in 1 ml sterile filtered phosphate buffered saline (PBS 1x; Thermo Fisher Scientific, Baltics UAB, Vilnius, Lithuania), aliquoted and stored at a concentration of 2.9 M at -20°C . For dose determination experiments, Gli36 cells were plated in a 6-well plate (Corning Costar Flat Bottom Cell Culture plates; Corning Incorporated, Corning, NY) at a seeding density of 250,000 cells per well on day 0. Cells were allowed to grow for 24 h in the incubator at 37°C .
10 On day 1, each well was washed with 1 ml PBS to remove floating cells and 1.8 ml of fresh DMEM/well was added. Cells were dosed with 200 μL of 5-ALA solutions from secondary stock concentrations to obtain final concentrations of 64 mM, 32 mM, 16 mM, 8.0 mM, and 0.8 mM in the first 5 wells. The last well was mock dosed with 200 μL filtered PBS. The final volume of each well was kept constant at 2.0 ml. Fluorescence intensity and viability were assessed 24 h after dosing. For the remaining experiments,
15 Gli36 cells and HMBVEC were plated in P15 plates (Nunc Dish 150 mm, Thermo Fisher Scientific, Waltham, MA) at a seeding density of 5 million cells/plate. On day 1, each plate was washed with PBS, dosed with 0.8 mM concentration of 5-ALA or mock (filtered PBS) after replacing DMEM with 15% EV depleted Fetal Bovine Serum (FBS; Life Technologies Corporation, Carlsbad, CA). Conditioned media was collected 24 h after dosing. All experiments were performed in a dim light with the plates covered
20 with aluminum foil at all times to avoid bleaching of PpIX fluorescence.

Cell viability

The cells were trypsinized (0.25% Trypsin-EDTA; Life Technologies Corporation, Carlsbad, CA) and the viability was assessed using Countess II FL Automated Cell Counter (Thermo Fisher Scientific,
25 Waltham, MA).

Confocal microscopy

Cells were plated in glass bottom 6-well plate (VRW, Radnor Headquarters, Radnor, PA) and dosed as described above (Dosing with 5-ALA). Twenty-four hours after dosing, confocal images were
30 acquired with Nikon A1R Confocal Microscope using 60X objective. Cells were excited at 405 nm and collected using 700/75 long pass filter. Fluorescence measurements from confocal images were analyzed using Fiji software (Schindelin et al., Nat Methods 9:676-82 (2012)). Confocal imaging was performed quickly and with minimal white light exposure to avoid bleaching of PpIX fluorescence.

EV isolation

EVs were isolated from the conditioned media using ExoEasy kit (Enderle et al., PLoS One 10:e0136133 (2015)) (ExoEasy Maxi Kit, Qiagen, Hilden, Germany). EVs were isolated from 20 ml of conditioned media. Briefly, media is allowed to reach room temperature and is mixed with a binding buffer in 1:1 ratio. The buffer-media is then loaded into affinity columns, washed and EVs are eluted using the
40 ExoEasy EV elution buffer. EVs are then suspended in elution buffer and used for ISX analysis.

EGFR conditional transgenic mice

All mouse procedures were performed in accordance with Beth Israel Deaconess Medical Center's recommendations for the care and use of animals, and they were maintained and handled under protocols approved by the Institutional Animal Care and Use Committee. Cre/Lox-mediated conditional expression of the human EGF receptor vIII was achieved by stereotactic intracranial injections of an adenovirus expressing Cre recombinase (Gene Transfer Vector Core, University of Iowa, Iowa City, IA) as described in Zhu et al., (Proc Natl Acad Sci USA. 106:2412-16 (2009)).

5-ALA dosing in mice

Mice were monitored by weight, body conditioning, and behavioral symptoms for tumor burden. Pre-5-ALA blood samples were collected by submandibular puncture, blood was collected into a tube containing 3.8% Sodium Citrate. 20 mg/kg 5-ALA was injected intraperitoneally and mice were kept in the dark for three hours prior to post 5-ALA bleeds. Mice were deeply anesthetized with Ketamine/Xylazine (ketamine 125 mg/kg, xylazine 12.5 mg/kg) and blood was collected by cardiac puncture. Plasma was then separated from the hematocrit, aliquoted and stored at -80°C until ISX analysis. Demographics of the mice are reported in Table 1.

Table 1 Mice demographics.

Mice	Age (days)	Sex	Diagnosis	Plasma collection post 5-ALA dosing (hours)	Tumor Fluorescence	Fold Change Post/Pre 5-ALA
CI	100	M	Control	3	N/A	0.6
C2	100	M	Control	3	N/A	1
C3	100	M	Control	3	N/A	1.4
T1	76	M	Glioblastoma	3	Fluorescent	5.4
T2	87	M	Glioblastoma	3	Fluorescent	1.7

Ethics approval and consent to participate

All the participating patients signed the informed consent form, and the study was approved by the Internal Review Board (IRB) ethical committee of the Massachusetts General Hospital (2017P001581).

Patient characteristics

The study population ($n = 6$) consisted of patients 18 years or older with a newly identified T1-weighted gadolinium-enhancing lesion concerning for malignant glioma who underwent FGS using 5-ALA at the Massachusetts General Hospital. Pathology confirmed GBM diagnosis for all patients ($n = 6$). Samples from four patients were collected as part of a clinical trial (ClinicalTrials.gov Identifier: NCT02632370; 5-Aminolevulinic Acid (5-ALA) to Enhance Visualization of Malignant Tumor) to evaluate the safety and efficacy of GLIOLAN (Trademark name for 5-ALA in the trial) as a surgical adjunct to malignant glioma tumor resection. Two patients were given GLEOLAN (Trademark name for FDA approved ALA: ALA HCl, NX Development Corp., Lexington, KY) independent of the trial. Patients with contrast enhancing lesions were given an oral dose of 20 mg/kg 5-ALA, 3–5 h prior to FGS for glioma surgery as a part of standard of care. Patient demographics, MRI tumor volume, pathological diagnosis

and the intensity of intraoperative fluorescence (as assessed by the operating neurosurgeon) is reported in Table 2. We characterized all avidly fluorescent tumors as 'avidly fluorescent' and the tumors with either no fluorescence or background level of fluorescence as seen in normal cerebral cortex as 'minimally fluorescent' (Fig. 12a). All protocols were approved by an institutional review board.

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Table 2 Patient demographics.

Patient	Age	Sex	Diagnosis	Tumor Volume (cm ³)	Plasma collection post 5-ALA dosing (hours)	Intraoperative Fluorescence	Fold Change Post/Pre 5-ALA
P1	55	M	Glioblastoma	37.7	3	Avidly fluorescent	3.1
P2	72	F	Glioblastoma	28.8	3.5	Avidly fluorescent	2.3
P3	63	M	Glioblastoma	9.23	3	Avidly fluorescent	2.0
P4	73	M	Glioblastoma	2.53	3	Avidly fluorescent	1.6
P5	42	F	Glioblastoma	18.1	5	Minimally fluorescent	0.6
P6	51	M	Glioblastoma	21.2	2	Minimally fluorescent	0.9

Patient plasma processing

Pre-5-ALA and the subsequent post 5-ALA plasma samples were collected prior to and 3 h after 5-ALA administration. Whole blood samples were collected using K2 EDTA tubes with an inert gel barrier (BD Vacutainer® blood collection tubes) and centrifuged at 1100 x g for 10 min to separate the hematocrit from the plasma. Samples were then filtered using 0.8 µm filter and aliquoted into amber-colored cryovials (Polypropylene Microtubes, Analytical Sales and Services Inc., Flanders, NJ) to reduce any fluorescence bleaching caused by direct light exposure. All plasma samples were processed within 2 h of collection and stored at -80°C until ISX analysis. Precautions were taken to minimize the exposure to light during sample collection and processing. For 4 of the 6 patients, blood samples were collected 3–4 h after the oral administration of 5-ALA. While for patients P5 and P6, the post 5-ALA samples were collected 2 h and 5 h after 5-ALA dosing, respectively. For patient P3, pre-5-ALA blood draw were not obtained, hence we obtained 2-week postoperative plasma sample that we used as baseline.

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ImageStream[®] imaging flow cytometer

We used an ImageStream[®] (ISX) MkII Imaging flow cytometer (IFC, Amnis Corporation, Seattle, WA, USA) equipped with two charged couple device (CCD) cameras and 12 spectral image detection channels (Ch 01- Ch 12): Ch 01 420–480 nm, Ch 02 480–560 nm, Ch 03 560–595 nm, Ch 04 595–660 nm, Ch 05 660–740 nm, Ch 06 740–800 nm, Ch 07 420–505 nm, Ch 08 505–570 nm, Ch 09 570–595 nm, Ch 10 595–660 nm, Ch 11 660–740 nm, Ch 12 740–800 nm; and 5 solid state excitation lasers: 405 nm laser, 488 nm laser, 561 nm laser, 592 nm laser, and 658 nm laser. Two channels (Ch 01 and Ch 09) were set to brightfield, permitting spatial coordination between cameras and Ch 06 was set to side scatter. INSPIRE version 200.1.620.0 instrument software was used for instrument setup, calibration and data acquisition. Upon each startup, the instrument calibration tool ASSIST[®] was performed to optimize

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performance and consistency. The advanced fluidic control of ISX, coupled with the presence of continuously running speed beads enable cell/particle enumeration using the “objects per ml” feature within the IDEAS® data analysis software. The samples were loaded and the events were collected as RIF files. 1,000 event single color compensation controls were collected (without BF or SSC) and later merged to create a compensation matrix for analysis. Once the RIF files were merged and compensated, a gating strategy was used for consistency. The ISX requires small volumes (25- 100 µL), in most cases we opted for 100 µL sample. Cells were centrifuged at 300 x g for 5 mins and resuspended in 100 µL of PBS for ISX analysis. For ISX analysis of EV samples, 30–100 µL conditioned media, ExoEasy isolated EVs, mice plasma and patient plasma was used. All samples were collected in eppendorf tubes covered with aluminum foil to minimize exposure to light. Detergent lysis of all samples was performed using 0.1% Triton™ X-100 (Thermo Fisher Scientific, Waltham, MA) for 15 min at room temperature.

Optimization and calibration for EV analysis

ApogeeMix (Apogee Flow Systems, UK) and fluorescent-labeled liposomes (Avanti Lipids, USA) were used to optimize and calibrate ISX for EV analysis. ApogeeMix contained green beads 110 nm and 500 nm in size. Six preparations of liposomes were custom manufactured. Alexa Fluor 405, Alexa Fluor 555 and Alexa Fluor 647 liposomes, each of 100 and 200 nm sizes. The ApogeeMix were analyzed undiluted, while the liposomes were analyzed after 1 in 500 dilution in filtered PBS.

ISX analysis of cells

Cells were analyzed with fluidics set at low speed, sensitivity set to high, magnification at 40X and core size 10 µm. The 405 nm laser was set at 120 mW and side scatter SSC/785 nm laser at 1.00 mW. 100 µL samples were loaded and multiple 10,000 event files were acquired. RIF files were generated, merged, compensated and a gating strategy (Fig. 2) was used for consistency.

Gating strategy for cells

Gate R1 (green): Aspect ratio and area of brightfield images (Ch 01) were used to gate for single cell events. Aspect ratio is the ratio of minor axis to major axis and is representative of shape and circularity of an event. Area is the measure of size. Single cells as detected by aspect ratio ~ 1.0 and uniform area. Gate R5 (red): Gradient RMS of brightfield images (Ch 01) was used to further analyze the R1 population to identify single cells in focus. Gradient RMS represents the sharpness quality of the image. Finally, R5 population was analyzed for fluorescence in Ch 11: λ 660–745 nm. The gated events were visually interrogated and verified (Fig. 2). Mean fluorescence intensity in Ch 11 in relative light units (RLU) was used for comparisons. For further details see Samsel et al., Cytometry B Clin Cytom. 84:379-89 (2013) and Basiji et al., Clin Lab Med. 27:653-70 (2007).

EV analysis on ISX

Analysis was performed with fluidics set at low speed, sensitivity set to high, magnification at 60X, core size 7 µm, and the “Hide Beads” option unchecked prior to every acquisition in order to visualize speed beads in analyses. All parameters are stored in acquisition template except the latter, which requires unchecking prior to each acquisition. The lasers were run at maximal power to ensure maximal

sensitivity: 405 nm (120 mW), 488 nm (200 mW), 561 nm (200 mW), and 642 nm (150 mW). To avoid the risk of coincident particle detection, EV samples were not run at concentrations greater than 1010 objects/ml (Lannigan et al., Methods. 112:55-67 (2017)). The Flow Speed was stably maintained at around 44 and the Flow Speed Error CV was maintained under 0.2, and priming was done when the Flow Speed became unstable. Samples were allowed to run for 1–2 min prior to acquisition, to reach a stable Flow Speed and a low Flow Speed Error CV (Fig. 6).

Collection strategy for EV analysis

R0 Collection Gate (white): Scatter plot of fluorescence intensity (Ch 11) vs. side scatter (Ch 06) was generated and R0 Collection Gate was Set to exclude the majority speed bead events. A minimum of 40,000 R0 Collection Gate events were collected. The runs were kept under 20 mins, even if 40,000 R0 events were not acquired (Fig. 6). RIF files were generated, merged, compensated and a gating strategy (Fig. 4) was used for consistency.

Gating strategy for EVs

Alexa Fluor 647 100 nm-sized liposomes were nanoparticles used to optimize for EV gating strategy. Gate R1 (green): Population with a stable ISX Flow Speed was selected. Unstable portions of the run were excluded to minimize the bias caused by the variations in instruments fluidics as well so to remove artifactual events. Gate R2 (grey) was created to exclude doublets, triplets, debris and large aggregates. Aspect ratio and area of bright field images was used to gate out the population with large area and variable aspect ratio. Speed beads were included in the Gate R2 to have a reference population. Finally, Gate R3 (red) was created to encompass low side scattering events, that did not produce a brightfield image but demonstrated fluorescence in Ch 11: λ 660–745 nm. Inspire masking was used to detect fluorescent events in Ch 11 and spot counting feature was used to exclude swarming. The gated events were visually interrogated and verified. The positive events were expressed as objects/ml (Fig. 4). See Mastoridis et al., Front Immunol. 9:1583 (2018), Ricklefs et al., J Extracell Vesicles. 8:1588555 (2019), Lannigan et al., Methods. 112:55-67 (2017), and Galbo et al., Oncotarget. 8:114722-35 (2017).

Nanoparticle tracking analysis (NTA)

Concentrations and particle sizes of EVs in conditioned media, ExoEasy isolated EVs, plasma of mice and humans were measured by NanoSight NTA (LM10, Malvern Inst. Ltd., UK). Samples were prepared in dilutions of 1:10 for media, 1:100 for EVs and 1:3000 for plasma of mice and humans. Dilutions were made with filtered PBS and measurements recorded in triplicates over 30 s, with manual monitoring of temperature and camera level set to 14. Analysis was performed using NTA v3.1 software, with detection threshold set to 7. All NTA EV concentration data was expressed in EVs / ml and EV size data in mode values.

Statistical analysis

Data are expressed as mean 95% confidence interval. Statistical analysis was performed using Microsoft Office Excel 2011 or Graph Pad Prism 8 software. The unpaired two-tailed Student's *t*-test was

used for comparisons between groups. * $p \leq 0.005$, ** $p \leq 0.01$, *** $p \leq 0.001$ was considered statistically significant. Comparisons were done between the samples of each patient before and after 5-ALA dosing. The same was done for the mice. Inter-patient or inter-mice comparisons were not performed due to heterogeneity in the pre-dosing background levels. The increase in the PpIX-positive EV counts was expressed as fold change due to heterogeneity in the background. The same unit of measurement was used for both pre and post-dosing patient/mice samples.

RESULTS

Optimization of 5-ALA concentration in glioma cells for maximum viability and fluorescence intensity

Dosing of 5-ALA for cells in culture was established via *in vitro* testing of the human glioma Gli36 cell line. Glioma cells were seeded in a 6-well plate (250,000 cells/well) and dosed with increasing concentrations of 5-ALA (mock, 0.8 mM, 8 mM, 16 mM, 32 mM and 64 mM). Twenty-four hours after dosage of cells, we assessed mean fluorescence intensity in relative light units (RLU) using confocal microscopy (Fig. 1a and Fig. 3a). We observed a decrease in fluorescence intensity with increasing concentrations of 5-ALA, and the maximum mean fluorescence intensity was observed with the 0.8 mM dose ($p = 0.0002$; Fig. 1b). The maximum cell viability was also noted with 0.8 mM (after the mock treated cells), and the viability showed an inverse dose-dependent correlation with increasing concentrations ($r^2 = 0.90$; Fig. 1c). Confocal microscopy also allowed us to visualize the increase in dead cells as 5-ALA dosage increased (Fig. 3a). The decreasing fluorescence intensity with increasing concentrations of 5-ALA, is possibly a consequence of increasing cell death with the increasing concentration of the compound.

We next used ISX to determine the brightest possible PpIX cell fluorescence and the lowest rate of cell death to minimize the possibility of apoptotic particles released in the media. We used gating strategy to capture a homogeneous population of single cells with fluorescence in Ch 11 ($\lambda 660$ -745 nm; Fig. 2a–c). From this cell population, ISX analysis confirmed maximum mean fluorescence intensity from cells dosed with the 0.8 mM concentration of 5-ALA (Fig. 2d) and a decreasing mean fluorescence intensity with increasing 5-ALA doses ($p = 0.001$; Fig. 3b). After performing these experiments in three biological replicates, the 0.8 mM dose of 5-ALA consistently resulted in the highest fluorescence intensity and the lowest cell death rate (after mock). Findings were congruent between ISX and confocal microscopy, therefore we used the 0.8 mM concentration for all our subsequent *in vitro* experiments (Fig. 3).

Optimization of ISX for EV analysis using nanoparticle-sized calibration beads and fluorophore-labeled liposomes

ISX is a high throughput and sensitive cytometer that allows interrogation of single fluorescence-positive nanoparticles with simultaneous visualization within brightfield, side scatter, and fluorescent channels. We used custom-made 100 and 200 nm sized Alexa Fluor 647 liposomes (excitation max $\lambda 650$ nm, emission max $\lambda 665$ nm) to optimize the gating strategy for single fluorescent EV detection. These liposomes represented nanoparticles of similar size and emission range of what would be expected with PpIX-labeled EVs, and therefore served as a positive control. Liposomes were excited with a 658 nm

laser (100 mW) and analyzed. We first selected the region of the run where the fluids were stable (Fig. 4a) and then gated out large aggregates with high area and low aspect ratio (Fig. 4b). Finally, we gated in our population of interest as low side-scattering fluorescent events in Ch 11 which were not captured in brightfield (Fig. 4c). Simultaneously, we analyzed all other channels and found no overlapping events with those observed in Ch 11, confirming the specificity of our Alexa Fluor 647 liposomes to Ch 11.

We based our fluorescence thresholds by first analyzing PBS buffer, our negative control (Fig. 4d, Fig. 5). ISX was able to detect 100 and 200 nm fluorescent liposomes, both separately and in combination, yet we were unable to clearly differentiate between 100 nm and 200 nm subpopulations, likely due to the similarity in size and limits in sensitivity of the instrument (Fig. 4d, Fig. 5). On the contrary, we were able to clearly visualize the subpopulations of the ApogeeMix calibration beads mix of 110 nm and 500 nm (Fig. 5a). In order to confirm the specificity of nanoparticle detection of different fluorescence spectra, we also analyzed 100 nm and 200 nm-sized Alexa Fluor 405 and Alexa Fluor 555 liposomes (Fig. 5b,c). Finally, we confirmed that we were unable to detect fluorescent events following detergent lysis of liposomes, which supports the notion that the fluorescent events that we had previously detected were indeed true liposomes (Fig. 4b, Fig. 5b,c). For all ISX runs of nanoparticles, we used the instrument settings as previously reported in the literature (Fig. 6) (Mastoridis et al., Front Immunol. 9:1583 (2018), Ricklefs et al., J Extracell Vesicles. 8:1588555 (2019), Lannigan et al., Methods. 112:55-67 (2017)).

Glioma cells, but not HBMVEC, dosed with 5-ALA release PpIX-positive EVs that can be detected using ISX

Next, we determined the specificity of PpIX-positive EVs released from 5-ALA dosed cancer cells (Gli36) cells as compared to non-cancer cells (HBMVEC). We first analyzed negative controls of PBS buffer alone, PBS buffer mixed with 5-ALA, as well as Elution Buffer from a commercial EV-isolation kit in order to establish background fluorescent signal (Fig. 7a). Next, we dosed both the glioma cells and HBMVEC with 0.8 mM of 5-ALA and PBS as control. Conditioned media was collected 24 h later, centrifuged at low speed (300 x g for 10 min) to remove debris, and filtered through a 0.8 µm filter to remove large particles. We then analyzed the purified media on ISX using our previously established gating strategy (see above). We found a 247-fold increase of PpIX-positive EVs in glioma cells dosed with 5-ALA compared to mock dosing (Fig. 7b), while HBMVEC dosed with 5-ALA yielded a 6-fold increase in PpIX-positive EVs compared to mock dosing (Fig. 7c). These findings suggest that the increased PpIX accumulation known to occur preferentially in glioma cells lead to a significantly higher release of PpIX-containing EVs, a phenomenon not as pronounced in healthy cells. To confirm that the fluorescent events were indeed extracellular vesicles, we performed a lysis step and found a significantly reduced signal (Fig. 8a,b).

To further validate that we were indeed working with EVs we used a commercial kit to isolate EVs from 5-ALA dosed cells (Enderle et al., PLoS One. 10:e0136133 (2015)). Twenty ml of media was used as input into the ExoEasy kit, which can isolate intact EVs by membrane affinity (Enderle et al., PLoS One. 10:e0136133 (2015)), and eluted them in 400 µl of elution buffer. We then analyzed the EVs on ISX and found a 20-fold increase of PpIX-positive EVs released from glioma cells dosed with 5-ALA

compared to mock dosing (Fig. 7d), while HBMVEC dosed with 5-ALA showed a 7-fold increase in PpIX-positive EVs compared to mock dosing (Fig. 7e). We again performed a lysis step and found a significantly reduced signal (Fig. 8c,d).

5 Exposure to white light leads to bleaching of PpIX-positive EVs and loss of fluorescent ISX signal

We then investigated why there was a lower yield of PpIX-positive EVs isolated using the kit as compared to direct analysis of the conditioned media. Bleaching of PpIX fluorescence from white light exposure has been well established for tumor tissue analysis (Stummer et al., Neurosurgery. 42:5118-25 (1998)). With this in mind, our main hypothesis was that the decrease in signal was due to the increase in
10 white light exposure that occurred with kit processing. To test this hypothesis, we exposed EVs from glioma cells dosed with 5-ALA to white light for 20 min prior to ISX analysis. We observed an 8-fold decrease in PpIX-positive EVs as compared to EVs isolated without prolonged exposure to white light (Fig. 9). Given the significant effect of white light on the loss of ISX signal intensity from PpIX-positive EVs, for our *in vivo* studies we chose to analyze EVs directly from unprocessed plasma.

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PpIX-positive EVs can be detected in plasma of glioma-bearing mice dosed with 5-ALA but not in control mice

We next sought to explore the specificity of PpIX-positive EV signal from the plasma of glioma-bearing mice as compared to control mice after dosage with 5-ALA (Table 2, Fig. 10a). We first sampled
20 baseline plasma (pre-5-ALA) from 3 control mice and 2 glioma mice. Three hours following weight-based 5-ALA dosing, the plasma of all mice was sampled again (post-5-ALA). The mice were kept in the dark following drug dosage, as were all plasma samples. Given the findings of diminished PpIX signal with *in vitro* use of EV-isolation kit, we decided to analyze pure plasma. ISX analysis demonstrated a significant increase ($p = 0.004$) in PpIX-positive EVs in the glioma-bearing mice following 5-ALA administration as
25 compared to pre-5-ALA levels while we did not observe a similar increase in the control mice (Fig. 10b–d, Fig. 11). The brains of the tumor-bearing mice were obtained immediately following the terminal plasma collection and were confirmed to contain avidly fluorescent tumor under blue light-filtered microscope. These findings suggest that, despite systemic uptake of 5-ALA, a rise in PpIX EVs occur only when a glioma is present, and therefore, the EVs are specific to the glioma itself. As determined by NTA, the size
30 and concentrations of EVs from the mice was similar between pre- and post-5-ALA dosing (Fig. 13a,b).

Patients with avidly fluorescent malignant gliomas observed during FGS release PpIX-positive EVs into circulating plasma

We interrogated the plasma of 6 patients who underwent FGS to determine the PpIX-positive EV
35 signal before and after dosage with 5-ALA. All patients enrolled in this study underwent surgical management at our institution. We observed tumors with avid fluorescence in 4 of 6 patients, whereas the remaining 2 patients had minimal fluorescence, all under blue light microscopy (Fig. 12a,b, Table 3).

ISX analysis of pure plasma in patients with avidly fluorescent tumors demonstrated a highly significant increase ($p = 0.00009$) in PpIX-positive EVs in the post-5-ALA samples as compared to pre-5-
40 ALA samples. We did not observe a similar rise from the intraoperative samples obtained from the patients whose tumors had minimal fluorescence (Fig. 12c). We also established that a 1.0-fold change or

greater increase of post-5-ALA EVs over baseline levels represented a true positive signal (Fig. 12d). Furthermore, this fold change in signal significantly and positively correlated with each patient's enhancing tumor volume ($r^2 = 0.888$, Fig. 12e).

We were also able to track Patient 2 longitudinally and observed a 2.3-fold increase in PpIX-positive EVs in post-5-ALA plasma compared to pre-5-ALA levels, followed by a sustained return to baseline levels at 2 weeks and 6 weeks post-operatively (Fig. 12f). The lack of avid fluorescence in 2 of our patient tumors was thought to be due to poor timing, as the tumor resection and plasma collection for these patients occurred at the shortest and longest intervals following 5-ALA administration. The fluorescence observed by the operating neurosurgeon in these 2 patients was consistent with either no fluorescence or a background fluorescence seen in normal cerebral cortex, which is very minimal compared to the usual avid fluorescence seen in the other 4 patients. As determined by NTA, the size and concentrations of EVs from the mice was similar between pre- and post-5-ALA dosing (Fig. 13c,d).

This study demonstrates that malignant gliomas with avid intraoperative PpIX fluorescence release PpIX-positive EVs that can be detected in circulating plasma using IFC. The ability to perform a minimally-invasive liquid biopsy for patients with malignant glioma, either through plasma or other biofluids, expands our ability to diagnose and track treatment response for this challenging and lethal disease. We have detected and quantified tumor-derived PpIX-positive sEVs released from 5-ALA dosed glioma cells in vitro, glioma bearing xenograft models dosed with 5-ALA, and malignant glioma patients undergoing 5-ALA based FGS.

We have used imaging flow cytometry to detect and quantify individual fluorescent EVs, allowing us to test using a liquid biopsy strategy in brain tumor patients. Prior to interrogating patient plasma, we first established a PpIX-positive EV signal specific to the glioma-bearing mouse plasma, with plasma from control mice remaining at background levels, both pre- and post-5-ALA dosing ($p = 0.004$). The preferential release of PpIX-containing EVs from glioma cells in vitro and in our pre-clinical in vivo rodent model set the stage for our efforts to clinically detect these PpIX-positive EVs in brain tumor patients undergoing FGS with 5-ALA as a proof-of-principle. In 6 patients analyzed, we demonstrated that patients with avidly fluorescent tumors (4/6; 66%) indeed contained circulating PpIX-positive EVs at levels significantly higher than their pre-dosing background ($p = 0.00009$), and this rise in signal correlated highly with enhancing tumor volumes ($r^2=0.888$). For 2 patients with minimally fluorescent tumors, the PpIX-positive EV signal remained at or below their baseline background levels (Fig. 12a–c, Fig. 14).

Our study is therefore consistent with previous reports that show circulating PpIX levels only rise after the Although background levels remained consistently low among both of our patient groups, there was some inter-patient variability which limited our ability to establish a confident background cutoff valid across all individuals. Because of this heterogeneity in background signal, a fold change of >1.0 (PpIX-positive EVs post/pre-5-ALA levels) is what we determined the cutoff for a true positive tumor signal (Fig. 12d). We also determined that for patients with this true positive signal, the tumor volumes significantly correlated with the resulting fold change, which indicates that these PpIX-positive EVs are truly glioma-derived. In one case (Patient 2), PpIX-positive EV levels increased 2.3-fold with fluorescent-guided tumor resection and this signal returned to background levels in the following weeks (Fig. 12f), suggesting that the rise in PpIX-positive EVs represented a true tumor signal and not a random phenomenon. This work

is an important proof-of-concept of plasma-based EV detection technology for potential clinical applications in both diagnosis and monitoring of malignant glioma.

Two of our cases lacked avid intraoperative fluorescence and, in turn, did not demonstrate a rise in PpIX-positive EV signal. This was an important observation because it demonstrated the critical importance of the timing of plasma sampling following administration of 5-ALA in order to optimize the potential of detecting PpIX EVs. Tumors lacking fluorescence, a false negative, usually occur when resection is undertaken too quickly following 5-ALA ingestion or in cases of high tumor necrosis (Stummer et al., Neurosurgery. 42:5118-25 (1998)). Our two cases likely fell within both of these categories, with Patient 6 plasma sampling and tumor resection occurring 2 h following dosing, which was likely too early. We also had a lack of tumor fluorescence for Patient 5, which was carried out at 5 h after dosing, the longest interval between dosing and surgery in our group. Furthermore, the tumor was highly necrotic, which could be contributing factor for the lack of fluorescence observed intraoperatively. This suggests that the optimal time window for plasma sampling for PpIX-positive EV signal occurs at 3–4 h post-5-ALA administration.

The detection of PpIX-positive EVs in patients with malignant glioma provides for analyzing clinical samples as a strategy for non-invasive brain tumor diagnosis and monitoring.

EXAMPLE 3: Secondary Labelling EGFRvIII glioma cell derived EVs

We also examined a secondary labelling strategy using EGFRvIII glioma cell derived EVs using ISX. Conditioned media derived from EGFRvIII glioma cells was stained with CFDA-SE stain, fluorescently labelled Tenascin C and EGFRvIII antibodies. Imaging flow cytometry was used for fluorescent EV analysis. As is shown in Fig. 15, glioma cell derived EVs were successfully stained/labelled with pan EV marker (CFDA-SE) and glioma specific markers (Tenascin C and EGFRvIII).

EXAMPLE 4: Detection of PpIX positive EVs in CSF and urine of GBM patient

Using ISX analysis, we detected EVs in the CSF and urine from GBM patient. CSF and urine from GBM patient were analyzed for the presence of PpIX positive EVs 3 hours after the administration of 5-ALA. As is shown in Fig. 16, we detected 48,394 PpIX positive EVs from CSF and 1,22,197 PpIX positive EVs from urine of GBM patient 3 hours post ALA administration.

EXAMPLE 5: Additional Malignant Neoplasms (Renal cancer and melanoma)

Relative Fluorescence in 5-ALA dosed cancer cells

We determined whether 5-ALA dosed cancer cells fluoresced. To this end, cancer cells (Glioma cell lines: Gli36 WT, V3, U87; Renal cancer cell line: HEK293T; Melanoma cell line: Yumel) were treated with 0.8mM 5-ALA or Mock. Fluorescence was analyzed by image flow cytometry. We found that 5-ALA dosed cancer cells fluoresced, while normal cells (e.g., fibroblasts) did not fluoresce (Fig. 17).

PpIX EVs derived from 5-ALA dosed cancer cells

Next we determined whether 5-ALA dosed cancer cells released PpIX positive EVs. Accordingly, cancer cells (Glioma cells: Gli36 WT, V3, U87; Renal cancer cells: HEK293T; Melanoma cells: Yumel)

were treated with 0.8 mM 5-ALA or Mock. Conditioned media was then analyzed for PpIX positive EVs by imaging flow cytometry. We found that 5-ALA dosed cancer cells release PpIX positive EVs, while normal control cells (fibroblasts) did not release PpIX positive EVs (Fig. 18).

5 **EXAMPLE 6: Detection and Sorting of PpIX-positive EVs**

Using an Astrios-EQ nano FACS (Beckman Coulter), we sorted 5-ALA EVs with high purity into low volumes of PBS (200 μ l) according to methods described in Morales-Kastresana et al., J Extracell Vesicles. 8(1):1597603 (2019).

10 **Glioma Cells**

To sort PpIX positive EVs from 5-ALA dosed cancer cells, glioma cells (Gli36 WT, EGFR V3) were treated with 0.8mM 5-ALA or Mock. Astrios-EQ NanoFACS was used to sort PpIX positive EVs from conditioned media for 1 hour. As is shown in Fig.19, 20,638 PpIX positive EVs were sorted from conditioned media of 5-ALA dosed Gli36WT cells, while there were 74 particles in the mock dosed
15 Gli36WT cells. Furthermore, 124,155 PpIX positive EVs were sorted from conditioned media of 5-ALA dosed EGFR V3 cells, while there were 1011 particles in the mock dosed V3 cells.

Glioblastoma Patient Plasma

We next sorted PpIX positive EVs from plasma of patients with glioblastoma undergoing 5-ALA
20 based fluorescence guided surgery. Plasma was collected from glioblastoma patients, pre and post 5-ALA administration. Astrios-EQ NanoFACS was used to sort PpIX positive EVs from plasma for 10 minutes (Run1) and 1 hour (Run 2). The results of this analysis are shown in Fig. 20. 403 PpIX positive EVs and 25,635 PpIX positive EVs were sorted from post-5-ALA plasma in run 1 and run 2, respectively. Significant background events were identified in pre-5-ALA plasma due to higher concentration, swarming
25 effect, unstable fluid dynamics and lenient gating.

Isolation of Nucleic Acids from PpIX positive EVs

We performed whole transcriptome RNA-seq of RNA extracted from PpIX positive EVs.. Briefly, PpIX positive EVs (from cell lines and plasma) were concentrated using an Amicon filter, direct Qiazol
30 lysis was used to extract RNA from these EVs, followed by extraction of RNA using ExoRNeasy kit. RNA is extracted from (1 mL) conditioned media of Gli36 WT cells dosed with 5-ALA or mock using ExoRNeasy kit. RNA is extracted from (0.5mL) pre and post 5-ALA plasma using ExoRNeasy kit. The quality of extracted RNA was analyzed using Bioanalyzer. RNA-seq was performed using UPX-whole transcriptome kit on MiSeq. Data analysis was performed using GeneGlobe platform.

We compared isolated RNAs obtained from sorted 5-ALA positive EVs with all the EVs in a patient's plasma post 5-ALA treatment (Fig. 21). Accordingly, we examined the contents of the fluorescent 5-ALA/PpIX positive EVs. We have found an RNA preferentially expressed in neural brain tissues; namely NMNAT2 (nicotinamide 5 -mononucleotide adenylyltransferase). NMNAT2 is also a key RNA in NAD⁺ pathway that is significantly enhanced in glioblastomas. NMNAT2 (p=0.00293) was
40 significantly enriched in PpIX positive EVs when compared to post-ALA-plasma of our glioblastoma patient. These results demonstrate a tumor-specific and neural specific marker in PpIX-positive EVs. Our

methods accordingly are useful for enriching for tumor specific biomarkers for detecting the presence of a neoplasm.

5 In sum, multiple cancer cells (e.g., glioma, renal cancer, melanoma cells) have been found to release PpIX positive EVs which can be detected using imaging flow cytometry. Astrios-EQ NanoFACS is useful for high purity sorting of fluorescent PpIX positive EVs from glioma cells and plasma of a glioblastoma patients. Whole transcriptome analysis of sorted 5-ALA EVs showed selective enrichment of RNA in sorted PpIX positive EVs from 5-ALA treated Gli36 WT cells and in plasma of a glioblastoma patient undergoing 5-ALA based fluorescent guided surgery. Furthermore, detection of neuronal specific and tumor specific marker, NMNAT2 ($p=0.00293$) which is a key RNA in NAD⁺ pathway (upregulated in
10 GBMs) in PpIX positive EVs demonstrates the usefulness of the methods described herein.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, other embodiments are also within the claims.

What is claimed is:

Claims

1. A highly purified population of protoporphyrin IX (PpIX)-positive extracellular vesicles (EVs).
2. The population of claim 1, wherein the EVs are stored in a container impervious to light.
3. The population of claim 1, wherein the EVs are obtained directly from the plasma of a subject.
4. The population of claim 3, wherein the subject is a human.
5. A method of identifying a neoplasm in a subject, the method comprising the steps of
 - a) administering a 5-aminolevulinic acid (5-ALA) to the subject; and
 - b) detecting, in a biofluid of the subject, PpIX-positive EVs by imaging flow cytometry, wherein an increase in the amount of PpIX-positive EVs compared to a control sample is taken as an indication that the subject has the neoplasm.
6. The method of claim 5, wherein the neoplasm, following administration of 5-ALA, releases PpIX-positive EVs.
7. The method of claim 5, wherein the biofluid is collected about 2 hours after administration of the 5-ALA.
8. The method of claim 5, wherein the biofluid is collected before administration of the 5-ALA to the patient.
9. The method of claim 5, further comprising sorting the PpIX-positive EVs.
10. The method of claim 9, wherein the sorting comprises laser-based sorting EVs of interest.
11. The method of claim 9, wherein one or more fluorescent markers is detected on the sorted EVs.
12. The method of claim 5, wherein the biofluid is a liquid biopsy, plasma, blood, cerebrospinal fluid or urine.
13. The method of claim 5, wherein the neoplasm is a cancer.
14. The method of claim 5, wherein the neoplasm is an aggressive tumor.
15. The method of claim 14, wherein the tumor is a high grade malignant glioma.
16. The method of claim 13, wherein the cancer is a low grade glioma, melanoma, a meningioma, non-small cell lung cancer, pancreatic cancer, or bladder cancer.
17. The method of claim 5, wherein said 5-ALA is 5-ALA hydrochloride.
18. The method of claim 5, wherein the subject is a mammal.
19. The method of claim 18, wherein the mammal is a human.
20. The method of claim 5, wherein the subject has undergone surgery.
21. The method of claim 20, wherein the surgery is fluorescence guided surgery (FGS).
22. The method of claim 5, wherein PpIX-positive EVs are detected by multidimensional analysis.
23. The method of claim 22, wherein the multidimensional analysis comprises identifying EVs using an EV-specific marker.
24. The method of claim 23, wherein the EV-specific marker is a stain.
25. The method of claim 24, wherein the stain is carboxyfluorescein diacetate succinimidyl ester stain (CFDA-SE).

26. The method of claim 23, wherein the EV-specific marker is an antibody.
27. The method of claim 26, wherein the antibody is a fluorescent-labeled antibody that specifically binds to CD81+ or CD63+ tetraspanins.
28. The method of claim 26, wherein the antibody is a fluorescent-labeled antibody that specifically binds to EGFR, EGFRvIII, or Tenascin C.
29. The method of claim 5, wherein the method distinguishes pseudoprogression from true progressive disease.
30. The method of claim 5, wherein the method monitors a subject's cancer.
31. The method of claim 5, wherein the method monitors a response of a subject's cancer treatment.
32. The method of claim 5, wherein the subject is suspected of having a cancer.
33. The method of claim 5, wherein the PpIX-positive EVs are fluorescent.
34. A method of detecting EVs in a subject, the method comprising the steps of
 - a) administering a 5-ALA to the subject; and
 - b) detecting, in a biofluid of the subject, PpIX-positive EVs by imaging flow cytometry.
35. The method of claim 34, wherein a neoplasm in the subject, following administration of 5-ALA, releases PpIX-positive EVs.
36. The method of claim 34, wherein the biofluid is collected about 2 hours after administration of the 5-ALA.
37. The method of claim 34, further comprising sorting the PpIX-positive EVs.
38. The method of claim 37, wherein the sorting comprises laser-based sorting EVs.
39. The method of claim 37, wherein one or more fluorescent markers is detected on the sorted EVs.
40. The method of claim 34, wherein the biofluid is a liquid biopsy, plasma, blood, cerebrospinal fluid or urine.
41. The method of claim 34, wherein said 5-ALA is 5-ALA hydrochloride.
42. The method of claim 34, wherein the subject is a mammal.
43. The method of claim 42, wherein the mammal is a human.
44. The method of claim 34, wherein PpIX-positive EVs are detected by multidimensional analysis.
45. The method of claim 44, wherein the multidimensional analysis comprises identifying EVs using an EV-specific marker.
46. The method of claim 45, wherein the EV-specific marker is a stain.
47. The method of claim 46, wherein the stain is CFDA-SE.
48. The method of claim 45, wherein the EV-specific marker is an antibody.
49. The method of claim 48, wherein the antibody is a fluorescent-labeled antibody that specifically binds to CD81+ or CD63+ tetraspanins.
50. The method of claim 48, wherein the antibody is a fluorescent-labeled antibody that specifically binds to EGFR, EGFRvIII, or Tenascin C.

51. A method of purifying EVs from a subject, the method comprising the steps of administering a 5-ALA to the subject; and isolating, from a biofluid of the subject, PpIX-positive EVs by imaging flow cytometry, thereby purifying EVs from the subject.

52. The method of claim 51, further comprising detecting the presence or absence of an EV marker.

53. The method of claim 52, wherein the EV marker is a nucleic acid.

54. The method of claim 53, wherein the nucleic acid is an RNA.

55. The method of claim 52, wherein the marker is a tumor specific marker.

56. The method of claim 52, wherein the EV marker is a protein.

57. The method of claim 52, wherein the EV marker is a lipid.

58. The method of claim 51, wherein the neoplasm, following administration of 5-ALA, releases PpIX-positive EVs.

59. The method of claim 51, wherein the biofluid is collected about 2 hours after administration of the 5-ALA.

60. The method of claim 51, wherein the biofluid is collected before administration of the 5-ALA to the patient.

61. The method of claim 51, further comprising sorting the PpIX-positive EVs.

62. The method of claim 61, wherein the sorting comprises laser-based sorting EVs of interest.

63. The method of claim 62, wherein one or more fluorescent markers is detected on the sorted EVs.

64. The method of claim 51, wherein the biofluid is a liquid biopsy, plasma, blood, cerebrospinal fluid or urine.

65. The method of claim 51, wherein the neoplasm is a cancer.

66. The method of claim 51, wherein the neoplasm is an aggressive tumor.

67. The method of claim 66, wherein the tumor is a high grade malignant glioma.

68. The method of claim 65, wherein the cancer is a low grade glioma, melanoma, a meningioma, non-small cell lung cancer, pancreatic cancer, or bladder cancer.

69. The method of claim 51, wherein said 5-ALA is 5-ALA hydrochloride.

70. The method of claim 51, wherein the subject is a mammal.

71. The method of claim 70, wherein the mammal is a human.

72. The method of claim 51, wherein the subject has undergone surgery.

73. The method of claim 72, wherein the surgery is FGS.

74. The method of claim 51, wherein PpIX-positive EVs are detected by multidimensional analysis.

75. The method of claim 74, wherein the multidimensional analysis comprises identifying EVs using an EV-specific marker.

76. The method of claim 75, wherein the EV-specific marker is a stain.

77. The method of claim 76, wherein the stain is CFDA-SE.

78. The method of claim 75, wherein the EV-specific marker is an antibody.

79. The method of claim 78, wherein the antibody is a fluorescent-labeled antibody that specifically binds to CD81+ or CD63+ tetraspanins.

80. The method of claim 78, wherein the antibody is a fluorescent-labeled antibody that specifically binds to EGFR, EGFRvIII, or Tenascin C.

81. The method of claim 51, wherein the method distinguishes pseudoprogression from true progressive disease.

82. The method of claim 51, wherein the method monitors a subject's cancer.

83. The method of claim 51, wherein the method monitors a response of a subject's cancer treatment.

84. The method of claim 51, wherein the subject is suspected of having a cancer.

85. The method of claim 51, wherein the PplX-positive EVs are fluorescent.

86. A method of counting EVs, the method comprising the steps of administering a 5-ALA to the subject; isolating, from a biofluid of the subject, PplX-positive EVs by imaging flow cytometry; and counting the EVs in the biofluid.

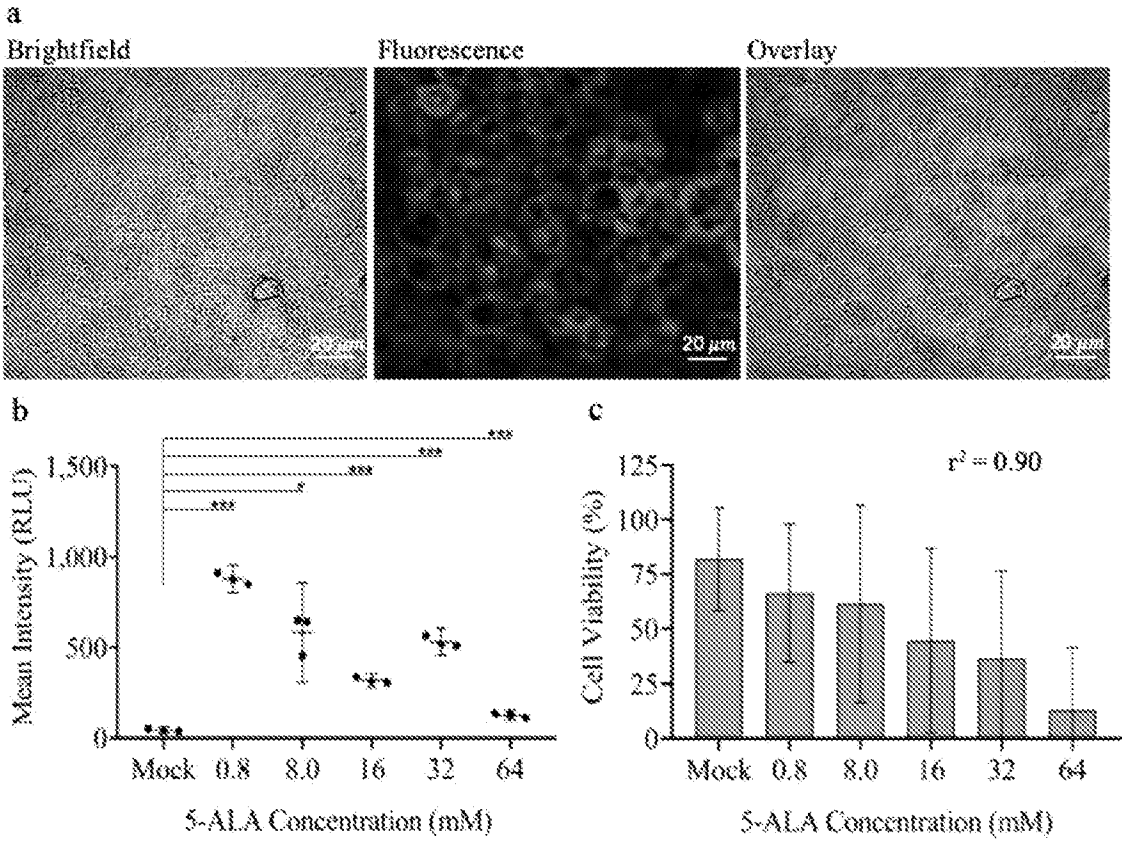


FIG. 1

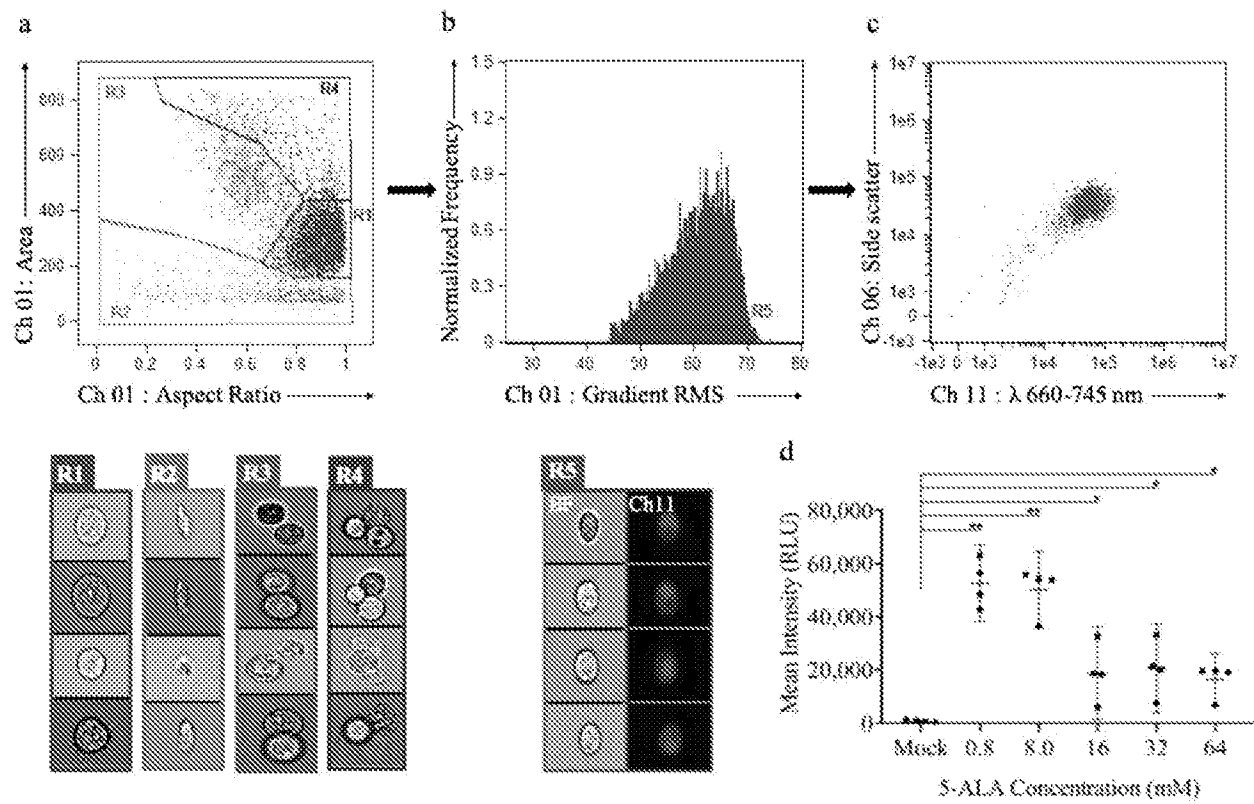


FIG. 2

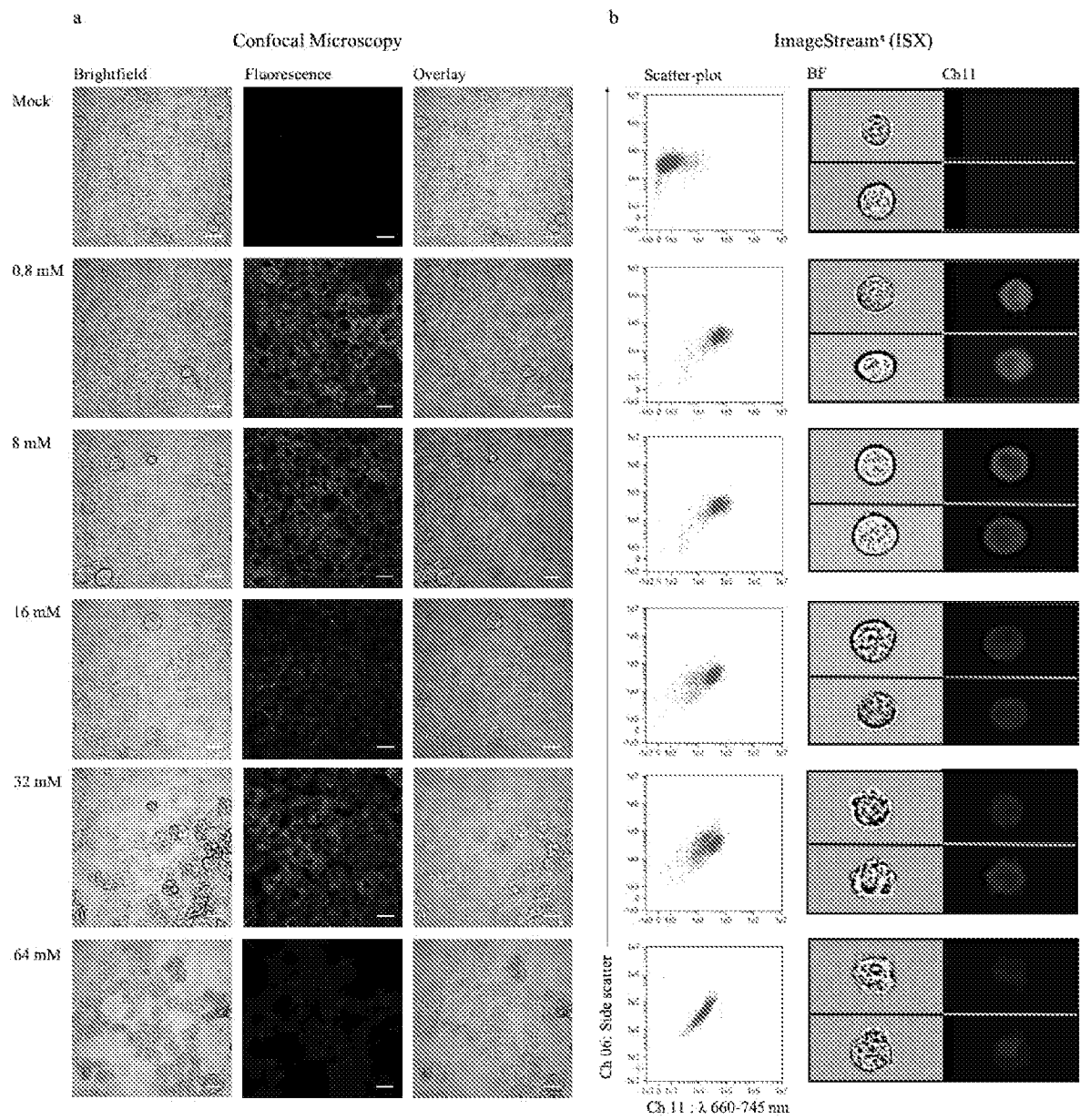


FIG. 3

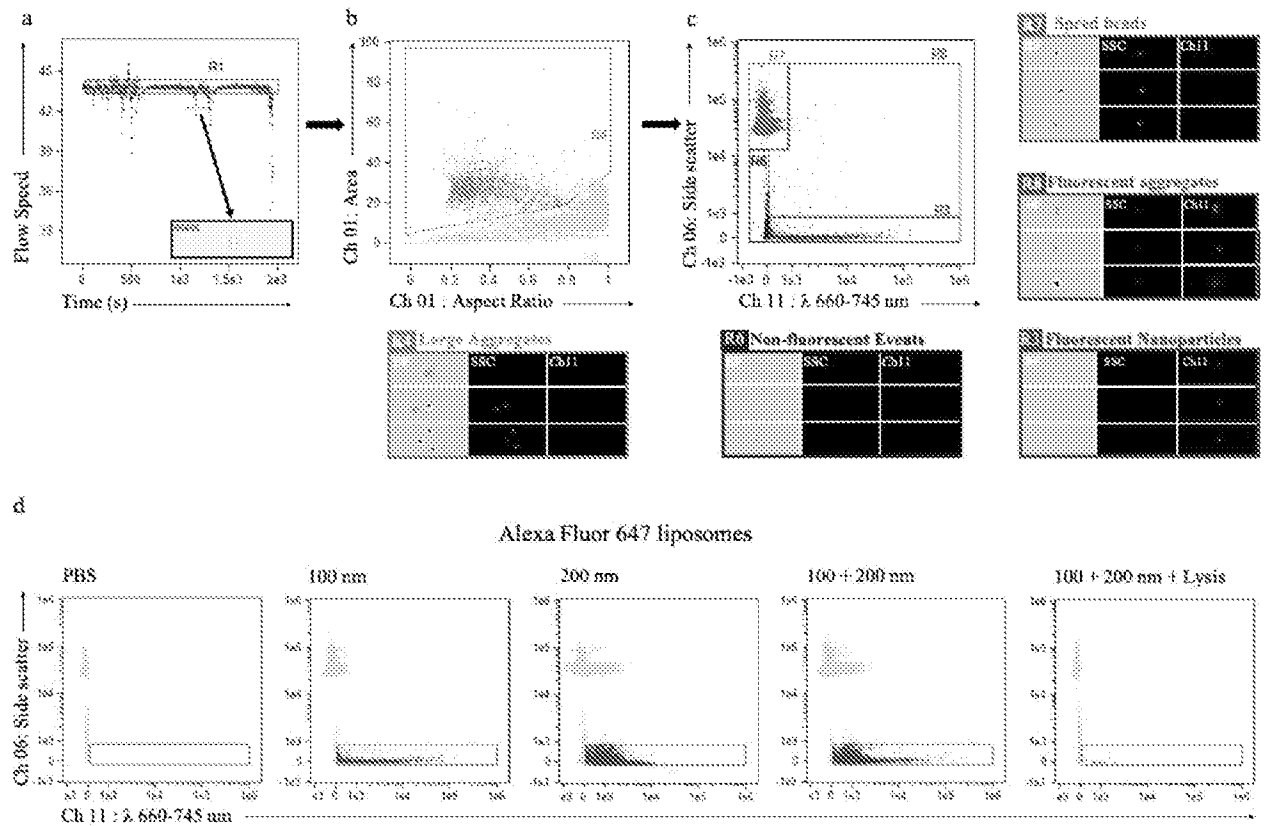
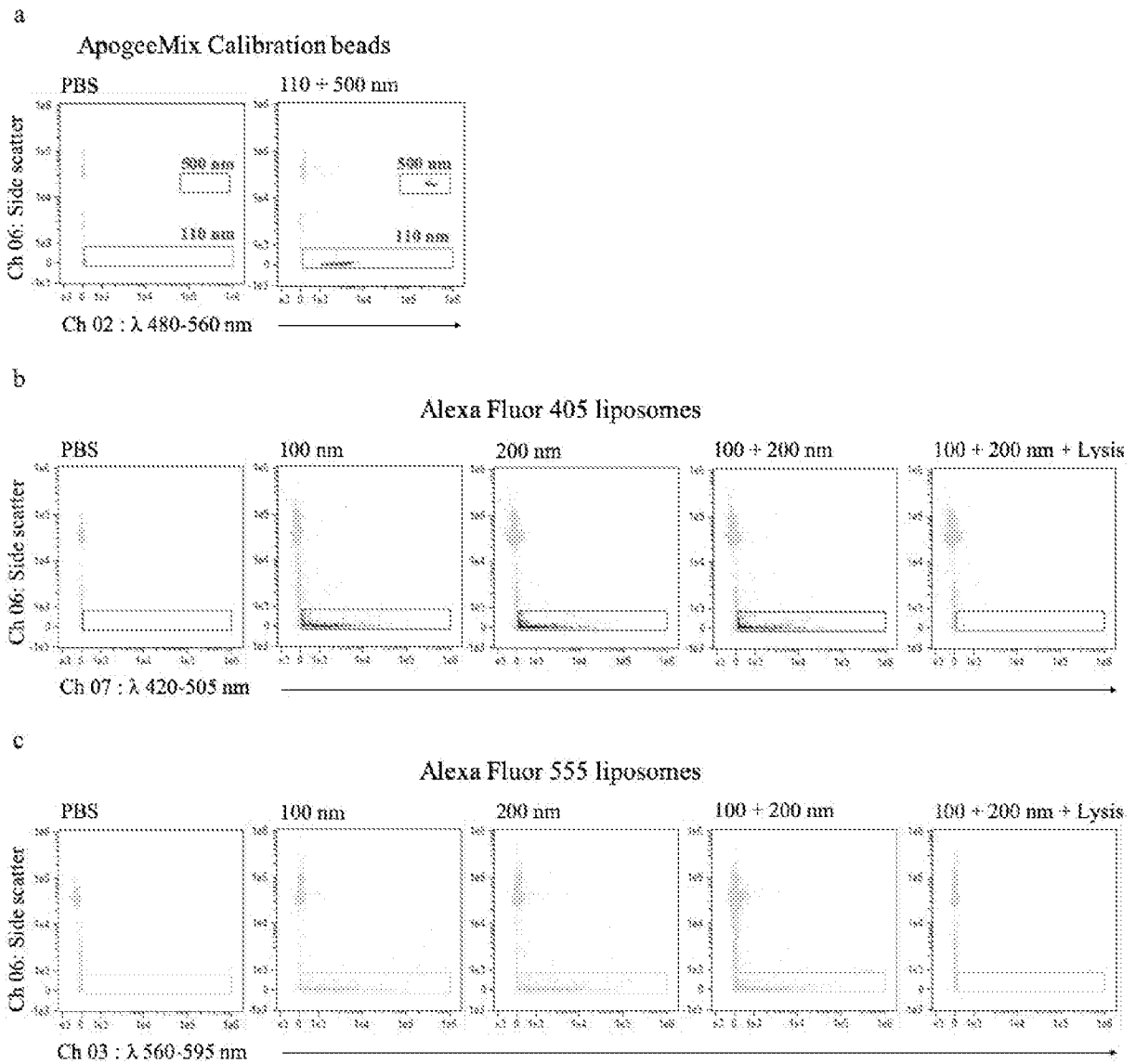


FIG. 4



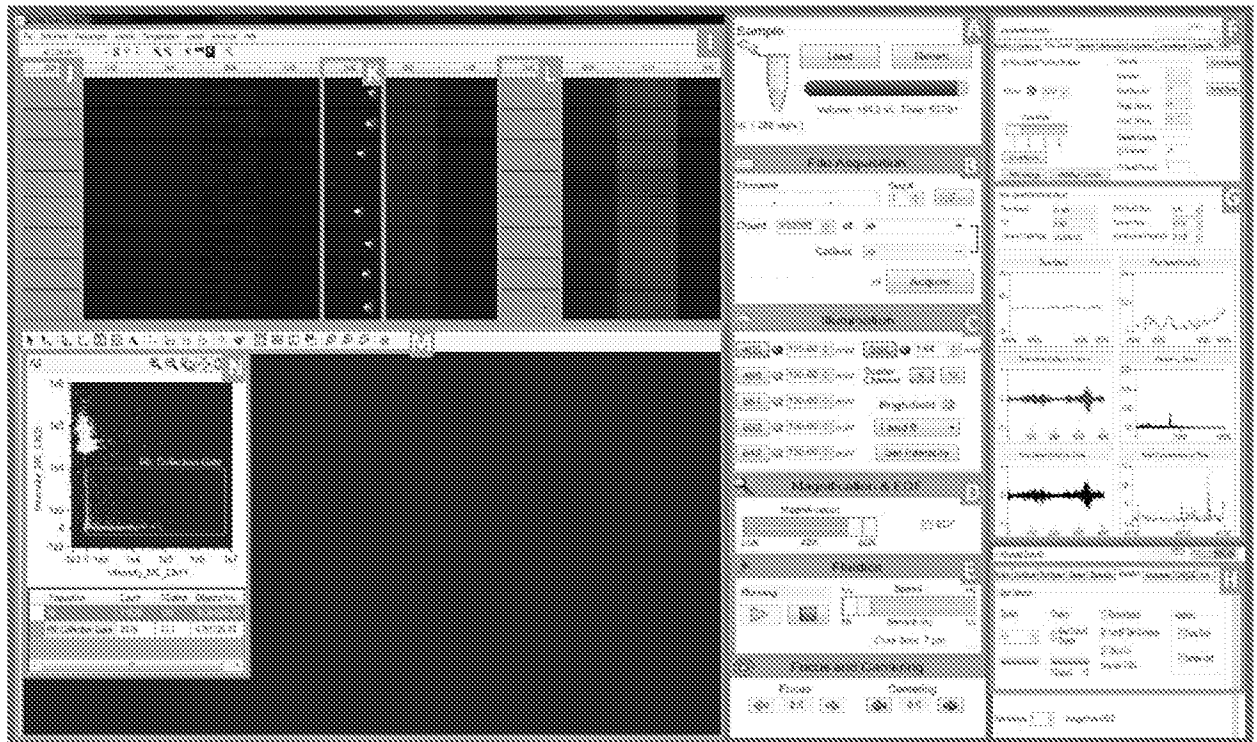


FIG. 6

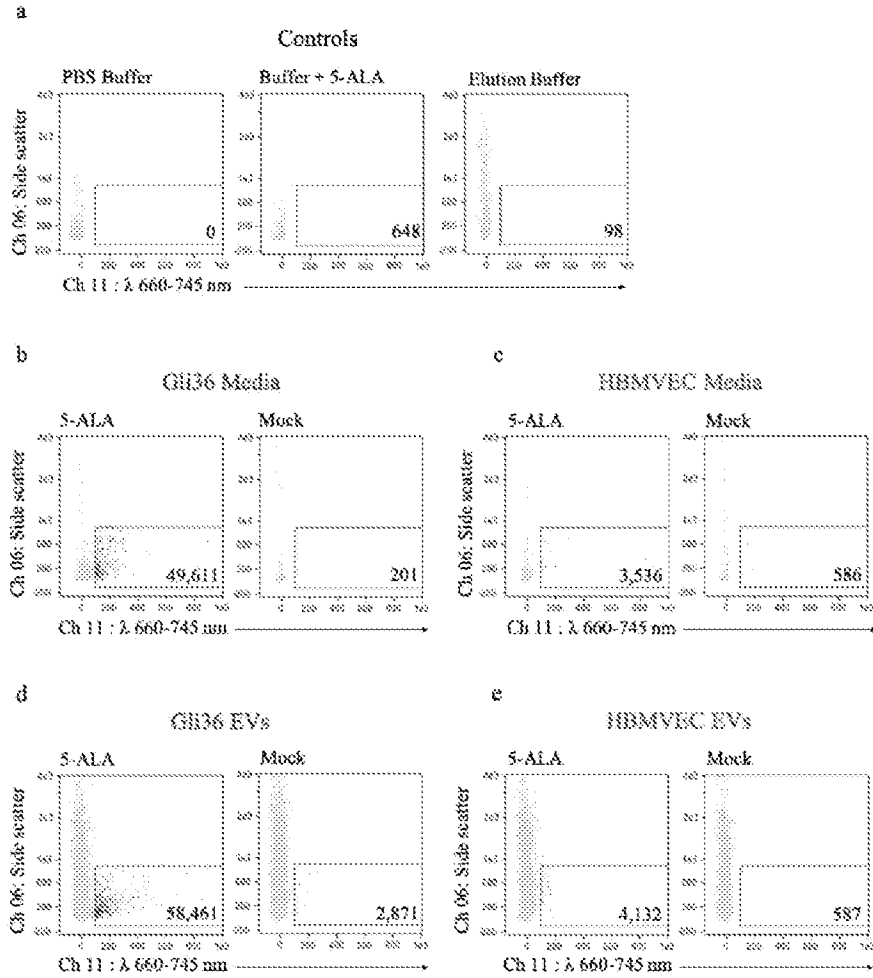


FIG. 7

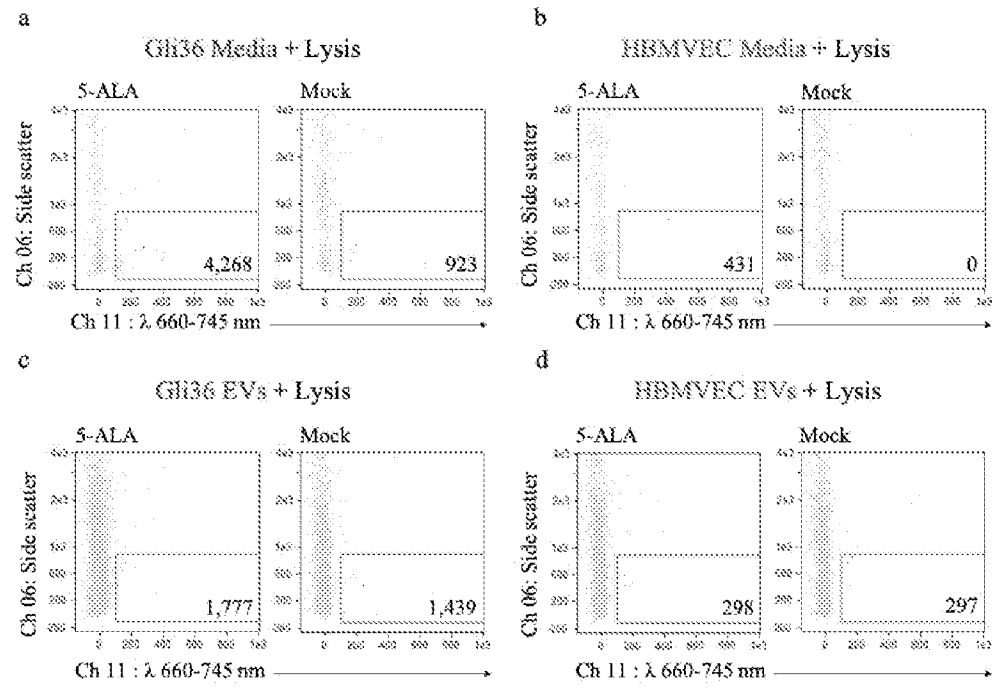


FIG. 8

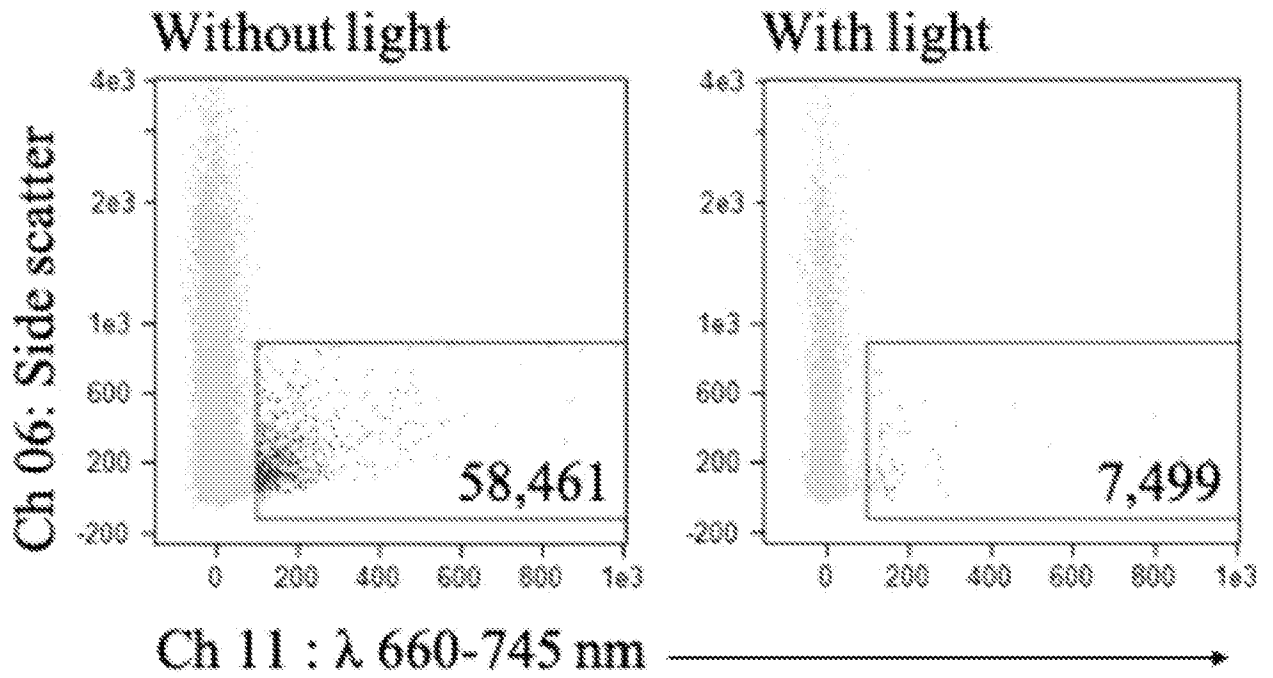


FIG. 9

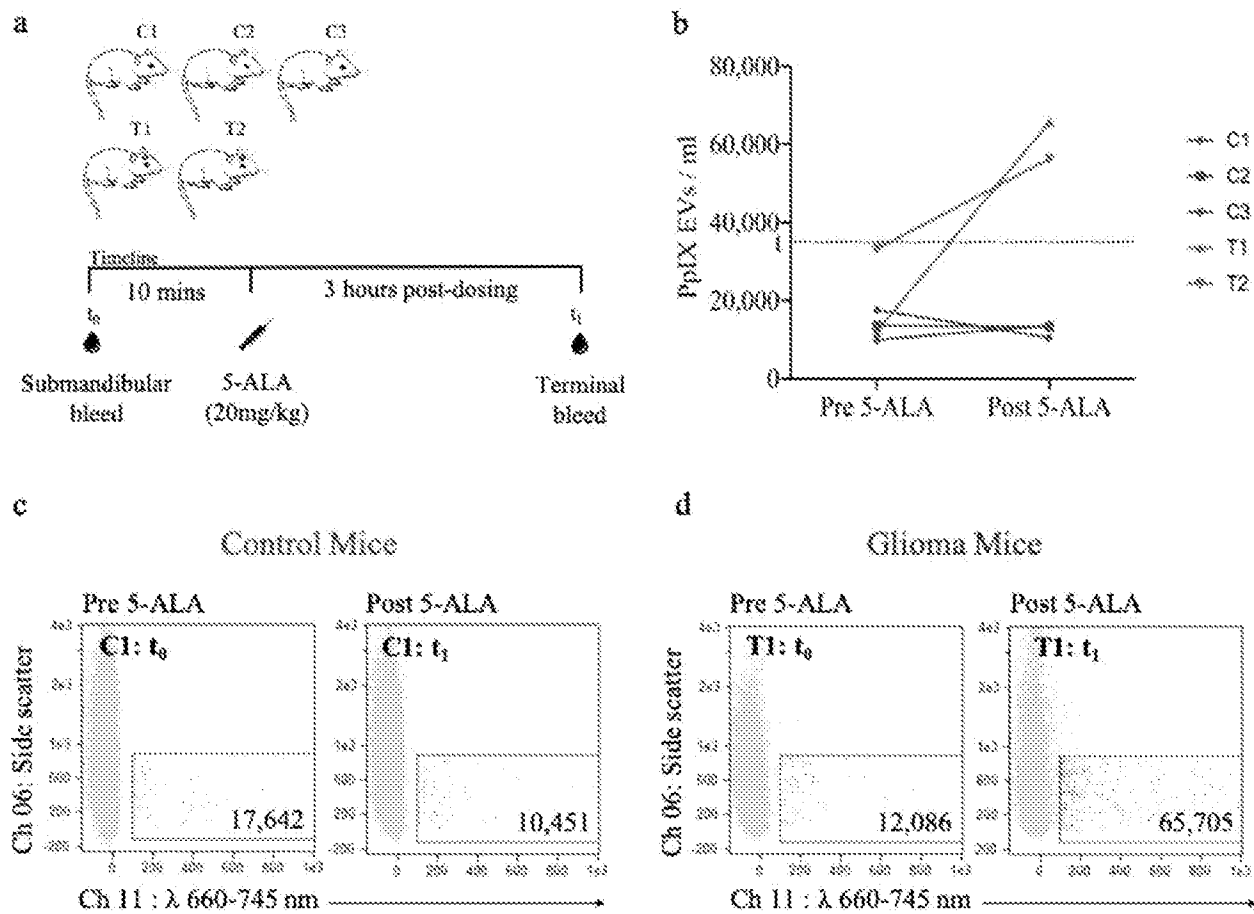


FIG. 10

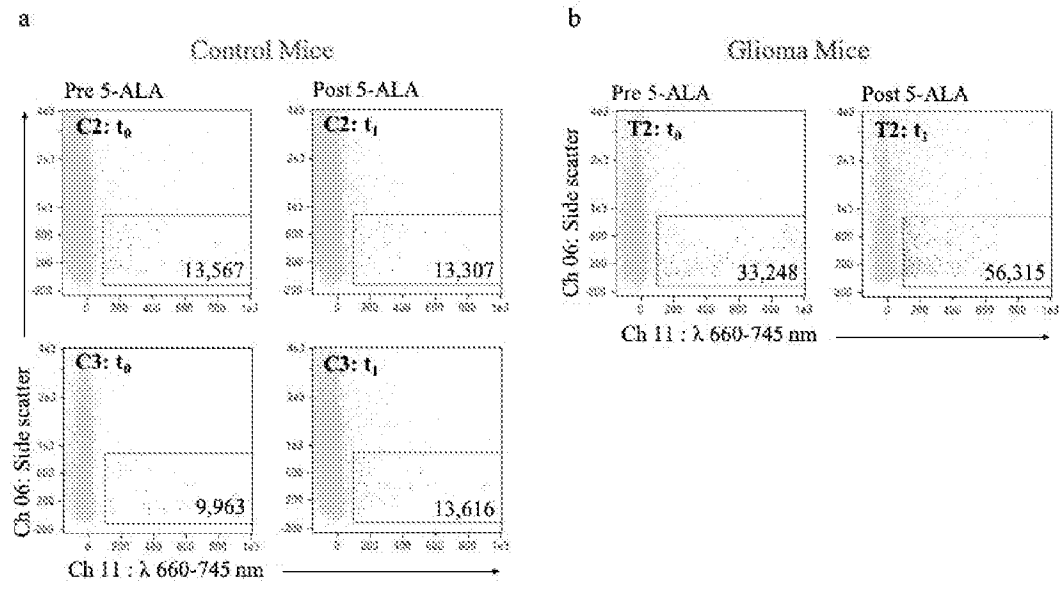


FIG. 11

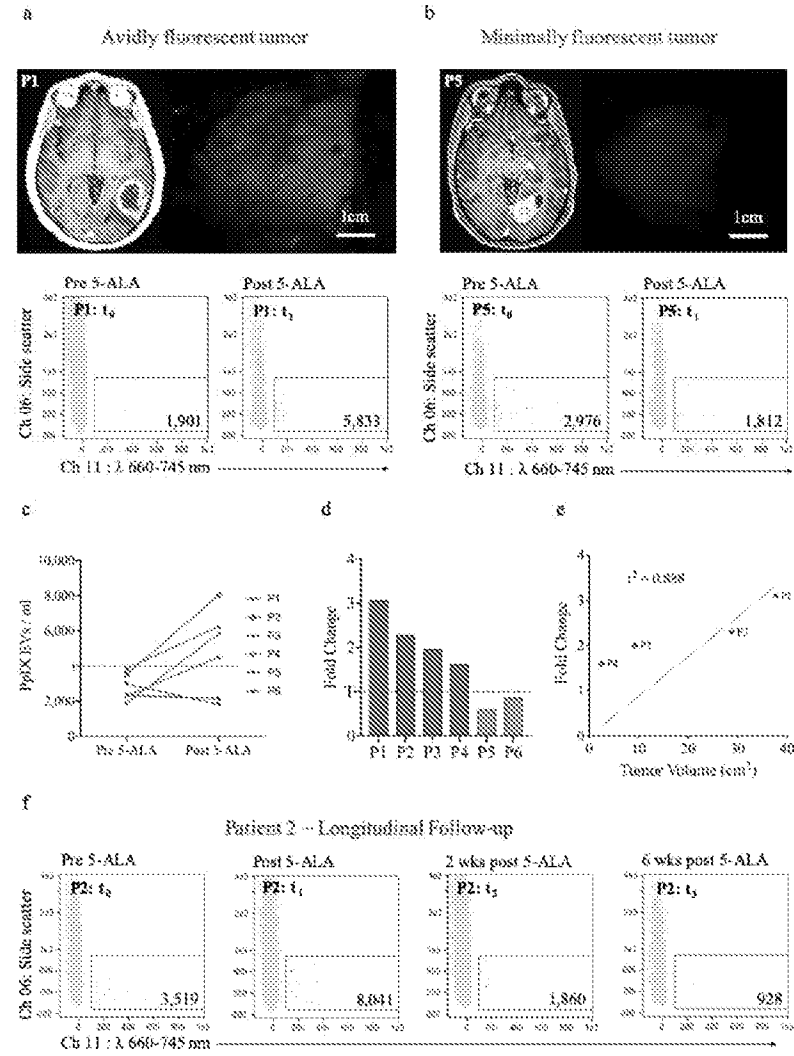


FIG. 12

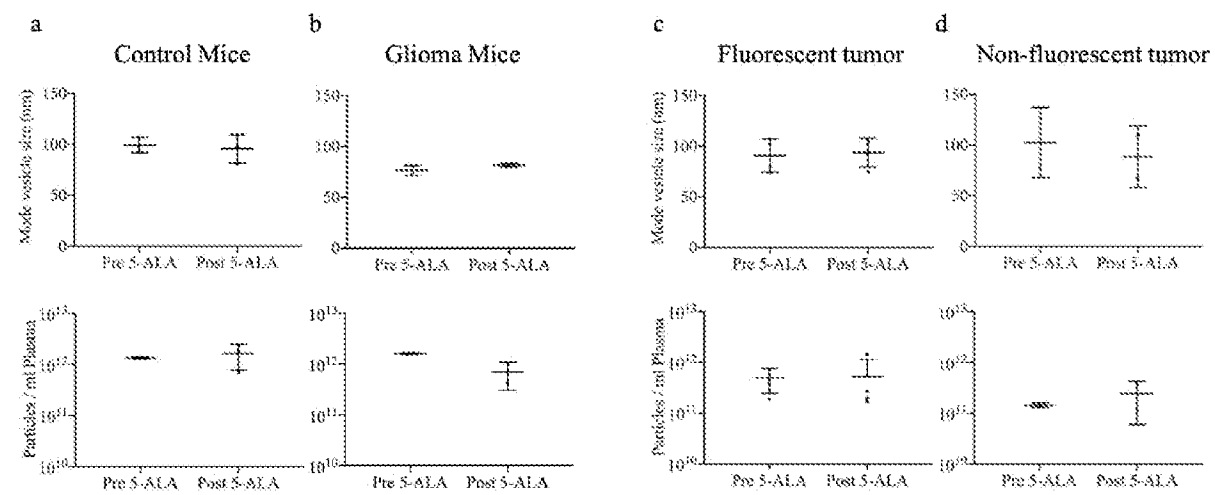


FIG. 13

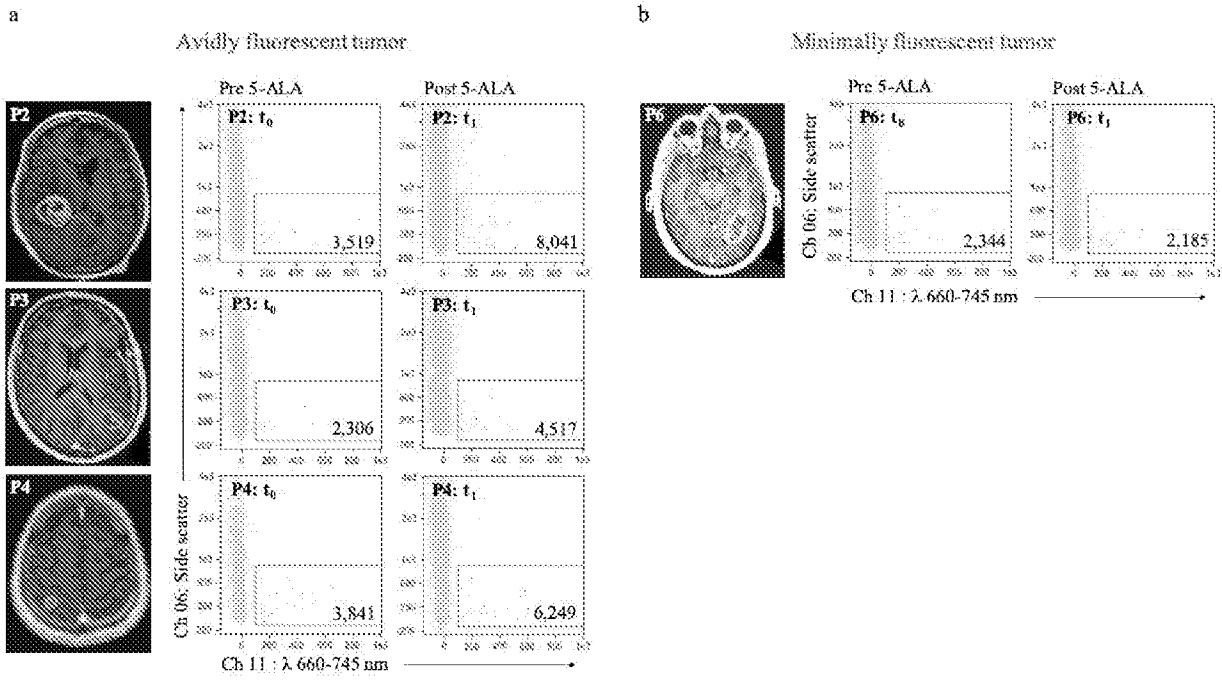


FIG. 14

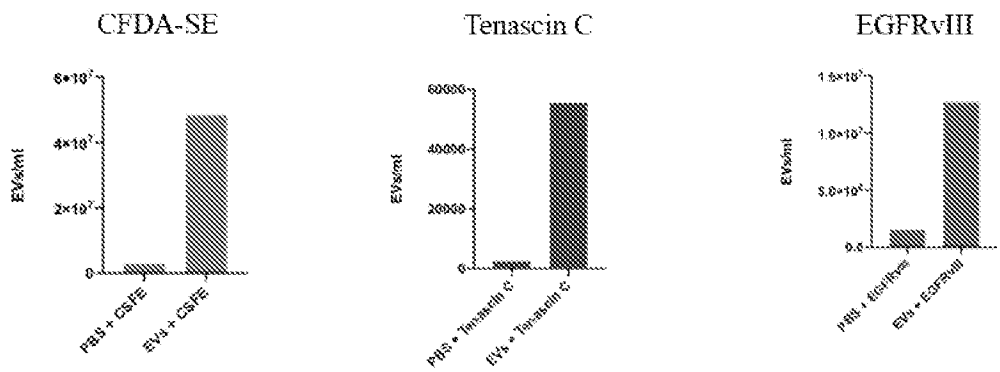


FIG. 15

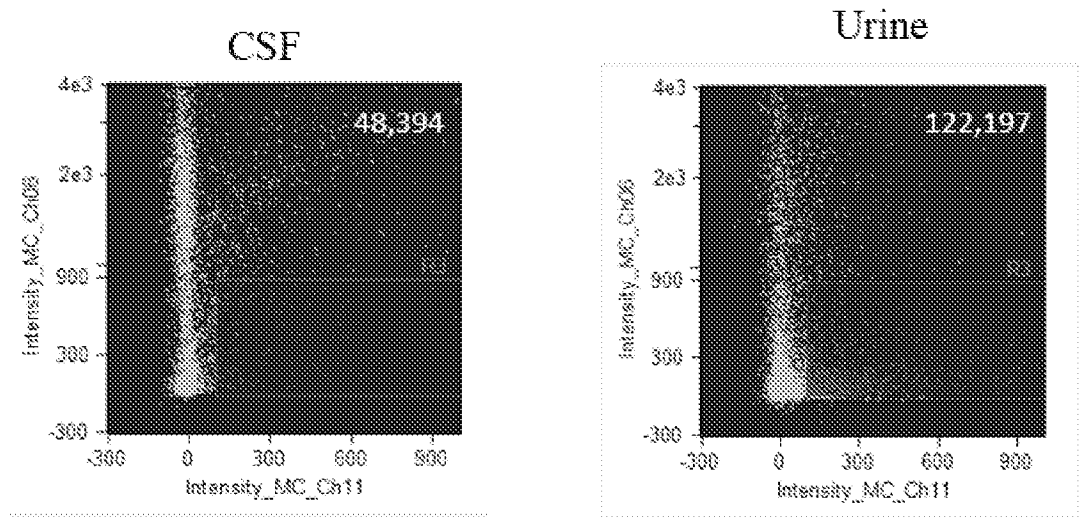


FIG. 16

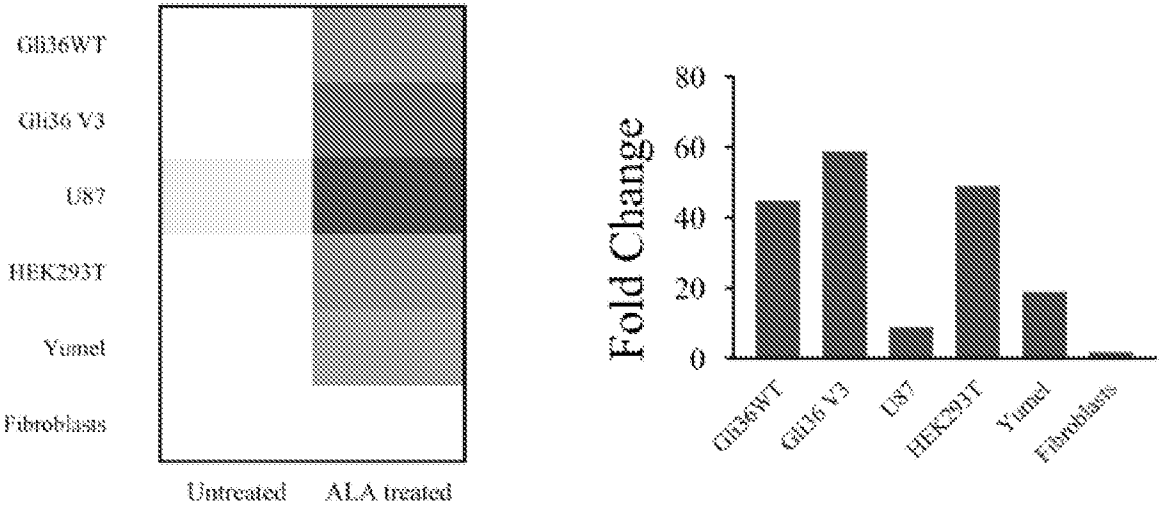


FIG. 17

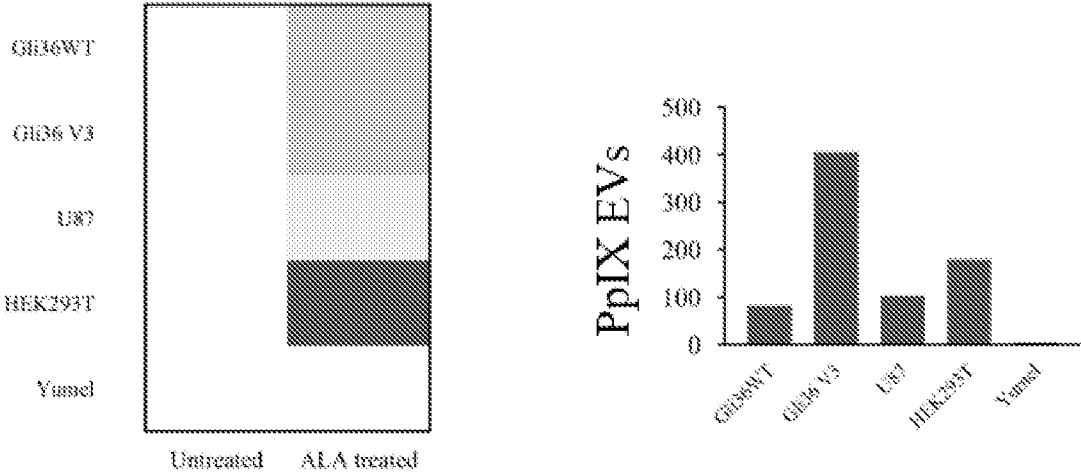


FIG. 18

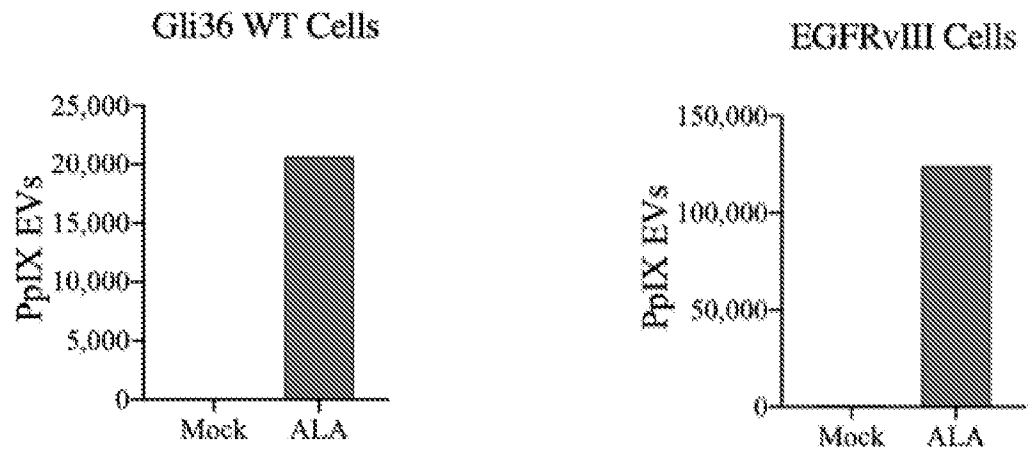


FIG. 19

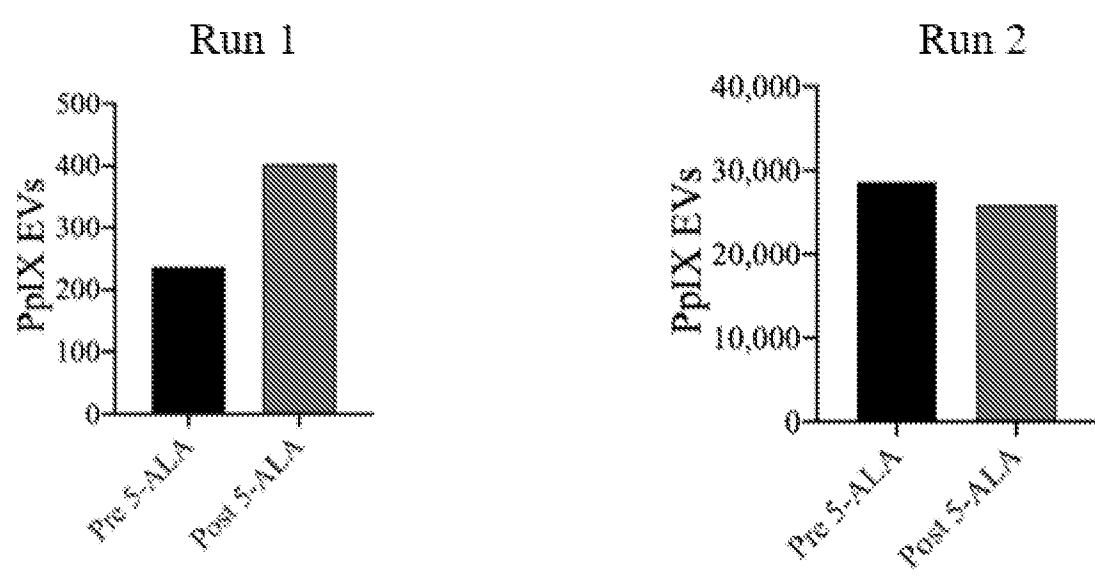


FIG. 20

Downregulated RNA in
ALA positive EVs

MT-FL1: tRNA-Leu (UUA/G) 1
MT-RNR1:12S rRNA
MT-ATP6: ATP Synthase Membrane Subunit 6
MT-CO2: Cytochrome C Oxidase I
MT-RNR2: 16S rRNA
MT-ND4: NADH, Ubiquinone Oxidoreductase Core Subunit 4
MT-NDL2: NADH, Ubiquinone Oxidoreductase Core Subunit 2
MT-CO3: Cytochrome C Oxidase III
MT-ND1: NADH, Ubiquinone Oxidoreductase Core Subunit 1
MT-ND5: NADH, Ubiquinone Oxidoreductase Core Subunit 5

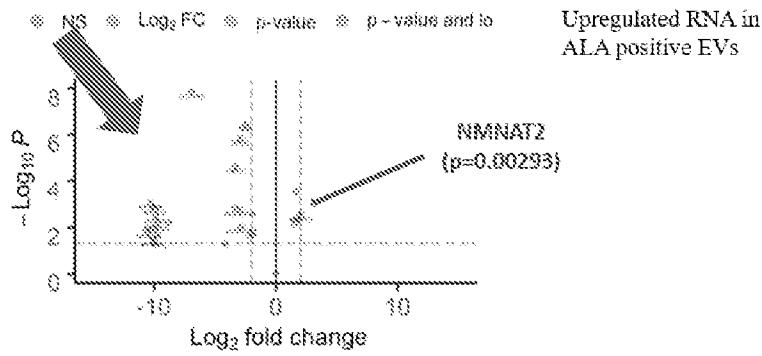


FIG. 21

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/30125

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.:
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:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I: claims 1-4, drawn to a highly purified population of protoporphyrin IX (PpIX)-positive extracellular vesicles.

Group II: claims 5-86, drawn to a method of identifying a neoplasm in a subject, a method of detecting EVs in a subject, and a method of purifying EVs from a subject.

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

*****Continued in Supplemental Box*****

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.:
1-4

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/US 20/30125

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 9/14, A61K 47/10, A61K 31/195, A61P 17/02 (2020.01)

CPC - A61K 8/06, A61K 8/44, A61K 8/553, B82Y 5/00, A61K 2800/21

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.
X	US 2017/0192005 A1 (PIOMA, INC) 6 July 2017 (06.07.2017); abstract; para [0019], [0039], [0070], [0073], [0105], [0106], [0122]	1, 3-4
----- Y		2
Y	US 2008/0194508 A1 (CHRISTENSEN et al.) 14 August 2008 (14.08.2008); para [0205]	2

☐ 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

24 August 2020

Date of mailing of the international search report

18 SEP 2020

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Authorized officer

Lee Young

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/US 20/30125

Continuation of Box III: Observations where unity of invention is lacking:

Special Technical Features

Group I includes the special technical feature of a composition which differs from the special technical feature of a method, as disclosed by Group II.

Common Technical Features

The inventions of Groups I and II share the technical feature of a purified EV comprising protoporphyrin IX (PpIX).

However, these shared technical features do not represent a contribution over prior art in view of US 2017/0192005 A1 to Pioma, Inc (hereinafter "Pioma").

Pioma teaches (instant claim 1) a purified population of protoporphyrin IX (PpIX)-positive extracellular vesicles (Evs) (Abstract "The present disclosure relates to methods for detecting brain tumors and assessing the recurrence of such tumors by administering a pharmaceutical composition comprising 5-aminolevulinic acid (5-ALA) and detecting the conversion of 5-ALA to protoporphyrin IX (PPIX) associated with brain-derived microparticles."; para [0039] "The term "microparticle" as used herein refers to any small vesicles released from any cell type. Microparticles include, for example, exosomes and microvesicles."; [0105] "Microparticles with a mode hydrodynamic diameter of 50-100 nm were isolated using size exclusion chromatography."; [0106] "microparticles with a mode diameter of 50-100 nm measured by light scattering are present in GBM patient serum"; [0122] "U87 Cell Line Converts 5-ALA to PPIX Via a CPOX-Mediated Conversion Process Which can be Monitored in Shed Microparticles. This example demonstrates that U87 cell cultures actively convert 5-ALA to PPIX, that these cells produce coproporphyrinogen III oxidase (CPOX protein), and that CPOX proteins and PPIX are present in shed microparticles in cancer cell lines.").

As said technical features were known in the art at the time of the invention, these cannot be considered special technical features that would otherwise unify the groups.

Groups I and II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.