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(54) Title: PHOTOSENSITISER BASED BLADDER CANCER DETECTION METHOD

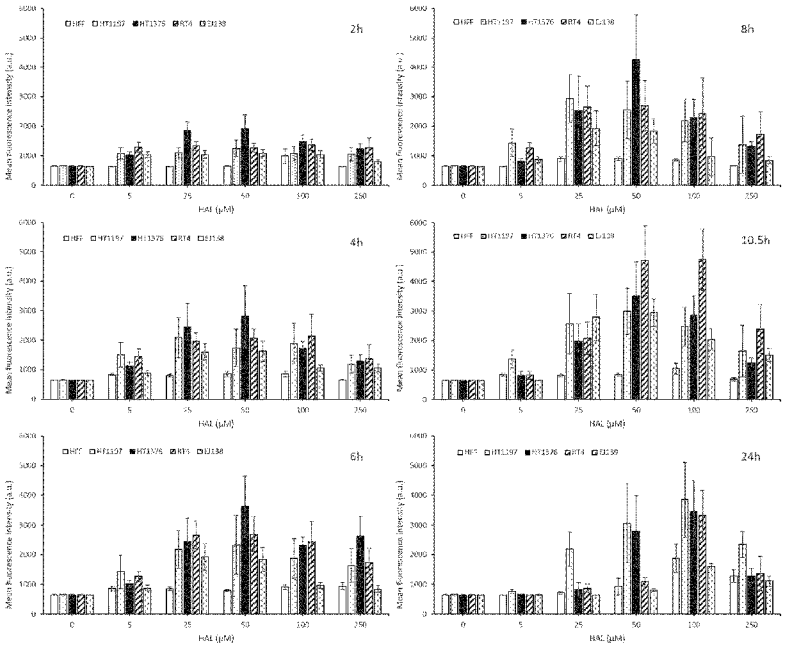

FIGURE 1

(57) Abstract: A method of selectively detecting bladder cancer cells in urine or a urine derived fluid, the method comprising: combining a sample of urine or a urine derived fluid to be analysed with a cancer cell specific photosensitiser composition and/or a cancer cell specific photosensitiser precursor composition contacting the treated sample with one or more bladder cancer selective cell capture surfaces under conditions to bind at least some of the target bladder cancer cells to the cell capture surface; exposing the cells captured on the cell capture surface to light having a first excitation wavelength for the cancer cell specific photosensitiser, measuring the intensity of light emitted at one or more selected cancer cell specific photosensitiser emission wavelengths from cells captured on the cell capture surface; and determining if bladder cancer cells are present on the cell capture surface using the measured light intensity.

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PHOTOSENSITISER BASED BLADDER CANCER DETECTION METHOD

TECHNICAL FIELD

The present disclosure relates to methods for detecting bladder cancer cells.

BACKGROUND

Bladder cancer is one of the most common forms of cancer diagnosed worldwide. In the United States, it is the seventh most common cause of cancer death (National Cancer Institute. Bethesda, M. *SEER Cancer Stat Facts: Bladder Cancer.* . 2018; Available from: https://seer.cancer.gov/statfacts/html/urinb.html). with 60,000 new cases and 12,000 resultant deaths in 2017 (Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer Statistics, 2017.* CA Cancer J Clin, 2017. **67**(1): p. 7-30). Cancers of the urinary bladder are usually urothelial carcinomas, and most patients are diagnosed with low grade non-muscle invasive bladder cancer (NMIBC), which has a high recurrence rate of up to 70%. Additionally, 50% of NMIBC are likely to progress to more aggressive and higher grade muscle-invasive bladder cancer (MIBC) (Witjes, J.A., The impact of recurrent non-muscle-invasive bladder cancer on progression. Eur Urol, 2013. **63**(1): p. 155-7).

Bladder cancer survivors have to undertake frequent follow-up tests for the rest of their lives. The nature of the follow-up tests used in clinical practice has earnt bladder cancer the title of most expensive cancer to treat from diagnostic to death per capita (Leal, J., et al., *Economic Burden of Bladder Cancer Across the European Union.* European Urology, 2016. **69**(3): p. 438-447). Cystoscopy, the gold standard diagnostic method, is invasive, costly and often fails to detect flat lesions (Geavlete, B., et al., Treatment changes and long-term recurrence rates after hexaminolevulinate (HAL) fluorescence cystoscopy: does it really make a difference in patients with non-muscle-invasive bladder cancer (NMIBC)? BJU Int, 2012. **109**(4): p. 549-56). The most common non-invasive test is cytology, which has disappointingly low sensitivity rates (Bell, M.D.Y., F.A.; Brimo, F.; Steinberg, J.; Aprikian, A.G.; Tanguay, S.; Kassouf, W. , Prognostic value of urinary cytology and other biomarkers for recurrence and progression in bladder

cancer: A prospective study. . World J. Urol. , 2016. **34**: p. 1405-1409). While several other non-invasive approaches have been proposed over the years, they have not proven accurate enough to supplant cystoscopies.

There is thus a need for improved non-invasive diagnostic methods for use in long term surveillance to improve bladder cancer management.

SUMMARY

According to a first aspect, the present disclosure provides a method of selectively detecting bladder cancer cells in urine or a urine derived fluid, the method comprising:

combining a sample of urine or a urine derived fluid to be analysed with a cancer cell specific photosensitiser composition and/or a cancer cell specific photosensitiser precursor composition;

contacting the treated sample with one or more bladder cancer selective cell capture surfaces under conditions to bind at least some of the target bladder cancer cells to the cell capture surface;

exposing the cells captured on the cell capture surface to light having a first excitation wavelength for the cancer cell specific photosensitiser,

measuring the intensity of light emitted at one or more selected cancer cell specific photosensitiser emission wavelengths from cells captured on the cell capture surface; and

determining if bladder cancer cells are present on the cell capture surface using the measured light intensity.

In certain embodiments, the method also comprises:

combining the sample of urine or a urine derived fluid with a luminescent cell nucleus stain composition to provide the treated sample;

exposing the cells captured on the cell capture surface to light having a second excitation wavelength for the luminescent cell nucleus stain; and

measuring the intensity of light emitted at one or more selected luminescent cell nucleus stain emission wavelengths from cells captured on the cell capture surface.

In certain embodiments, the cancer cell specific photosensitiser precursor composition is a PpIX precursor composition. The PpIX precursor composition may be any compound that is metabolized by cells to produce fluorescent PpIX but which is metabolized by cancer cells faster than healthy cells, leading to increased production of PpIX in cancer cells relative to healthy cells and, therefore, increased fluorescence in cancer cells relative to healthy cells. In certain specific embodiments, the PpIX precursor

is selected from one or more of the group consisting of 5-aminolevulinic acid (5-ALA) and hexaminolevulinate (HAL).

In embodiments in which the cancer cell specific photosensitiser precursor composition is a PpIX precursor composition, the process may comprise maintaining the treated sample in a low light environment for a time and at a temperature suitable for production of PpIX by cells present in the urine or urine derived fluid.

In certain embodiments, the PpIX precursor composition comprises a PpIX precursor and a serum free medium. A serum free medium is advantageously used in the present method because lipophilic fluorescent PpIX diffuses out of the cells faster in culture media containing serum.

As an alternative to using a cancer cell specific photosensitiser precursor composition, such as a PpIX precursor composition, the method may involve using a cancer cell specific photosensitiser composition comprising a cancer cell specific photosensitiser that does not need to be metabolised by cells to produce the photosensitiser. In certain embodiments, the cancer cell specific photosensitiser is hypericin. Several studies have demonstrated that hypericin mediated photodynamic therapy (Hyp-PDT) has high tumor specific cytotoxicity and minimal side effects.

In the method of the first aspect, the cancer cell specific photosensitiser composition and/or a cancer cell specific photosensitiser precursor composition is combined with a sample of urine or a urine derived fluid. Cells are in suspension in the urine or urine derived fluid sample and are not bound to a surface. As such, the cells are free and this provides maximum exposure of the cells to the cancer cell specific photosensitiser composition and/or a cancer cell specific photosensitiser precursor composition before diagnosis.

Advantageously, the method of the first aspect may also utilize at least one secondary luminescent cell nucleus stain for improved classification of cells containing a nucleus (ie. a nuclear stain).

In certain embodiments, the luminescent cell nucleus stain is fluorescent.

In certain embodiments, one or more additional fluorescent tags are used as luminescent cell nucleus stain.

In certain embodiments, the luminescent cell nucleus stain composition comprises a cell penetrative solvent. In these or other embodiments, the luminescent cell nucleus stain composition comprises an iron chelating solvent. In specific embodiments, the luminescent cell nucleus stain comprises dimethyl sulfoxide (DMSO).

In certain embodiments, the luminescent cell nucleus stain is nuclear red (ie. 4-amino-9,10-dihydro-1,3-dihydroxy-9,10-dioxo-2-anthracenesulfonic acid sodium salt or kernechtrot).

In certain embodiments, the cancer cell specific photosensitiser has a first excitation wavelength, the luminescent cell nucleus stain has a second excitation wavelength and the first and second wavelengths are spectrally separated from one another.

In certain embodiments, the sample of urine or a urine derived fluid is diluted with a buffer solution before the contacting step. The sample may be incubated after the contacting step.

The method of the first aspect thus provides improved methods for the *ex-vivo* or *in-vitro* detection of bladder cancer cells in urine or urine derived fluids. By using this improved method, bladder cancer cells and normal cells can be differentiated at a single cell level using fluorescence of the cancer cell specific photosensitiser and the luminescent cell nucleus stain.

The method of the first aspect also provides a method for detecting single cancer cells.

BRIEF DESCRIPTION OF DRAWINGS

Embodiments of the present invention will be discussed with reference to the accompanying drawings wherein:

Figure 1 shows a series of plots showing the mean fluorescence intensity values of different monolayer cells after HAL incubation;

Figure 2 shows the effect of time and concentration on the 5-ALA induced fluorescence of cell monolayers. a) Schematic of the monolayer *in vitro* experiment. b) time-dependent and concentration-dependent graphs of mean fluorescence intensities accumulated in different cell lines after incubation with various concentrations of HAL. c) intracellular localization of PpIX in bladder cancer cells (HT1376 and HT1197) and non-cancer cells (HFF) after incubation with 50μM HAL for 6h. Bright red fluorescent

observed in both cancer cells but none in non-cancer cells under fluorescence microscopy, magnification 10x. Brackets: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$;

Figure 3 shows PpIX production measurements over 24 h in different monolayer cells incubated with 50 μM HAL under Olympus inverted fluorescence microscope IX83 and PerkinElmer Operetta high-content imaging system;

Figure 4 shows a series of plots showing the effect of different vessels for imaging. Cells were incubated with different concentrations of HAL in various time points. After HAL incubation, cells in suspension were aliquoted to plasma polymer coated slides, uncoated PMMA slides and 96 well plates respectively;

Figure 5 shows the effect of time and concentration for cells in suspension. a) Schematic of the ex vivo experiment. b) time-dependent and concentration-dependent graphs of PpIX accumulated in different cell lines after incubation with various concentrations of HAL. c) intracellular localization of PpIX in bladder cancer cells (HT1376 and HT1197) and non-cancer cells (HFF) after incubation with 50μM HAL for 1h. Bright red fluorescent observed in both cancer cells but none in non-cancer cells under fluorescence microscopy, magnification 5x. Brackets: **** $p < 0.0001$;

Figure 6 shows plots showing the mean fluorescence intensity values of different cells in suspension after HAL incubation;

Figure 7 shows the effect of temperature on PPIX fluorescence in bladder cancer HT1376 cells and human foreskin fibroblast cells after incubation with various concentrations of HAL in a) 0.5h, b) 1h and c) 2h time. Note only for RT and 37°C, p-value was calculated as *** $p < 0.001$;

Figure 8 shows the combined effect of PpIX and nuclear red. a) PpIX has an excitation at 405 nm and emission at 635 nm. Nuclear red has an excitation and emission at 622 nm and 645 nm respectively. Nuclear red increased cancer cell sensitivity to PpIX accumulation after HAL incubation. b) Fluorescence microscopy images of different cell lines after incubation with HAL. Cells were incubated in serum-free medium with 100μM HAL for 1h. Cells were stained with 0.5 μM nuclear red for 15 min before observation. HAL-induced PpIX red fluorescence were observed in HT1197 and HT1376 cells but not in HFF cells. Original magnification 5x;

Figure 9 shows the effect of nuclear red on cell fluorescence intensity changes after 1 or 2 h incubated with various HAL concentrations in serum-free medium. Cells were measured under the custom made

inverted fluorescent microscope. b) cell viability of cells after 1 h 100μM HAL incubation with or without nuclear red staining, different types of cells were counted with the LUNA-II™;

Figure 10 shows plots showing the mean fluorescence intensity values of different cells in suspension after 1 and 2 h HAL incubation and nuclear red treatment;

Figure 11 shows histograms of the mean statistical distribution of the PpIX fluorescence intensity of non-cancer HFF and bladder cancer HT1376 cells. Cells were incubated with 50μM HAL in PBS for 1h and 2h respectively. After HAL incubation, cells were pipetted into POx coating channels without antibody and block; and

Figure 12 shows specific capture rate and actual cell number in microchannels of all substrates in a) bright field; b) PpIX; and c) cy5 filter. d) Schematic of the functionalized microchannel slide for cell capture experiment. Histogram data from the PpIX fluorescence images of anti-EpCAM functionalized substrate before and after wash and image of single cell captured via EpCAM affinity and PpIX positive was shown in e).

DESCRIPTION OF EMBODIMENTS

The following definitions are provided for specific terms which are used in the following written description.

As used in the specification and claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a PpIX precursor" includes a plurality of precursors, including mixtures thereof.

As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but do not exclude other elements. "Consisting essentially of" shall mean excluding other elements of any essential significance to the combination.

The term "about" or "approximately" means within an acceptable range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, e.g., the limitations of the measurement system. For example, "about" can mean a range of up to 20%, preferably up to 10%, more preferably up to 5%, and more preferably still up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean

within an order of magnitude, preferably within 5 fold, and more preferably within 2 fold, of a value. Unless otherwise stated, the term 'about' means within an acceptable error range for the particular value, such as ± 1-20%, preferably ± 1-10% and more preferably ±1-5%.

Where a range of values is provided, it is understood that each intervening value, between the upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within this disclosure. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within this disclosure, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either both of those included limits are also included in this disclosure.

The present disclosure arises from the inventors' studies of cancer cell specific photosensitiser-induced fluorescence (such as 5-ALA and HAL-induced fluorescence) in several human bladder cancer and non-cancer control cell lines with the aim to maximise the difference in fluorescence intensity between malignant and benign cell types. Different variables such as time, concentration and temperature were optimised as well as the addition of second fluorescent dye adjuvant to identify optimum conditions for the selective identification of cancer cells from voided urine samples.

Disclosed herein is a method of selectively detecting bladder cancer cells in urine or a urine derived fluid. The method comprises combining a sample of urine or a urine derived fluid to be analysed with a cancer cell specific photosensitiser composition and/or a cancer cell specific photosensitiser precursor composition to provide a treated sample. The treated sample is then contacted with one or more bladder cancer selective cell capture surfaces under conditions to bind at least some of the target bladder cancer cells to the cell capture surface. The cells captured on the cell capture surface are then exposed to light having a first excitation wavelength for the cancer cell specific photosensitiser. The intensity of light emitted at one or more selected cancer cell specific photosensitiser emission wavelengths from cells captured on the cell capture surface is then measured and the measured light intensity is used to determine if bladder cancer cells are present on the cell capture surface.

The methods disclosed herein provide significant improvements in the reliability and accuracy of bladder cancer cell detection compared to prior art methods.

The urine or urine derived fluid may be diluted before processed in the inventive method, for example with a suitable buffer solution. Dilution may be to 25%, 30%, 40% or 50% concentration or more. In one embodiment the sample is diluted to a 50% concentration.

The cancer cell specific photosensitiser can be formed ex-situ and the sample of urine or a urine derived fluid can be exposed to a composition containing the cancer cell specific photosensitiser under conditions for the cancer cell specific photosensitiser to be uptaken by some or all of any bladder cancer cells present in the sample. Hypericin is an example of a cancer cell specific photosensitiser that can be used in these embodiments. Conditions under which hypericin is uptaken by cancer cells are known in the art and an example is described in Berlanda *et al* (2010) *J Photochem Photobiol* B 100: 173–180. Other cancer cell specific photosensitisers are known in the art and can also be used for this purpose.

As an alternative, the sample of urine or a urine derived fluid can be exposed to a composition containing a cancer cell specific photosensitiser precursor composition. A cancer cell specific photosensitizer precursor is a compound, suite of compounds or composition that is metabolized at least by target cancer cells to produce a photosensitiser *in-situ*. An example of a photosensitiser that can be formed in this way is protoporphyrin IX (PpIX). In these embodiments, the cancer cell specific photosensitiser precursor composition is a PpIX precursor composition. The PpIX precursor can be any compound that is metabolized by cells to produce fluorescent PpIX but which is metabolized by cancer cells faster than healthy cells, leading to increased production of PpIX in cancer cells relative to healthy cells and, therefore, increased fluorescence in cancer cells relative to healthy cells. In certain specific embodiments, the PpIX precursor is selected from one or more of the group consisting of 5-aminolevulinic acid (5-ALA), hexaminolevulinate (HAL). In one embodiment, the PpIX precursor is 5-ALA. In another embodiment, the PpIX precursor is HAL.

When a PpIX precursor composition or other cancer cell specific photosensitiser precursor composition is used, the process may comprise maintaining the treated sample in a low light environment for a time and at a temperature suitable for production of PpIX by cells present in the urine or urine derived fluid.

5-ALA is a natural amino acid synthesized from succinyl-CoA and glycine in haem biosynthesis. 5-ALA does not fluoresce but is a precursor of the natural photosensitizing metabolite, PpIX (Ponka, P., *Cell Biology of Heme.* The American Journal of the Medical Sciences, 1999. **318**(4): p. 241-256; Yang, X., et al., Effects of Silencing Heme Biosynthesis Enzymes on 5-Aminolevulinic Acid-mediated Protoporphyrin IX Fluorescence and Photodynamic Therapy. Photochem Photobiol, 2015. **91**(4): p. 923-30; Spikes, J.D., The Historical Development of Ideas on Applications of Photosensitized Reactions in the Health Sciences, in Primary Photo-Processes in Biology and Medicine, R.V. Bensasson, et al., Editors. 1985, Springer US: Boston, MA. p. 209-227). Exogenous administration of 5-ALA increases the accumulation of PpIX primarily in tumour tissues. When excited by blue light, tumour cells emit a bright red fluorescence, reportedly several fold brighter than normal healthy cells (Kennedy, J.C., R.H. Pottier, and

D.C. Pross, Photodynamic therapy with endogenous protoporphyrin IX: basic principles and present clinical experience. J Photochem Photobiol B, 1990. **6**(1-2): p. 143-8; Kriegmair M, B.R., Knüchel R, Ehsan A, Steinbach P, Lumper W, Hofstädter F, Hofstetter A., *Photodynamic diagnosis of urothelial neoplasms after intravesicular instillation of 5-aminolevulinic acid.* Urologe A, 1994. **33**((4)): p. 270-5).

In some circumstances the use of 5-ALA can give rise to non-specific fluorescence displayed by urinary bacteria after incubation (Fotinos, N., et al., Effects on gram-negative and gram-positive bacteria mediated by 5-aminolevulinic Acid and 5-aminolevulinic acid derivatives. Antimicrob Agents Chemother, 2008. **52**(4): p. 1366-73). The presence of a significant number of background cells and urine metabolites (Fu, C.Y., et al., Fluorescence detection of bladder cancer using urine cytology. Int J Oncol, 2007. **31**(3): p. 525-30) in conjunction with a low number of cancer cells actually being shed in urine as well as lengthy sample processing can place some limitations on the usefulness of 5-ALA as a PpIX precursor as a cancer specific photodiagnosis compound.

Hexaminolevulinate (HAL) has been successfully used as a 5-ALA derivative, and has been approved for *in vivo* photodynamic detection of bladder cancer by the FDA and European Union. It has been reported to increase PpIX fluorescence intensity in lower concentrations and with shorter exposure than 5-ALA itself (Lange, N., et al., Photodetection of early human bladder cancer based on the fluorescence of 5-aminolaevulinic acid hexylester-induced protoporphyrin IX: a pilot study. British Journal of Cancer, 1999. **80**(1-2): p. 185-193; Abdel-Kader, M.H., *History of Photodynamic Therapy*, in *Photodynamic Therapy: From Theory to Application*, M.H. Abdel-Kader, Editor. 2014, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 3-22). HAL is more lipophilic than 5-ALA, which enhances the cellular uptake through the plasma membrane. Several research groups (Čunderlíková, B., et al., Detection of urinary bladder cancer with flow cytometry and hexaminolevulinate in urine samples. Cytopathology, 2007. **18**(2): p. 87-95; Nakai, Y., et al., Spectrophotometric photodynamic detection involving extracorporeal treatment with hexaminolevulinate for bladder cancer cells in voided urine. Journal of Cancer Research and Clinical Oncology, 2017. **143**(11): p. 2309-2316) reported that the fluorescence intensity of cancer cells was higher and stronger than normal cells after HAL incubation. As a result, lower dosage and shorter incubation time are needed, and to achieve higher PpIX fluorescence selectivity in tumour cells (Marti, A., et al., Comparison of Aminolevulinic Acid and Hexylester Aminolevulinate Induced Protoporphyrin IX Distribution in Human Bladder Cancer. The Journal of Urology, 2003. **170**(2): p. 428-432; Dragoescu, O., et al., Photodynamic diagnosis of non-muscle invasive bladder cancer using hexaminolevulinic acid. Rom J Morphol Embryol, 2011. **52**(1): p. 123-7).

Thus, the problem of non-specific fluorescence of bacteria may be partially resolved by using HAL instead of 5-ALA. Indeed, Fotinos et al. indicated that no fluorescent porphyrins were produced by bacteria when HAL is used. This finding has since been supported by Nakai et al.. Thus, in certain specific embodiments, the PpIX precursor is HAL.

In certain embodiments, the PpIX precursor composition comprises the PpIX precursor and a serum free medium. A serum free medium is advantageously used in the present method because lipophilic fluorescent PpIX diffuses out of the cells faster in culture media containing serum. The term "serum-free" is used herein to mean that all whole serum is excluded from the medium. A wide range of serum free media are commercially available and can be used. Suitable serum free media include but are not limited to phosphate buffer saline (PBS), Iscove's modified Dulbecco's medium (IMDM), McCoy's 5a, Dulbecco's modified Eagle's Medium (DMEM), and Ham's-F12.

The concentration of the PpIX precursor in the PpIX precursor composition may be from about 10 μM to about 150 μM, such as about 10 μM, about 20 μM, about 30 μM, about 40 μM, about 50 μM, about 60 μM, about 70 μM, about 80 μM, about 90 μM, about 100 μM, about 110 μM, about 120 μM, about 130 μM, about 140 μM, or about 150 μM. For example, the concentration of the PpIX precursor in the PpIX precursor composition may be from about 25 μM to about 125 μM, from about 25 μM to about 100 μM or from about 50 μM to about 100 μM. In certain specific embodiments, the concentration of the PpIX precursor in the PpIX precursor composition is about 50 μM.

In the methods of the present disclosure, the PpIX precursor composition is combined with a sample of urine or a urine derived fluid. In these methods, the cells are in suspension in the urine or urine derived fluid sample and are not bound to a surface. As such, the cells are free and this provides maximum exposure of the cells to the PpIX precursor composition before diagnosis. Indeed, a significant increase of PpIX fluorescence intensities in cells in suspension was observed when compared to monolayer cells.

Advantageously, the methods of the present disclosure may also utilize at least one (i.e. one or more) secondary luminescent cell nucleus stain for improved classification of cells containing a nucleus (i.e. a nuclear stain). In these embodiments, the sample of urine or a urine derived fluid is combined with a luminescent cell nucleus stain composition to provide a treated sample. The urine or a urine derived fluid may be combined with the luminescent cell nucleus stain at the same time as the urine or a urine derived fluid is combined with the cancer cell specific photosensitiser composition and/or a cancer cell specific photosensitiser precursor composition. Alternatively, the urine or a urine derived fluid may be combined with the luminescent cell nucleus stain before the urine or a urine derived fluid is combined with the

cancer cell specific photosensitiser composition and/or a cancer cell specific photosensitiser precursor composition. Alternatively, the urine or a urine derived fluid may be combined with the luminescent cell nucleus stain after the urine or a urine derived fluid is combined with the cancer cell specific photosensitiser composition and/or a cancer cell specific photosensitiser precursor composition.

In certain embodiments, the cancer cell specific photosensitiser has a first excitation wavelength, the luminescent cell nucleus stain has a second excitation wavelength and the first and second wavelengths are spectrally separated from one another.

When a luminescent cell nucleus stain is used, the cells captured on the cell capture surface are exposed to light having a second excitation wavelength for the luminescent cell nucleus stain. The cells can be exposed to light of a second wavelength at the same time as they are exposed to the light having the first excitation wavelength for the cancer cell specific photosensitiser. Alternatively, the cells can be exposed to light of a second excitation wavelength for the luminescent cell nucleus stain before they are exposed to the light having the first excitation wavelength for the cancer cell specific photosensitiser. Alternatively, the cells can be exposed to light of a second excitation wavelength for the luminescent cell nucleus stain after they are exposed to the light having the first excitation wavelength for the cancer cell specific photosensitiser.

When a luminescent cell nucleus stain is used, the intensity of light emitted at one or more selected luminescent cell nucleus stain emission wavelengths from cells captured on the cell capture surface is measured. The intensity of light emitted at one or more selected luminescent cell nucleus stain emission wavelengths can be measured at the same time as the intensity of light emitted at one or more selected cancer cell specific photosensitiser emission wavelengths is measured. Alternatively, the intensity of light emitted at one or more selected luminescent cell nucleus stain emission wavelengths can be measured before the intensity of light emitted at one or more selected cancer cell specific photosensitiser emission wavelengths is measured. Alternatively, the intensity of light emitted at one or more selected luminescent cell nucleus stain emission wavelengths can be measured after the intensity of light emitted at one or more selected cancer cell specific photosensitiser emission wavelengths is measured.

In certain embodiments, the luminescent cell nucleus stain is fluorescent.

In certain embodiments, the luminescent cell nucleus stain is nuclear red (ie. 4-amino-9,10-dihydro-1,3-dihydroxy-9,10-dioxo-2-anthracenesulfonic acid sodium salt or kernechtrot). The use of nuclear red may provide certain advantages because its excitation wavelength is well separated from the excitation

wavelength of the cancer cell specific photosensitisers used, and thus the cancer cell specific photosensitiser and the luminescent cell nucleus stain can be used simultaneously without any cross over between optical channels. In the present work, the inventors surprisingly found that emissions of a range of known luminescent cell nucleus stain were not published as being similar to the PpIX emissions but, in practice, there were significant emission of a number of nuclei stains in the expected PpIX emission range, thereby leading to the possibility of false PPIX signals. Furthermore, the present inventors also surprisingly found that the fluorescence is stronger in cancer cells when 5-ALA and nuclear red stain are used concurrently in the method.

In one specific embodiment two luminescent cell nucleus stains are used. For example, nuclear read and a secondary fluorescent tag may be used. The excitation of the secondary tag may be at ~480nm and emission may be at ~513nm.

In certain embodiments, the luminescent cell nucleus stain composition comprises a cell penetrative solvent. In these or other embodiments, the luminescent cell nucleus stain composition comprises an iron chelating solvent. In specific embodiments, the luminescent cell nucleus stain comprises dimethyl sulfoxide (DMSO).

The concentration of luminescent cell nucleus stain in the luminescent cell nucleus stain composition may be from about 0.1 μM to about 10 μM. As will be appreciated, the concentration of luminescent cell nucleus stain used will depend on the nature of the stain used. For example, the concentration of Nuclear red stain may be about 10μM. The concentration of Nuclear red stain may be from about 0.1 μM to about 1 μM, such as about 0.1 μM, about 0.2 μM, about 0.3 μM, about 0.4 μM, about 0.5 μM, about 0.6 μM, about 0.7 μM, about 0.8 μM, about 0.9 μM or about 1.0 μM. In certain embodiments, the concentration of luminescent cell nucleus stain in the luminescent cell nucleus stain composition is about 0.5uM.

The treated sample is maintained in a low light environment for a time and at a temperature suitable for production of PpIX by cells present in the urine or urine derived fluid to provide an incubated sample.

A range of time and temperatures can be used for the incubation. Temperature is important. At 4 °C there is no fluorescence whereas 37 °C is better for PpIX precursor metabolism. Incubation may be from 4 °C to 37 °C, such as from room temperature to 37 °C.

The incubation time is also important. The contrast is better at 1 hour than, for example, 4 hours. Thus, in certain embodiments the treated sample is maintained in a low light environment for a period of from

about 0.5 hour to about 6 hours, from about 0.5 hour to about 5 hours, from about 1 hour to about 5 hours, from about 1 hour to about 3 hours, such as from about 0.5 hour to about 1.5 hours. For example, as the incubation time may be about 30 mins, about 1 hour, about 1 hour 30 mins, about 2 hours, about 2 hours 30 mins, about 3 hours, about 3 hours 30 mins, about 4 hours, about 4 hours 30 mins or about 5 hours.

Incubation is preferably carried out without any movement of the sample.

In certain embodiments the treated sample is maintained in a low light environment for about 1 hour at about room temperature (i.e. 15 °C to 25 °C).

In certain embodiments a certain amount of the top of the solution is removed after the incubation step. For example, about 50%, about 75%, about 80%, about 85%, about 90% or about 95% of the top of the solution is removed. In one embodiment about 90% of the top of the solution is removed. A relevant volume of the remaining liquid is used in the next step.

The incubated sample is then contacted with one or more bladder cancer selective cell capture surfaces under conditions to bind at least some of the target bladder cancer cells to the cell capture surface. This is an important step because the rarity of target cells can limit the applicability or use of some prior art processes. These limitations can be overcome by using microfluidic devices designed to selectively immobilise cancer cells from mixed cells in suspension (Macgregor-Ramiasa, M., et al., *A platform for selective immuno-capture of cancer cells from urine.* Biosensors and Bioelectronics, 2017. **96**(Supplement C): p. 373-380). Such enriching microdevices used in combination with markers like HAL and Nuclear red™ significantly improve the sensitivity of cancer detection in urine, including down to single cell sensitivity.

Microfluidic approaches for the non-invasive selective capture of cancer cells in voided urine have previously been reported by at least some of the present inventors (Macgregor-Ramiasa, M., et al.). Carcinoma specific EpCAM antibodies were immobilized on polyoxazoline (POx) plasma polymers by covalent bonds in microfluidic channels. The anti-EpCAM functionalized POx film facilitates the selective capture of cancer cells in urine samples. Thus, in certain embodiments, the cell capture surface comprises a plasma polymerised polyoxazoline (PPOx) coating as described in published international patent application WO 2017/035566. An Epithelial Cell Adhesion molecule (EpCAM) anti-body cell capture agent is covalently or non-covalently bound to the PPOx coating using the methods described in WO 2017/035566.

The bladder cancer selective cell capture surface may be part of a microfluidic device described in co-pending Australian Provisional Patent Application No. 2018903556 titled "Diagnostic Device". Briefly, the device comprises a first fluid source in the form of a preparation chamber, at least one microchannel, wherein the or each microchannel comprises a fluid inlet and a fluid outlet and a capture surface for selective capturing the target analyte, and a fluid control means configured to transfer of a specific volume of fluid from the first fluid source to the or each microchannel. In use, a patient urine sample is placed in the preparation chamber, where it is exposed to 50 μM of HAL reagent and 0.5 μM of Nuclear red nuclei stain and incubated for 1 hour at room temperature. After the incubation period, a set volume of patient sample is dispensed to the at least one microchannel containing an Epithelial Cell Adhesion molecule (EpCAM) anti-body cell capture agent covalently or non-covalently bound to at least one inner surface of the microchannel. After a second incubation period, a PBS rinse is dispensed into the microchannel and allowed to flow through the microchannel.

The microchannel is then imaged using fluorescent microscopy (with specific imaging conditions described below) to identify cells likely to indicate presence of bladder tumor. Specifically, the cells captured on the cell capture surface are then exposed to light having a first excitation wavelength for the cancer cell specific photosensitiser (eg. PpIX) and a second excitation wavelength for the luminescent cell nucleus stain (eg. nuclear red). The intensity of light emitted at one or more selected cancer cell specific photosensitiser emission wavelengths and, optionally, at one or more selected luminescent cell nucleus stain emission wavelengths from cells captured on the cell capture surface is measured and the measured light intensity or intensities is/are used to determine if bladder cancer cells are present on the cell capture surface.

More specifically, to visualise PpIX fluorescence the cell capture surface can be imaged on a fluorescent microscope fitted with a light source capable of emitting with a peak in the range of 390-410nm. Light sources that can be used include laser, LED light source or white light with appropriate filters, but are not limited to. A filter set can be used with a narrow the range of the excitation filter at 400nm. The narrow excitation range used to induce PpIX fluorescence is important as, otherwise nuclear red could be excited and thus visible in the PpIX optical channel. The light intensity used may vary from 10 to 100%, and the exposure time from 1 to 1000ms. A long range emission filter at 620 nm can be used to detect the emitted light. The cell capture surface can be imaged in multiple optical channels, such as a first optical channel for detecting PpIX fluorescence, a second optical channel to detect nuclear red fluorescence. Optionally, a third bright field channel could be included in the images. The order of the optical channels may be important to minimise the bleaching of the PpIX. The skilled person will appreciate that there is some

flexibility with filtering and peaks used and, for example, changing a filter or filter type by small amounts or a different brand of light source with slightly different peaks or bandwidths i.e. 395 to 405nm peak light source may still be sufficient to excite fluorophores. Similarly, small changes to the full half width maximum (FHWM) of various filters, i.e. the difference between a 80nm FWHM filter compared to a 120nm FWHM would also likely be sufficient. Additionally the PpIX emission could also be single bandwidth instead of a long pass filter.

In one specific embodiment the inventive method may comprise the following specific steps:

1. Dilute the urine sample to 50% concentration using buffer solution
2. Add 50uM HAL and 0.5uM Nuclear red stain
3. Incubate at room temperature for 0.5-1.5 hour without movement
4. Remove the top ~90% of solution
5. Apply a relevant volume of the remaining liquid to the capture surface
6. Incubate at room temperature for 10-45 minutes without movement in dark environment
7. Rinse capture surface gently with buffer solution
8. Image fluorescent response of objects remaining on the capture surface
9. Apply image analysis to identify relevant objects of interest.

The methods disclosed herein thus provide improved methods for the ex-vivo or in-vitro detection of bladder cancer cells in urine or urine derived fluids. By using these improved methods, bladder cancer cells and normal cells can be differentiated at a single cell level using fluorescence of PpIX and the luminescent cell nucleus stain.

The method of the first aspect also provides a method for detecting single cancer cells. This method can be used to detect single cancer cells for further evaluation or diagnosis.

EXAMPLES

Materials and Methods

Cell Culture

Human foreskin fibroblasts (HFFs) (Cell Lines Service, DKFZ, Heidelberg, Germany) were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Life Technologies, Australia) supplemented with 10%

(v/v) fetal calf serum, 100 IU/mL penicillin, 100 µg/mL streptomycin and 1% L-glutamine (200mM). Four human Caucasian bladder transitional-cell carcinoma cell lines were obtained from European Collection of Authenticated Cell Cultures (ECACC). EJ138 (ECACC No. 85061108); HT 1376 (ECACC No.87032402); HT 1197 (ECACC No.87032403) and were cultured in Minimum Essential Medium Eagle (MEME) (Sigma-Aldrich, Australia) supplemented with 10% (v/v) fetal calf serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, 1% L-glutamine (200mM) and 1% 100x MEM Non-essential Amino Acid Solution. RT4 (ECACC No. 91091914) was cultured in McCoy's 5A medium (Sigma-Aldrich, Australia) supplemented with 10% (v/v) fetal calf serum, 100 IU/mL penicillin, 100 µg/mL streptomycin and 1% L-glutamine (200mM). All cells were cultured at 37°C with 5% CO_2 in a humidified atmosphere. Cells/viabilities were measured with an automated cell counter LUNA-II™ (Logos Biosystems, South Korea) after staining with trypan blue.

Chemicals

Hexaminolevulinate (HAL) hydrochloride was purchased from Sigma-Aldrich (NSW, Australia). HAL was supplied as a powder and was dissolved in phosphate-buffered saline (PBS). Solutions of different concentrations (from 0 to 250µM) were prepared. Phosphate buffer saline (PBS) tablets were purchased from Sigma-Aldrich. Nuclear red™ LCS1 (Cat# 17542) which is a cell-permeant nucleic acid detection dye was obtained from AAT Bioquest® (CA 94085, USA). Dimethylsulphoxide (DMSO) was used as a reconstitution solvent for Nuclear red™ LCS1 stock solution. Polyclonal goat anti-human EpCAM antibody (AF960) was purchased from R&D Systems. Chemicals were stored in aliquots at -20°C until use.

PpIX fluorescence microscopy

Cell Monolayer In vitro

Cells were seeded in 96-well plates (Corning, NY) and cultured at different time points with serum-free cultured medium containing different concentrations of HAL (0-250 µM) for 2-24h (Fig. 1) at 37°C. Serum-free media is necessary because lipophilic fluorescent PpIX diffuses out of the cells faster in culture media containing serum (Stepp, H.G., et al. Bladder tissue diagnostics utilizing Protoporphyrin IX fluorescence detection. in International Symposium on Biomedical Optics Europe '94. 1995. SPIE). The 96-well plates were kept in the CO_2 incubator during the whole incubation period. After incubation, the HAL containing medium was discarded, and the cells were washed with PBS (Fig. 2a).

PpIX intensity was measured using inverted fluorescent microscopes (Olympus, IX83, Japan or a custom made imaging instrument) in both of which the light excitation wavelength was set to 405 nm and emission wavelength to 600 nm, using a specially designed filter cube (Chroma). An Operetta high-content imaging system (PerkinElmer Inc. USA) with suitable light source and emission filter was also used.

Cells in suspension

Cells were removed from the culturing flasks after trypsinization, then resuspended in the serum-free medium to dilute the trypsin and adjusted to the targeted cell density. The cells in suspension were incubated in the dark from 30 min to 6 hours with serum-free mediums containing different concentrations of HAL at 37°C in a humidified 5% CO_2 incubator. After incubation, the cells were centrifuged at 1500rpm for 5 mins and the HAL containing medium discarded. Cells were resuspended with PBS, and 100µl per well was aliquoted into 96-wells plates.

PpIX fluorescence was imaged using appropriate LED lamps and custom filters, referred to as PpIX optical channel in the following. Images at x 5 magnification were recorded and the fluorescence intensity measured using CellSens software (Olympus, Japan). Fluorescence images were captured and used for visualizing the cellular localization of PpIX.

Temperature

To evaluate the effect of temperature on PpIX fluorescence, cells were stored in the dark at different temperature settings (4°C, RT ~23°C, and 37°C) in closed test tubes following the exogenous HAL incubation described above.

Nuclear red

Human bladder cancer cells HT1376 and non-cancer foreskin fibroblasts cells HFF were incubated in the dark at 37°C with 0, 10, 50 and 100µM HAL in serum-free medium for 1 and 2 hours. The selected HAL concentrations were based on the "cells in suspension" results. Cells were then centrifuged, and HAL-containing medium decanted before incubating for another 15 minutes with nuclear red stain diluted in DMSO. Prior to imaging, cells were washed with PBS twice. Nuclear red fluorescence was imaged using appropriate LED lamps and custom filters, referred to as Cy5 optical channel in the following.

Cell Capture

Plasma surface deposition

Polymethylmethacrylate (PMMA) slides with 3 microchannel grooves were coated with a plasma deposited oxazoline thin films using a custom made parallel plate plasma reactor, as described previously (Macgregor-Ramiasa, M., et al.). 2-Methyl-2-oxazoline (Sigma Aldrich Australia) precursor was introduced in the reactor with a $1.3e^{-1}$ mbar working pressure. The plasma was ignited for 3 minutes with a continuous radiofrequency power of 50W. The coated microfluidic slides (chips) were kept in the dark under vacuum until further use. Not all channels may actually be used in the diagnosis but some of the channels may be used as a quality check.

Functionalization of the polyoxazoline-coated substrate

One microchannel of the POx coated slides was used, unmodified, as positive control. POx biocompatibility (Ramiasa, M.N., et al., *Plasma polymerised polyoxazoline thin films for biomedical applications.* Chemical Communications, 2015. **51**(20): p. 4279-4282) allows non-specific attachment of all cell types. The test channel was functionalized with anti-EpCAM antibodies (EpCAM). 60µL of 10µg/mL anti-EpCAM antibody solution in PBS was added to the microchannel and stored in 4°C overnight for binding. The next day, the microchannel slide was incubated at 37°C for 1h, then incubated for 45 min with 1mg/mL skim milk solution to block the remaining POx surface. The last microchannel was also blocked with 1mg/mL skim milk solution, to be used as a negative control on which cell binding is inhibited. The microchannels were gently rinsed with PBS three times to remove any unbound protein and fresh PBS was added.

Selective cancer cell capture assay

Non-cancer HFF and bladder cancer HT1376 monolayer cells were trypsinized. Both cells in suspension were incubated with 50µM HAL at 37°C for first 30 mins and 23°C for the next 30 mins. Cancer HT1376 cells were stained with 0.25µM nuclear red for 10 mins. The cell densities of both cell lines were adjusted to range between $2x10^5$ to $3x10^5$ cells per mL, then mixed together in 1:1 ratio. 50µL of the mixed cell solution was pipetted into each microchannel for fluorescence microscope imaging. After 45 minutes, unbound cells were rinsed off with fresh PBS in all microchannels and the surfaces were imaged a second time. The fluorescence micrographs were used to determine the number of cell captured and PpIX fluorescence intensities.

Statistical analysis

Mean values and standard deviation (SD) of fluorescence intensity values for each concentration at each time point were calculated and analysed for each cell line. The student's t-test (GraphPad and Minitab 18 software) was used to establish the significance of differences. Statistical significance was considered as $p < 0.05$.

Results and Discussion

PpIX fluorescence in monolayer cell in vitro

Both bladder cancer cells lines monolayers (HT1197 and HT1376) displayed a bright red fluorescence when incubated with serum-free mediums containing various concentrations (10-250 μM) of HAL over 2 to 24 h incubation period. The differences in PpIX fluorescence intensity and intracellular localization of PpIX in each cell monolayer are shown in Fig. 2b and 2c. Non cancer HFF cells had lower PpIX fluorescence intensity than the cancer cells which remained rather constant for all HAL concentrations and incubation times. In contrast, the mean fluorescence intensities of cancer cell lines HT1197 and HT1376 demonstrated a time- and concentration-dependent pattern. The HAL-induced PpIX fluorescence in HT1197 and HT1376 increased with time from 2 to 8h before decreasing from the 10.5h time point (Fig. 1). A comparable time dependence was observed for two additional bladder cancer cell lines EJ138 and RT4, (Fig. 1). This trend was independent from the imaging systems used to measure the fluorescence intensity (Fig. 3). Furthermore, the fluorescence intensity was greatest for HAL concentrations between 25 to 100 μM. Lower HAL concentrations did not induce significant PpIX biosynthesis in the cancer cells compared to the healthy HFF cells. On the other hand, HAL concentrations higher than 100mM seemed to initiate cytotoxicity and damage to the cells as noted by a decrease in fluorescence intensity and the formation of extracellular vesicles (Fig. 4). Again, the observed trends in fluorescence intensity were consistent amongst all bladder cancer cell lines and all instruments, thus confirming the robustness of the process (Fig. 1 and 3). Finally, HAL-induced fluorescence of the cancer cell monolayer was tested on different cell growth substrates, namely plasma POx coated and non-coated microfluidic channels, Fig. 5. Again, the fluorescence intensity of cancer cells was higher than that of healthy cells and it increased with time just the same as on tissue culture plate. From these results, we deemed the HAL concentrations of 50 and 100 μM to be the optimum for further experiments.

The patterns of intracellular PpIX localization (Fig. 2c) observed by fluorescence microscopy were different between bladder cancer H1197 and HT1376 cells. PpIX was not only visible in discreet cell

organelles but also extended to the cytoplasm and nuclear region. Fluorescence in HT1376 and HT1197 cancer cells was also distributed in a few very bright red spots and in the plasma membrane.

It is also worth noting that PpIX production by exogenous HAL administration was found to be a cell density dependent phenomenon in the monolayer configuration. The PpIX fluorescence increased with increasing monolayer cell numbers, in agreement with previous studies (Steinbach, P., et al., Cellular fluorescence of the endogenous photosensitizer protoporphyrin IX following exposure to 5-aminolevulinic acid. Photochem Photobiol, 1995. **62**(5): p. 887-95; Moan, J., et al.[1], Protoporphyrin IX accumulation in cells treated with 5-aminolevulinic acid: dependence on cell density, cell size and cell cycle. Int J Cancer, 1998. **75**(1): p. 134-9; Casas, A., et al., Photosensitization and mechanism of cytotoxicity induced by the use of ALA derivatives in photodynamic therapy. Br J Cancer, 2001. **85**(2): p. 279-84). It is well known that cancer cells tend to grow and divide densely which increases their cell to cell interaction (Moan et al.[1]). One manifestation of this cancer specific behaviour is an increased intercellular sharing of metabolites such as ions, molecules and likely in this case, PpIX (Casas, A., et al.).The HAL-induced PpIX molecules are synthesized in the mitochondria via the haem biosynthesis pathway. ABCG2 exports PpIX from the mitochondria to the cytosol and reduced ABCG2 levels have been reported to boost PpIX fluorescence in human bladder cancer T24 and human histiocytic lymphoma U937 cells (Kobuchi, H., et al., Mitochondrial Localization of ABC Transporter ABCG2 and Its Function in 5-Aminolevulinic Acid-Mediated Protoporphyrin IX Accumulation. PLoS ONE, 2012. **7**(11): p. e50082). Some studies have pursued the role of other membrane transporters such as heme exporter feline leukemia virus subgroup C receptor (FLVCR) and translocator protein (TSPO) on PpIX intra and intercellular transport. Rosenberg et al. showed that the suppression of TSPO protein can cause PpIX accumulation (Rosenberg, N., et al., In vitro catabolic effect of protoporphyrin IX in human osteoblast-like cells: possible role of the 18 kDa mitochondrial translocator protein. Journal of Bioenergetics and Biomembranes, 2013. **45**(4): p. 333-341), while others have demonstrated that PpIX can be exported out of the cells by FLVCR1 (Yang, Z., et al., Kinetics and specificity of feline leukemia virus subgroup C receptor (FLVCR) export function and its dependence on hemopexin. J Biol Chem, 2010. **285**(37): p. 28874-82). The presence of PpIX fluorescence in the cytoplasm and along the intercellular space observed here for HT1376 and HT1197 cell lines therefore warrant further investigation as it could be indicative of enzymatic activity and/or protein expression level unique to cancer cells.

PpIX Fluorescence in cells in suspension

The purpose of our current studies is to evaluate the feasibility of HAL for the PDD of cancer cells in patient urine samples. For the non-invasive diagnostic of bladder cancer, HAL would be applied to urine

samples in which cancer cells may be shed. In this practical situation, the cancer cells are not in the form of an ideal monolayer, but rather are dispersed in suspension. To test the real use scenario as accurately as possible we used cells in suspension for further experiments. Also, in an effort to mimic realistic time and reagent constraints, we tested a lower HAL concentration range (0 – 150 μM) and extended the investigation towards shorter incubation time (0.5 – 6 h).

A significant increase of PpIX fluorescence intensities in cells in suspension was observed when compared to monolayer cells. This could be due to the additional cell surface area available to uptake HAL for cells in suspension. At comparable incubation time (2h) and HAL concentration (50μM) the fluorescence of cancer cells in suspension was three time higher than that of cell monolayers. The accumulation of PpIX increased in a time-dependent manner for both cancer cells HT1197 & HT1376. The absolute fluorescence intensity for cancer HT1376 cells increased with time from 3500 a.u. at 0.5h to 6000 a.u. at 2h. Non-cancer HFF cells expressed a slight increase in the PpIX level over 2h HAL incubation but not at 0.5h and 1h. No significant difference in fluorescence emission was observed over the range of HAL concentrations investigated (10 μM to 150 μM). For all cell lines in suspension PpIX fluorescence is time dependent but seemingly not dose independent. Similar to the monolayer configuration, the fluorescence intensities variance from the mean was greater for longer HAL incubation time in cancer cells (Fig. 5b and 6). Overall, the difference of mean fluorescence intensities between both cancer cells and non-cancer cells is statistically significant ($p < 0.0001$).

Based on our data as depicted in Fig. 5b, we propose that the optimum condition for *ex vivo* diagnostics is 50uM HAL and 1h incubation.

Effect of temperature

PpIX is synthesized through the heme biosynthesis pathway. This multi-step process can be affected by many factors, including temperature. Tumours have a higher temperature compared to neighbouring normal tissues (Dang, C.V., *Links between metabolism and cancer.* Genes Dev, 2012. **26**(9): p. 877-90; DeBerardinis, R.J. and N.S. Chandel, *Fundamentals of cancer metabolism.* Sci Adv, 2016. **2**(5): p. e1600200) and studies have shown that more cellular PpIX is formed at higher temperatures in human cells (Moan, J., et al.[2], *The Temperature Dependence of Protoporphyrin IX Production in Cells and Tissues.* Photochemistry and Photobiology, 1999. **70**(4): p. 669-673; van den Akker, J.T.H.M., et al., Effect of elevating the skin temperature during topical ALA application on in vitro ALA penetration through mouse skin and in vivo PpIX production in human skin. Photochemical & Photobiological Sciences, 2004. **3**(3): p. 263-267). This is because the heme biosynthesis is an enzymatic process and the

rate of enzymatic activities increases as the temperature is raised, until the optimum temperature of around 37°C in human cells (Moan et al.[2]; Willey, A., R.R. Anderson, and F.H. Sakamoto, Temperature-modulated photodynamic therapy for the treatment of actinic keratosis on the extremities: a pilot study. Dermatol Surg, 2014. **40**(10): p. 1094-102). This cancer specific phenomenon is readily used in blue light cystoscopy for the identification of tumors *in situ*. To determine if temperature is a factor affecting the PpIX fluorescence *ex situ*, we used the same experimental setup as for cells in suspension but incubated the cells in HAL at 4°C and room temperature of 23°C instead of 37°C. Our data (Fig. 7) indicates that the cancer specific PpIX fluorescence is not only dependent on HAL incubation time but also temperature. The PpIX fluorescence intensities of HT1376 cells incubated at 23°C was comparable to that observed at 37°C, and significantly brighter than in healthy cells. However, for incubation conducted at 4°C, the fluorescence in the cancer cells was just as low as that of healthy HFF cells, and so at all three time points and all concentrations. HFF did not express significant differences in fluorescence intensity at any of the temperatures and time conditions examined.

During the first 0.5 hour of HAL application, the PpIX fluorescence was almost 2 times lower than after 2 hours incubation at 37°C, similarly to what was observed for cells in suspension. The difference in mean fluorescence intensities between HFF and HT1376 cells was highly statistically significant when incubated at 37°C [95% CI p=0.00008 (0.5h), p=0.00002 (1h), p= 0.000000002 (2h)] and statistically different when incubated at room temperature [95% CI p= 0.00007 (0.5h), p= 0.00001 (1h), p= 0.0000001 (2h)]. Therefore, we concluded the best approach for use in *ex vivo* would be to incubate HAL at 37°C for 2h.

Several studies investigated how the temperature influences 5-ALA uptake and its conversion to PpIX *in vitro* or *in vivo*. Most of the previous *in vivo* research focused on skin cancer (van den Akker et al.; Juzeniene, A., et al., Temperature effect on accumulation of protoporphyrin IX after topical application of 5-aminolevulinic acid and its methylester and hexylester derivatives in normal mouse skin. Photochem Photobiol, 2002. **76**(4): p. 452-6; Mamalis, A., et al., Temperature dependent impact of thermal aminolaevulinic acid photodynamic therapy on apoptosis and reactive oxygen species generation in human dermal fibroblasts. British Journal of Dermatology, 2016. **175**(3): p. 512-519). Their data proves that PpIX production in the skin cells is temperature-dependent. No or little PpIX production occurs when the skin temperature was below 15°C. In good agreement with the results presented here, these works reported that PpIX fluorescence increased as the temperature increased between 23°C and 37°C. Other research groups investigating different types of cancer cells monolayers *in vitro* also indicated that a rise in temperature increases the PpIX production (Moan et al.[2]; Rud, E., et al., 5-aminolevulinic acid, but not

5-aminolevulinic acid esters, is transported into adenocarcinoma cells by system BETA transporters. Photochem Photobiol, 2000. **71**(5): p. 640-7). Here we demonstrated for the first time that this trend also holds for cells in suspension which is an important finding for the use of HAL in *ex-vivo* diagnosis of cancer from body fluids.

Effect of nuclear red

Nuclear red was chosen as a tool for the subcellular localization of PpIX. It is a cell-permeant nucleic acid detection dye which stains nuclei in live cells, and shows red fluorescence significantly enhanced upon binding to DNA. The fluorescence spectrum of PpIX and nuclear red are shown in Fig. 9a. The Soret band of the excitation spectrum has a maximum at 405 nm and emission at 635 nm for PpIX. However, nuclear red exhibits excitation at 622 nm and emission at 645 nm. As the excitation wavelengths are well separated, there is no cross over from one fluorescent dye to the other when appropriate narrow band excitation filters are used.

After 1 hour of HAL incubation and 15 minutes nuclear red treatment in 37°C, both bladder cancer HT1376 and HT1197 cells showed clear red fluorescence when observed in the nuclear red and HAL fluorescence channels. Fluorescence microscopy revealed that, while the nuclear red localised in the cell nucleus as expected, accumulated PpIX mainly localized in other cell organelles in both bladder cancer HT1197 and HT1376 cells (Fig. 8b). Based on current knowledge of the Heme biosynthesis pathway, it is likely that PpIX is accumulating in the mitochondria, however, further investigation is warranted to confirm PpIX localisation and determine if it differs from one cell type to another.

Most remarkably, we observed that the addition of nuclear red increased, over time, the specificity of the HAL-induced fluorescence towards cancer cells. Indeed, after 2 hours HAL incubation, the accumulation of PpIX in both cancer HT1197 and HT1376 cells was markedly higher in cells treated with nuclear red than HAL alone. In contrast, the much less fluorescent HFF did not exhibit any significant difference in the presence or absence of nuclear red. Similar results were observed after just 1h HAL incubation, but only for HT1376. (Fig.9a.) Quantitatively, after 2h incubation with 50uM HAL, the PpIX fluorescence intensity increased by 1/5 and 1/3 for HT1197 and HT1376 cancer cells, respectively, when incubated with nuclear red. In the same conditions, no increase in fluorescence is observed for the healthy HFF cells. Overall, the fluorescence of HT1376, was after 2h incubation with 50uM HAL and nuclear red, 4.5 times higher than that of healthy HFF (Fig. 10).

This result indicates that the post-treatment with nuclear red increased the PpIX accumulation specificity so significantly that it could be used as an adjuvant to better discriminate between healthy and cancer cells. To shed light on the mechanism that could explain this enhanced cancer specificity, we investigated the cell viability in the relevant experimental conditions (HAL 100µM, 1 h, and nuclear red 15 min incubation). Results are shown in Fig. 9b. Both HAL and nuclear red have moderate toxicity for HT1376 and HT1197. HFF, on the other hand, bear a 50% decrease in cell viability when treated with HAL and nuclear red. Interestingly, the % of viability for both cancer cell lines are slightly lower after 1 h HAL incubation alone.

The difference observed, in both viability and PpIX fluorescence, between cancer and healthy cells could be the result of distinctive enzymatic activities, which can in part be explained by existing literature investigating the heme biosynthesis pathway triggered by the administration of exogenous HAL. It has been shown that excess free heme causes the formation of cytotoxic lipid peroxide which increases membrane permeability and sequentially may trigger cell lysis and death (Kumar, S. and U. Bandyopadhyay, *Free heme toxicity and its detoxification systems in human.* Toxicology Letters, 2005. **157**(3): p. 175-188; Chiabrando, D., et al., Heme in pathophysiology: a matter of scavenging, metabolism and trafficking across cell membranes. Front Pharmacol, 2014. **5**: p. 61). Yet, at least two distinct aberrant enzymatic expressions have been reported that could explain why heme production is hindered and PpIX accumulates in bladder cancer cells.

First, iron deficiency, or more precisely the lack of available mitochondrial iron is very common in cancer (Ludwig, H., et al., Prevalence of iron deficiency across different tumors and its association with poor performance status, disease status and anemia. Annals of oncology : official journal of the European Society for Medical Oncology, 2013. **24**(7): p. 1886-1892) and has been attributed, in bladder carcinoma, to low level of mitochondrial iron transporters Mitoferrin 1 and 2 (Paradkar, P.N., et al., Regulation of mitochondrial iron import through differential turnover of mitoferrin 1 and mitoferrin 2. Molecular and cellular biology, 2009. **29**(4): p. 1007-1016; Hayashi, M., et al., The effect of iron ion on the specificity of photodynamic therapy with 5-aminolevulinic acid. PLoS One, 2015. **10**(3): p. e0122351). Second, low levels of (ferrochelatase)FECH, an enzyme that catalyzes the formation of heme through the integration of iron into PpIX, has been reported in bladder cancer tissue (Hagiya, Y., et al., Expression levels of PEPT1 and ABCG2 play key roles in 5-aminolevulinic acid (ALA)-induced tumor-specific protoporphyrin IX (PpIX) accumulation in bladder cancer. Photodiagnosis and Photodynamic Therapy, 2013. **10**(3): p. 288-295; Nakai, Y., et al., Expression of ferrochelatase has a strong correlation in protoporphyrin IX accumulation with photodynamic detection of bladder cancer. Photodiagnosis and

Photodynamic Therapy, 2016. **13**(Supplement C): p. 225-232). Low FECH level means that PpIX accumulates rather than forming peroxide triggering heme. Taken together, low level of FECH and iron could reduce the free heme toxicity in cancer cells compared to HFF cells.

While the exact mechanism by which the nuclear red affected the PpIX production is not clear, the role played by the solvent DMSO should not be overlooked. DMSO is a highly membrane penetrative 5-ALA enhancer (De Rosa, F.S., et al., A vehicle for photodynamic therapy of skin cancer: influence of dimethylsulphoxide on 5-aminolevulinic acid in vitro cutaneous permeation and in vivo protoporphyrin IX accumulation determined by confocal microscopy. Journal of Controlled Release, 2000. **65**(3): p. 359-366) but also acts as an iron chelator (Conder, L.H., S.I. Woodard, and H.A. Dailey, Multiple mechanisms for the regulation of haem synthesis during erythroid cell differentiation. Possible role for coproporphyrinogen oxidase. Biochem J, 1991. **275 (Pt 2)**: p. 321-6) in conjunction with 5-ALA. In fact, it has been shown that DMSO (Conder et al.; Malik, Z., et al., Topical application of 5-aminolevulinic acid, DMSO and EDTA: protoporphyrin IX accumulation in skin and tumours of mice. Journal of Photochemistry and Photobiology B: Biology, 1995. **28**(3): p. 213-218; De Rosa et al.; Casas, A., et al., ALA and ALA hexyl ester-induced porphyrin synthesis in chemically induced skin tumours: the role of different vehicles on improving photosensitization. Br J Cancer, 2001. **85**(11): p. 1794-800; Zhang, L.-W., Y.-P. Fang, and J.-Y. Fang, *Enhancement techniques for improving 5-aminolevulinic acid delivery through the skin.* Dermatologica Sinica, 2011. **29**(1): p. 1-7) can enhance the 5-ALA-induced PpIX accumulation in cells. Our results further suggest that DMSO actually increases PpIX fluorescence in cancer cell more than in healthy cells, thus enhancing the specificity of HAL.

Cell capture experiment

In order to determine the efficiency of HAL fluorescence in captured cell experiments, both healthy HFF and cancer cells were stained with HAL: 50μM for 30 mins at 37°C and 30 mins at 23°C. To confirm the nature of the captured cells, the cancer cells were also stained with nuclear red which is imaged in the cy5 optical channel. The mixture of healthy and cancer cells in suspension were dispensed inside three microfluidic channels, a negative control 'block' channel, a positive control 'plasma Pox' coated channel, and the test EpCAM functionalised' channel. Images of the channels before and after PBS rinse were taken in bright field, and using custom fluorescent filters for 5-ALA and Cy5. The number of cells observed in bright field images corresponded to the total number of cancer and non-cancer cells, while the number of cells observed with the Cy5 fluorescent filter correspond to cancer cells only. Finally, we investigated the number of fluorescent cells observed with the 5-ALA fluorescent filter set, to determine the capacity of HAL at discriminating between healthy and cancer cells in the captured cell population.

The number of cells present in the channels before rinse as well as the number of cells captured in the three channels are shown in Fig. 11. From the cell counts before rinse we can see that the number of Cy5 positive cells (Fig 12c, blue bars) correspond to the number of cells visible in the PpIX optical channel (Fig 12b, blue bars). Since non-cancer cells are expected to express either no or very low PpIX fluorescence intensity after HAL-treatment, these results confirms that the cells visible and accounted for with the PpIX optical filters are indeed cancer cells.

For all optical filters, less than 3 % of the cells remained bound to the block surface after rinse and more than 95% of the cell population remain bound to the POx coated channel, thus indicating that both negative and positive control surfaces performed as expected.

The channel functionalized with anti-EpCAM antibody captured 87% of the cells stained with Nuclear red, demonstrating that the selective capture of cancer cell was successful. Most importantly, when observed through the PpIX filter, an 88% capture rate was also achieved.

A histogram representing the mean statistical distribution of the PpIX fluorescence intensity in anti-EpCAM functionalized substrate before and after wash is shown in Fig. 11d. The peak of the intensity profile occurs at a higher intensity value after rinse than before 2100 and 1850 a.u., respectively. This is in good agreement with our results presented above which indicated that the PpIX fluorescence intensity increases from 1 to 2h incubation time. Since the fluorescence intensity of HFF is shifted towards lower values (Fig. 11), detailed interrogation of the cell fluorescence intensity histogram could be used to determine the nature of the captured cells.

Together, these results show that the immuno-capture platform is highly selective towards cancer cells and that HAL-induced PpIX fluorescence can be used to gain a second level of confidence on the malignant nature of the capture cell population. In fact, combining the selective immune-capture platform to the cancer specific 5-ALA induced PpIX accumulation allow for single cells to be individually identified in the microfluidic device, Fig. 12e.

Conclusion

Overall, our findings show that the HAL based fluorescence markedly increased in cancer cells rather than non-cancer cells regardless of whether these were cells in a monolayer or cells in suspension. Optimal conditions for achieving cancer cell fluorescence were with cells in suspension incubated with 50 μM HAL in 37°C for 2 hours along with concurrent or subsequent nuclear red treatment. This fluorescent technique has been successfully optimised and applied to the capture and detection of bladder cancer cells

with a high 88% capture rate. This represents an attractive method for diagnosing the presence of bladder cancer cells in urine to be used as a diagnostic test in urine from patients with suspected bladder cancer or who are undergoing follow up surveillance.

Throughout the specification and the claims that follow, unless the context requires otherwise, the words "comprise" and "include" and variations such as "comprising" and "including" will be understood to imply the inclusion of a stated integer or group of integers, but not the exclusion of any other integer or group of integers.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgement of any form of suggestion that such prior art forms part of the common general knowledge.

It will be appreciated by those skilled in the art that the invention is not restricted in its use to the particular application described. Neither is the present invention restricted in its preferred embodiment with regard to the particular elements and/or features described or depicted herein. It will be appreciated that the invention is not limited to the embodiment or embodiments disclosed, but is capable of numerous rearrangements, modifications and substitutions without departing from the scope of the invention as set forth and defined by the following claims.

Please note that the following claims are provisional claims only, and are provided as examples of possible claims and are not intended to limit the scope of what may be claimed in any future patent applications based on the present application. Integers may be added to or omitted from the example claims at a later date so as to further define or re-define the invention.

CLAIMS

1. A method of selectively detecting bladder cancer cells in urine or a urine derived fluid, the method comprising:

combining a sample of urine or a urine derived fluid to be analysed with a cancer cell specific photosensitiser composition and/or a cancer cell specific photosensitiser precursor composition;

contacting the treated sample with one or more bladder cancer selective cell capture surfaces under conditions to bind at least some of the target bladder cancer cells to the cell capture surface;

exposing the cells captured on the cell capture surface to light having a first excitation wavelength for the cancer cell specific photosensitiser,

measuring the intensity of light emitted at one or more selected cancer cell specific photosensitiser emission wavelengths from cells captured on the cell capture surface; and

determining if bladder cancer cells are present on the cell capture surface using the measured light intensity.

2. The method of claim 1, wherein the cancer cell specific photosensitiser precursor composition is a PpIX precursor composition.

3. The method of claim 2, wherein the PpIX precursor is selected from one or more of the group consisting of 5-aminolevulinic acid and hexaminolevulinate.

4. The method of claim 3, wherein the PpIX precursor is 5-aminolevulinic acid.

5. The method of claim 3, wherein the PpIX precursor is hexaminolevulinate.

6. The method of any one of claims 1 to 5, wherein the PpIX precursor composition comprises a PpIX precursor and a serum free medium.

7. The method of any one of claims 1 to 6, wherein the method comprises maintaining the treated sample in a low light environment for a time and at a temperature suitable for production of PpIX by cells present in the urine or urine derived fluid.

8. The method of claim 1, wherein the cancer cell specific photosensitiser is hypericin.

9. The method of any one of claims 1 to 8, further comprising:

combining the sample of urine or a urine derived fluid with a luminescent cell nucleus stain composition to provide the treated sample;

exposing the cells captured on the cell capture surface to light having a second excitation wavelength for the luminescent cell nucleus stain; and

measuring the intensity of light emitted at one or more selected luminescent cell nucleus stain emission wavelengths from cells captured on the cell capture surface.

10. The method of claim 9, wherein the luminescent cell nucleus stain is fluorescent.

11. The method of claim 10, wherein the luminescent cell nucleus stain is 4-amino-9,10-dihydro-1,3-dihydroxy-9,10-dioxo-2-anthracenesulfonic acid sodium salt.

12. The method of claim 10 or 11, wherein one or more additional fluorescent tags are used as luminescent cell nucleus stain.

13. The method of any one of claims 9 to 12, wherein the luminescent cell nucleus stain composition comprises a cell penetrative solvent.

14. The method of any one of claims 9 to 13, wherein the luminescent cell nucleus stain composition comprises an iron chelating solvent.

15. The method of any one of claims 9 to 14, wherein the luminescent cell nucleus stain comprises dimethyl sulfoxide.

16. The method of any one of claims 9 to 15, wherein the PpIX has a first excitation wavelength, the luminescent cell nucleus stain has a second excitation wavelength and the first and second wavelengths are spectrally separated from one another.

17. The method of any one of claims 1 to 16, wherein the sample of urine or a urine derived fluid is diluted with a buffer solution before the contacting step.

18. The method of any one of claims 1 to 17, wherein the sample is incubated after the contacting step.

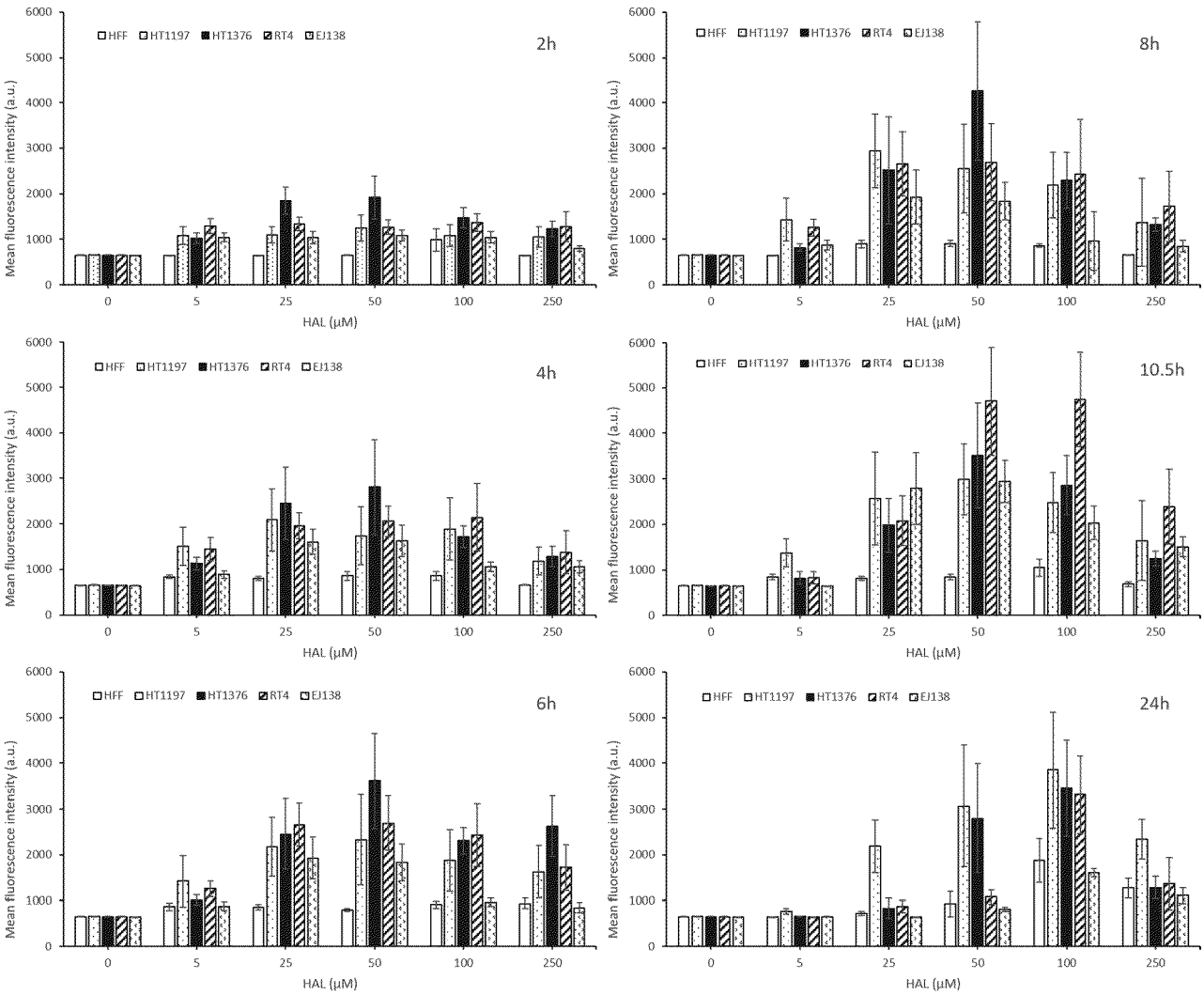

HFF HT1197 HT1376 RT4 EJ138
2h
8h
4h
10.5h
6h
24h
Mean fluorescence intensity (a.u.)
HAL (μM)
0
1000
2000
3000
4000
5000
6000
0
5
25
50
100
250

FIGURE 1

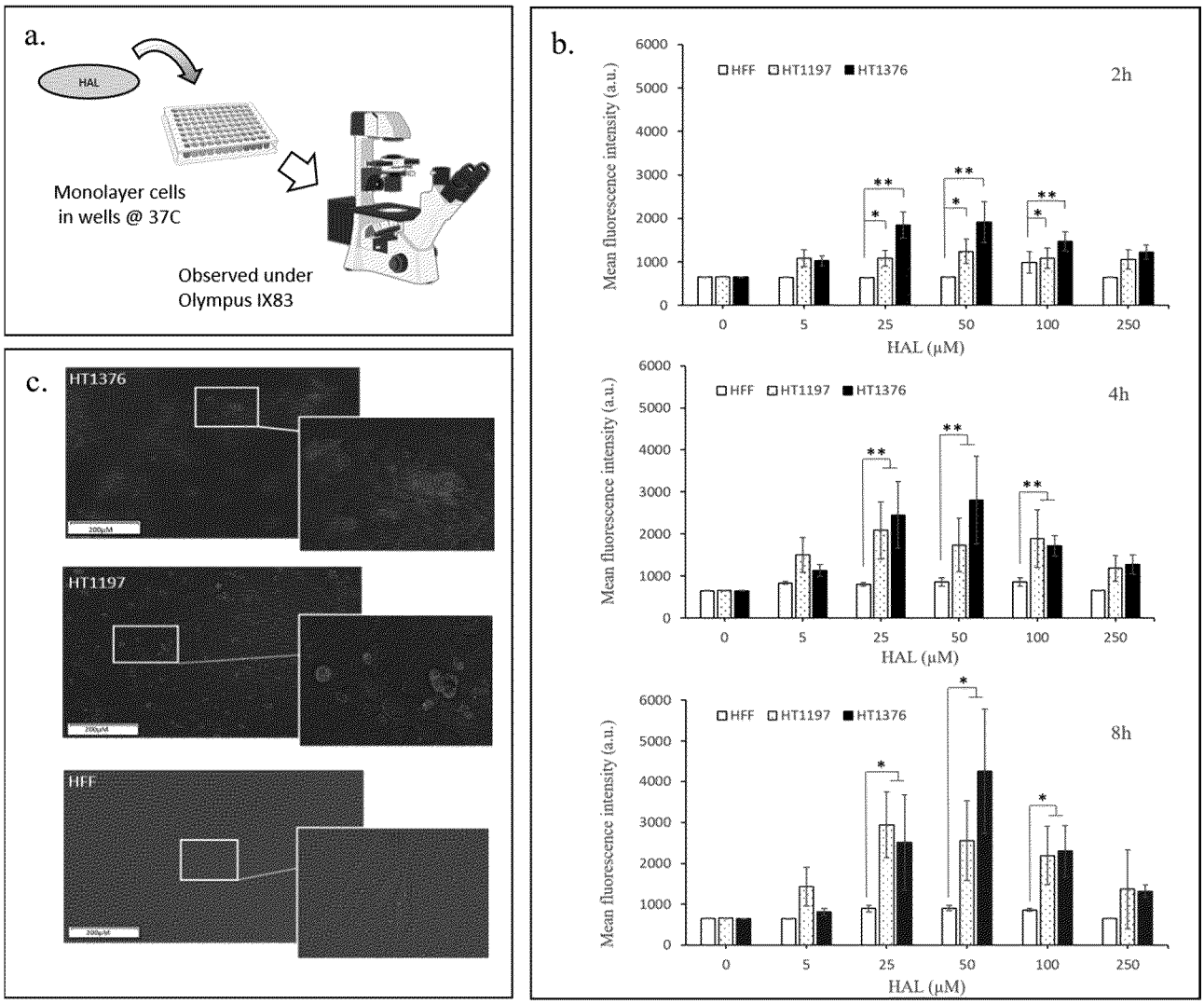

a.
HAL
Monolayer cells
in wells @ 37C
Observed under
Olympus IX83
b.
HFF HT1197 HT1376
2h
4h
8h
Mean fluorescence intensity (a.u.)
HAL (µM)
0
5
25
50
100
250
c.
HT1376
HT1197
HFF

FIGURE 2

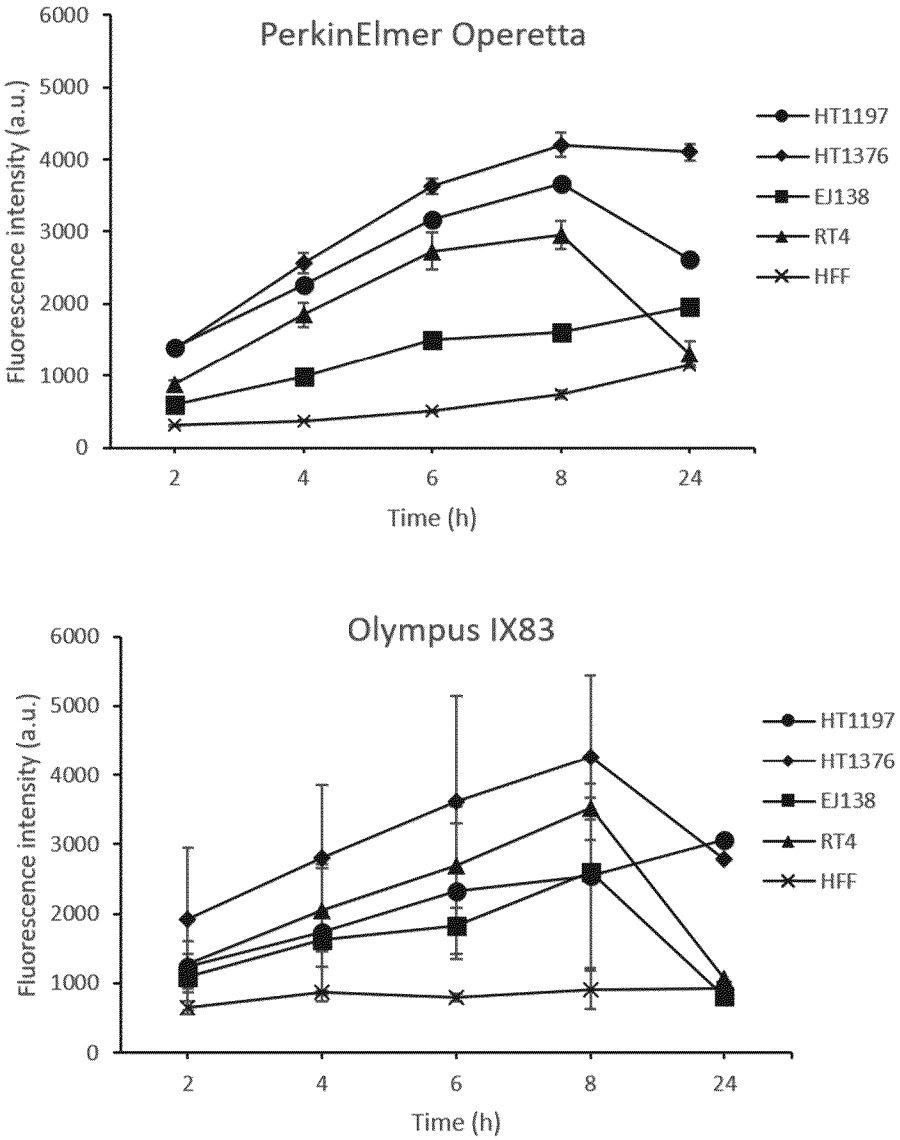

PerkinElmer Operetta
Fluorescence intensity (a.u.)
6000
5000
4000
3000
2000
1000
0
2
4
6
8
24
Time (h)
HT1197
HT1376
EJ138
RT4
HFF
Olympus IX83
Fluorescence intensity (a.u.)
6000
5000
4000
3000
2000
1000
0
2
4
6
8
24
Time (h)
HT1197
HT1376
EJ138
RT4
HFF

FIGURE 3

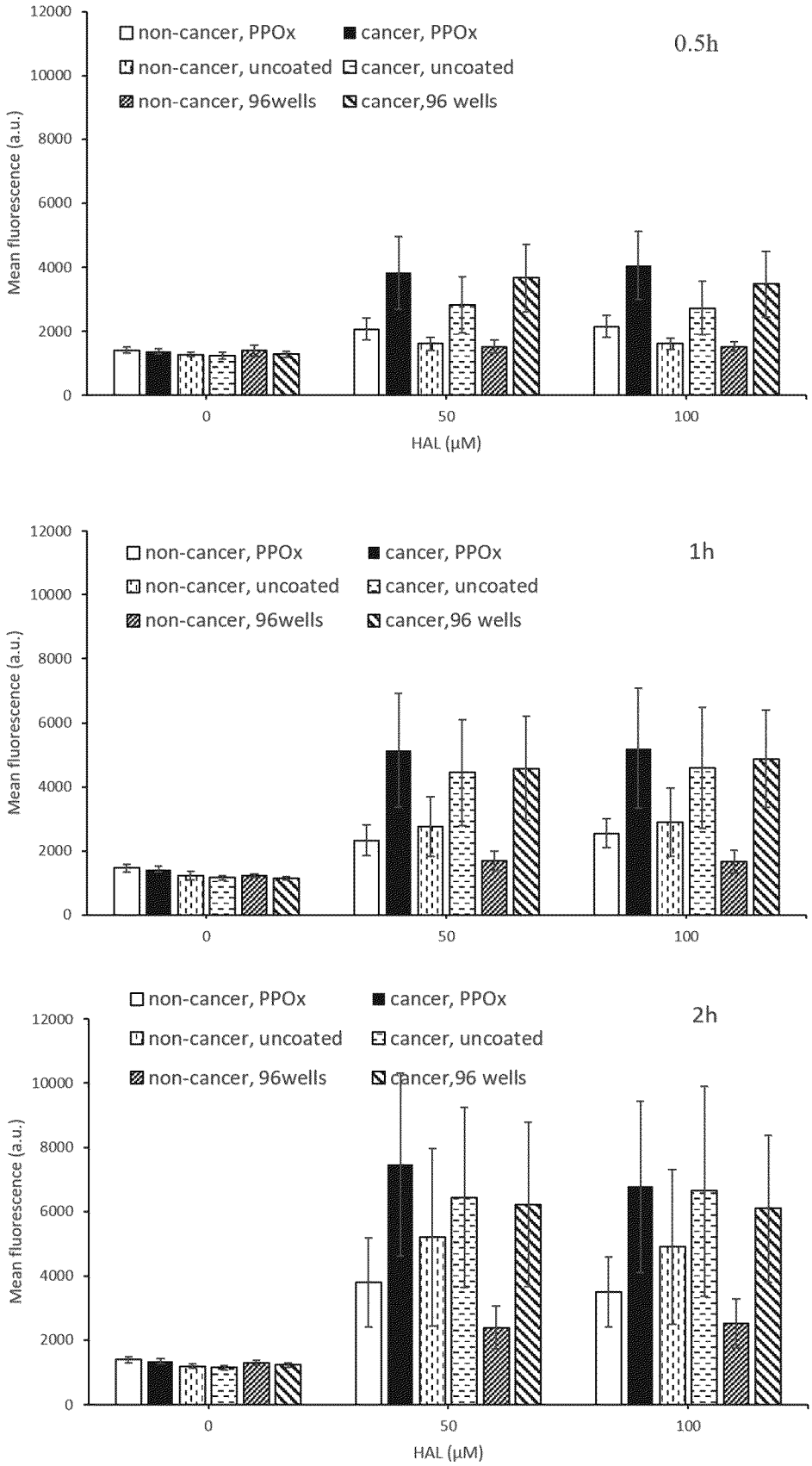

0.5h
non-cancer, PPOx
cancer, PPOx
non-cancer, uncoated
cancer, uncoated
non-cancer, 96wells
cancer,96 wells
Mean fluorescence (a.u.)
12000
10000
8000
6000
4000
2000
0
0
50
100
HAL (µM)
1h
non-cancer, PPOx
cancer, PPOx
non-cancer, uncoated
cancer, uncoated
non-cancer, 96wells
cancer,96 wells
Mean fluorescence (a.u.)
2h
non-cancer, PPOx
cancer, PPOx
non-cancer, uncoated
cancer, uncoated
non-cancer, 96wells
cancer,96 wells
Mean fluorescence (a.u.)
HAL (µM)

FIGURE 4

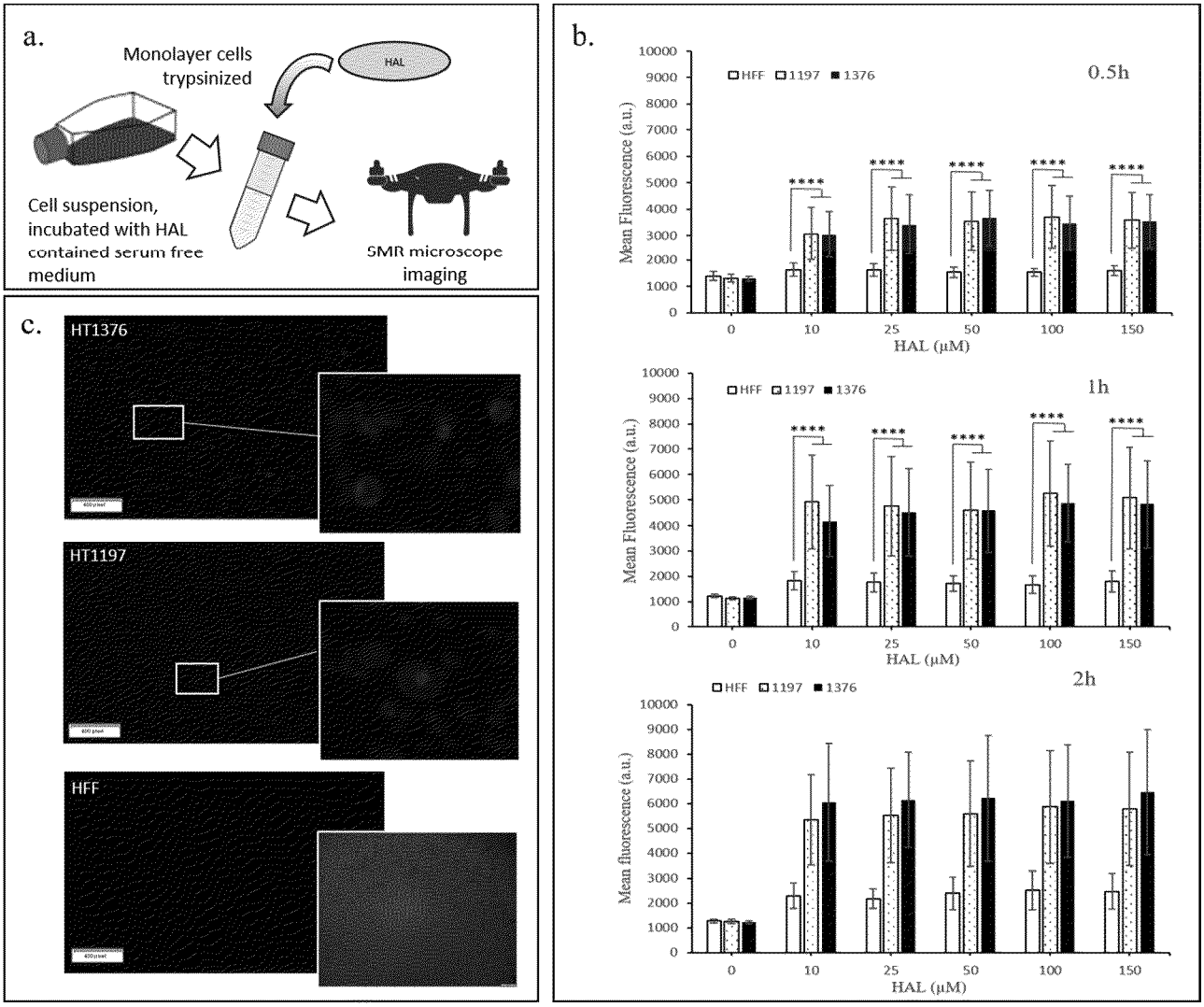

a.
Monolayer cells trypsinized
HAL
Cell suspension, incubated with HAL contained serum free medium
SMR microscope imaging
b.
HFF 1197 1376
0.5h
1h
2h
Mean Fluorescence (a.u.)
Mean fluorescence (a.u.)
HAL (μM)
0
10
25
50
100
150
****
c.
HT1376
HT1197
HFF

FIGURE 5

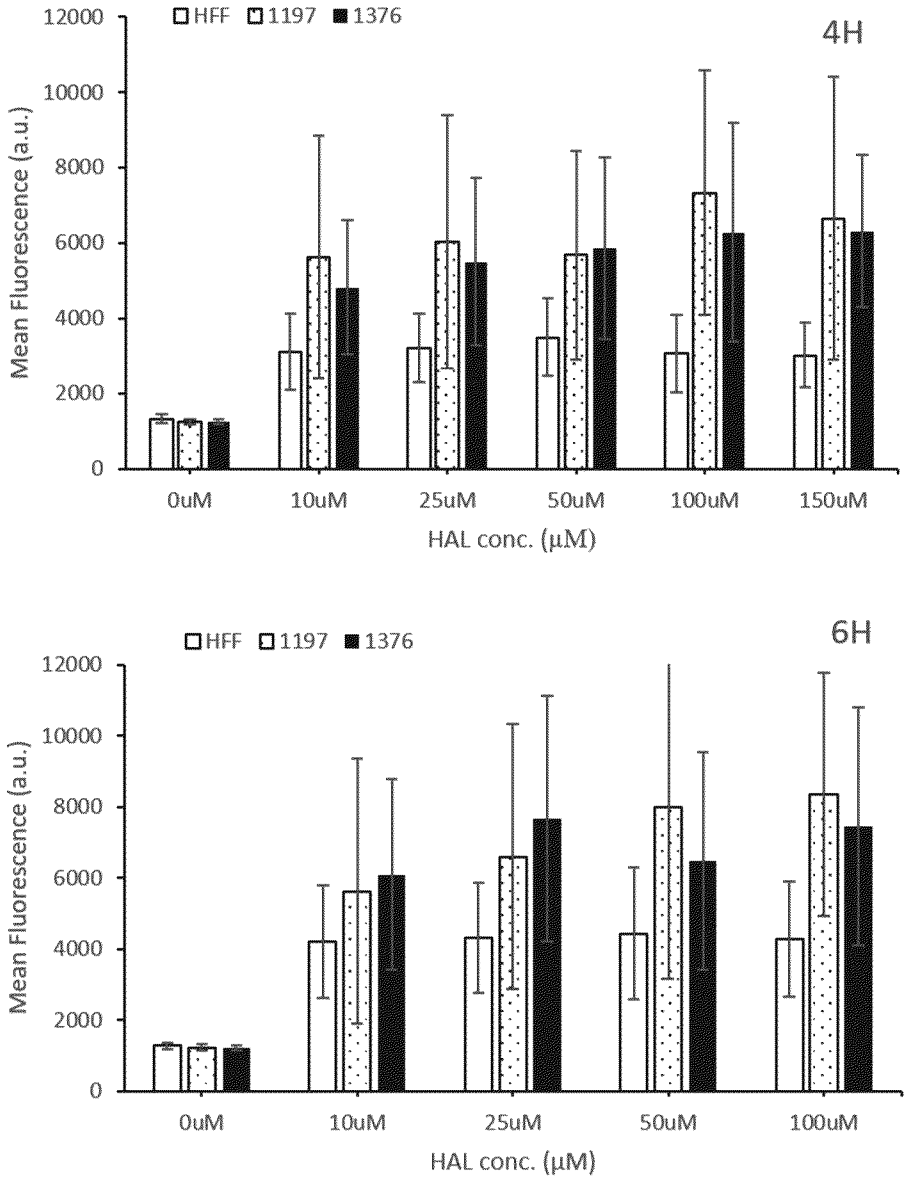

HFF
1197
1376
4H
Mean Fluorescence (a.u.)
12000
10000
8000
6000
4000
2000
0
0uM
10uM
25uM
50uM
100uM
150uM
HAL conc. (µM)
HFF
1197
1376
6H
Mean Fluorescence (a.u.)
12000
10000
8000
6000
4000
2000
0
0uM
10uM
25uM
50uM
100uM
HAL conc. (µM)

FIGURE 6

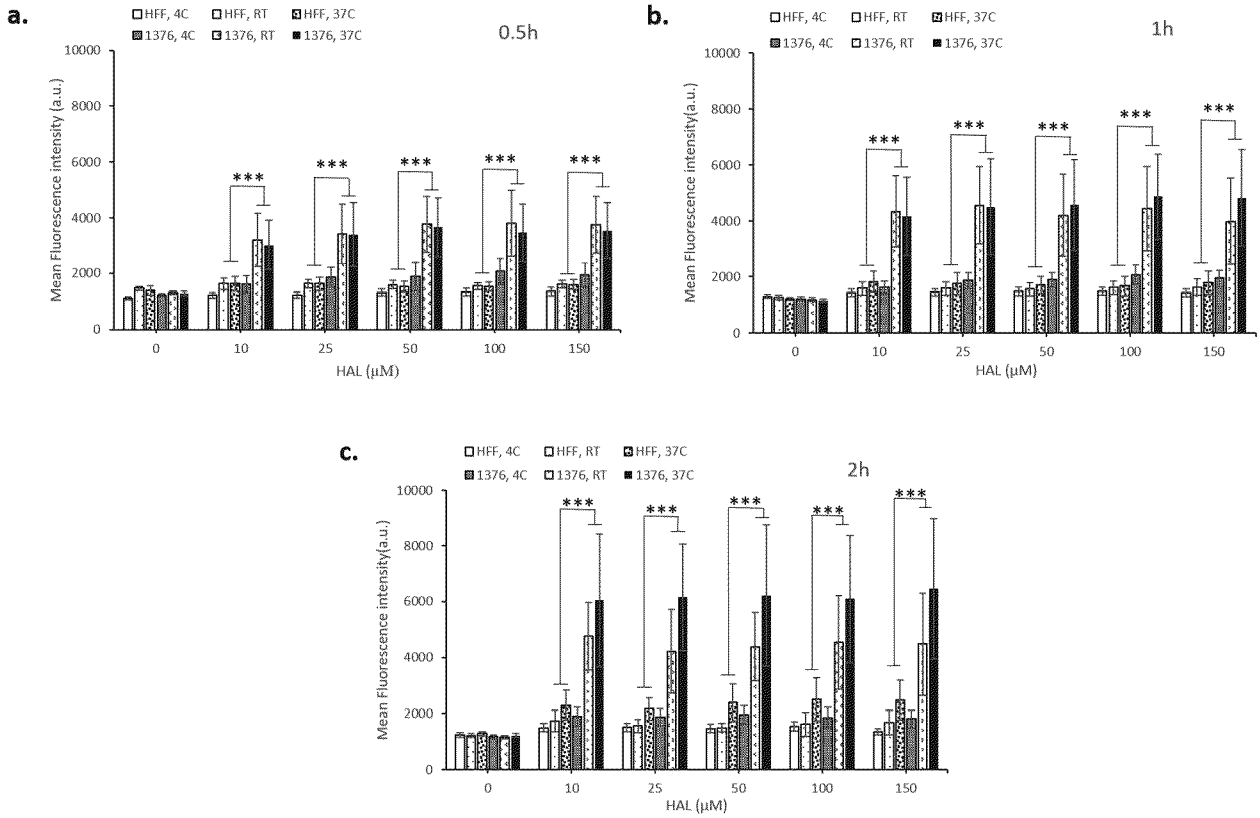
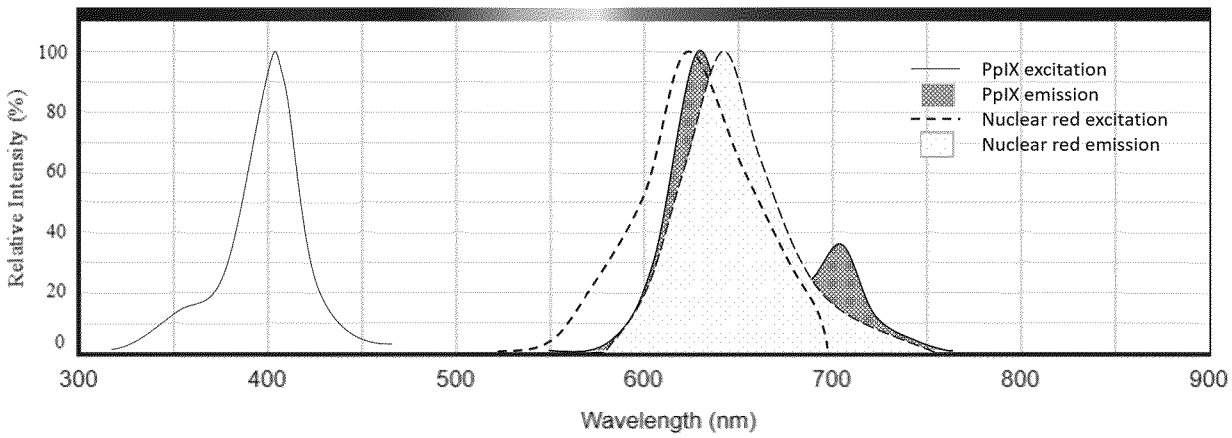

a.
HFF, 4C
HFF, RT
HFF, 37C
1376, 4C
1376, RT
1376, 37C
0.5h
Mean Fluorescence intensity (a.u.)
10000
8000
6000
4000
2000
0
***
0
10
25
50
100
150
HAL (µM)
b.
1h
c.
2h

FIGURE 7

a.


Relative Intensity (%)
100
80
60
40
20
0
300
400
500
600
700
800
900
Wavelength (nm)
PpIX excitation
PpIX emission
Nuclear red excitation
Nuclear red emission

b.

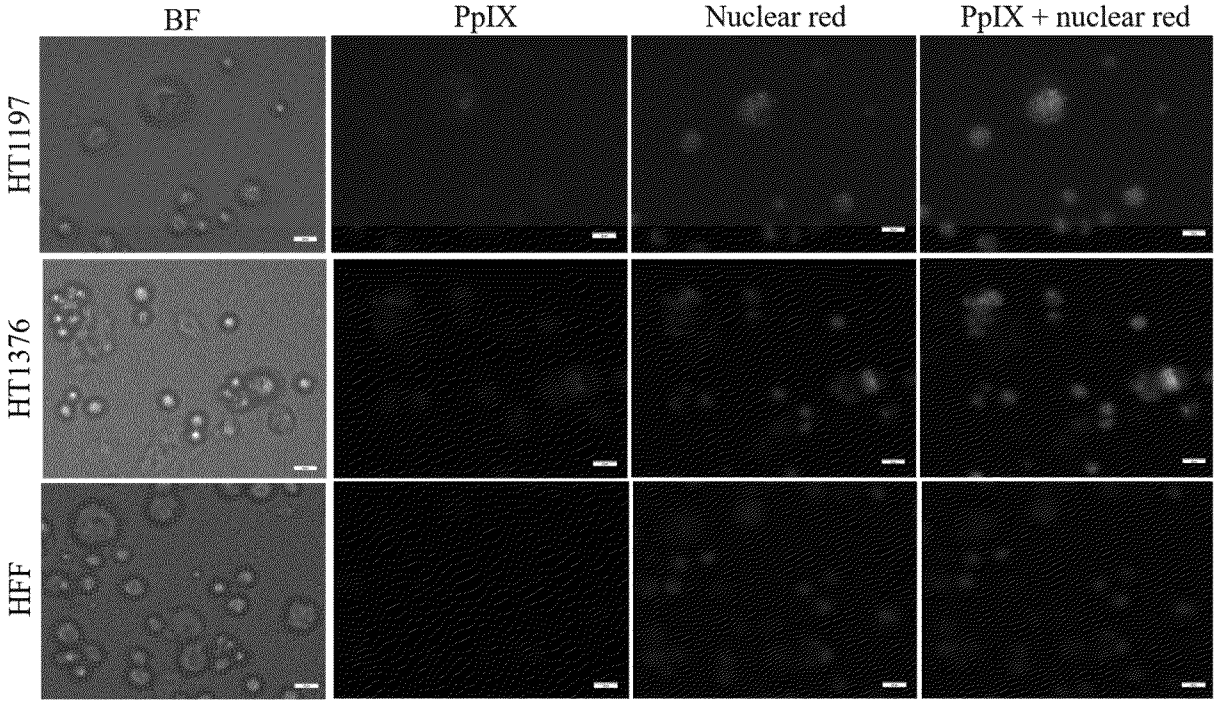

BF
PpIX
Nuclear red
PpIX + nuclear red
HT1197
HT1376
HFF

FIGURE 8

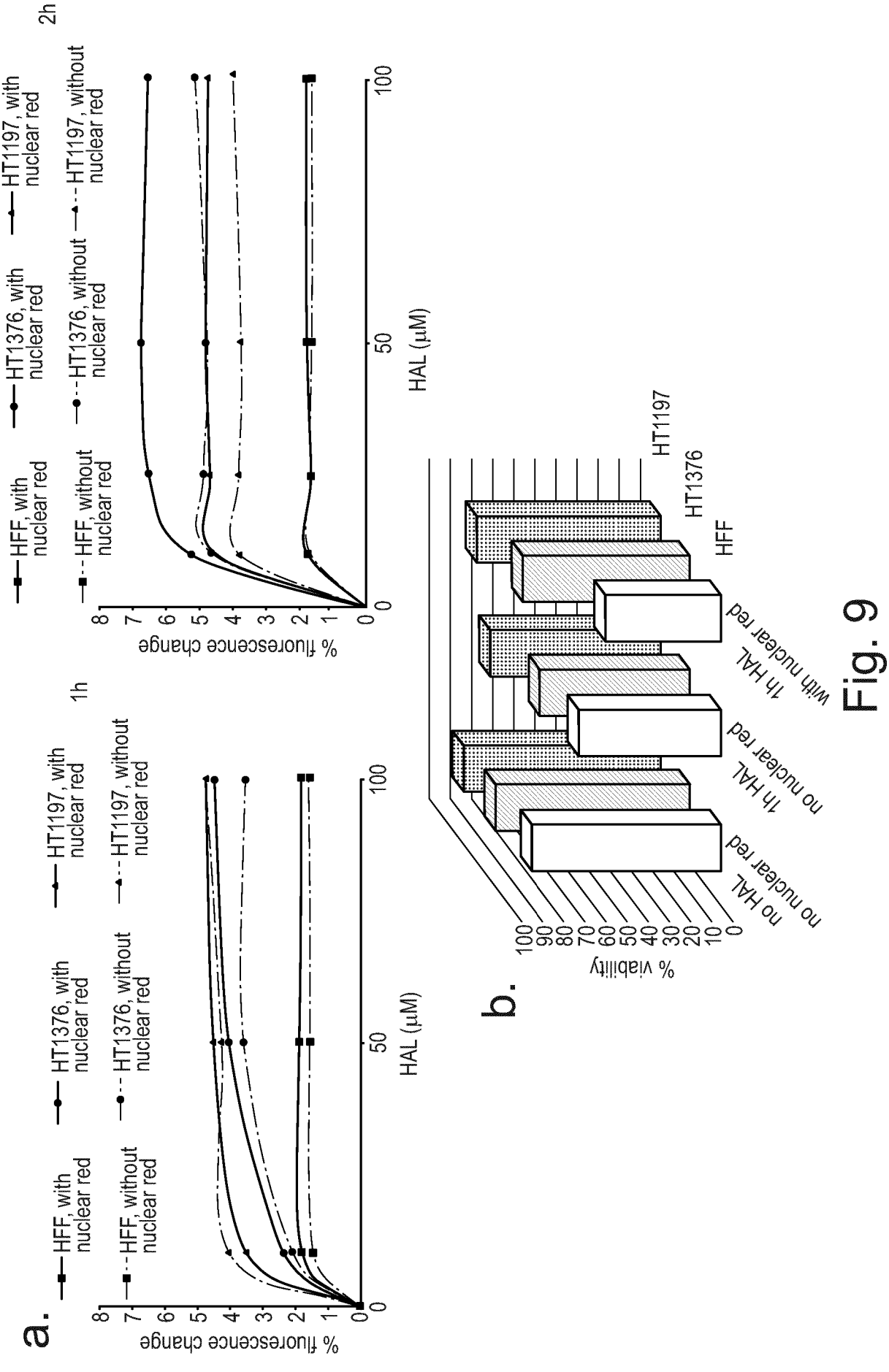

a.
1h
2h
HFF, with nuclear red
HFF, without nuclear red
HT1376, with nuclear red
HT1376, without nuclear red
HT1197, with nuclear red
HT1197, without nuclear red
% fluorescence change
HAL (µM)
b.
% viability
no HAL
no nuclear red
1h HAL
no nuclear red
1h HAL
with nuclear red
HFF
HT1376
HT1197

Fig. 9

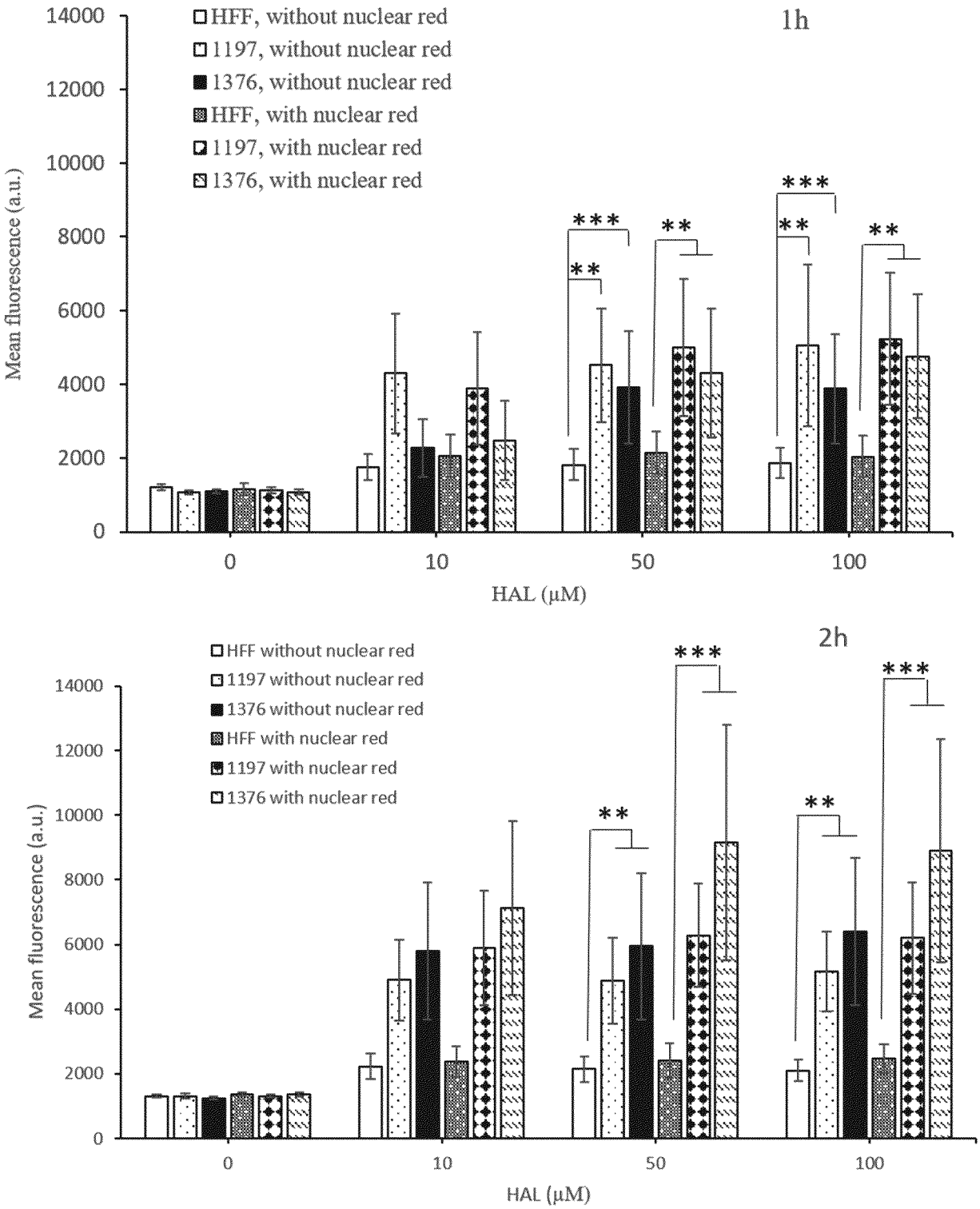

1h
HFF, without nuclear red
1197, without nuclear red
1376, without nuclear red
HFF, with nuclear red
1197, with nuclear red
1376, with nuclear red
Mean fluorescence (a.u.)
14000
12000
10000
8000
6000
4000
2000
0
0
10
50
100
HAL (µM)
***
**
2h
HFF without nuclear red
1197 without nuclear red
1376 without nuclear red
HFF with nuclear red
1197 with nuclear red
1376 with nuclear red

FIGURE 10

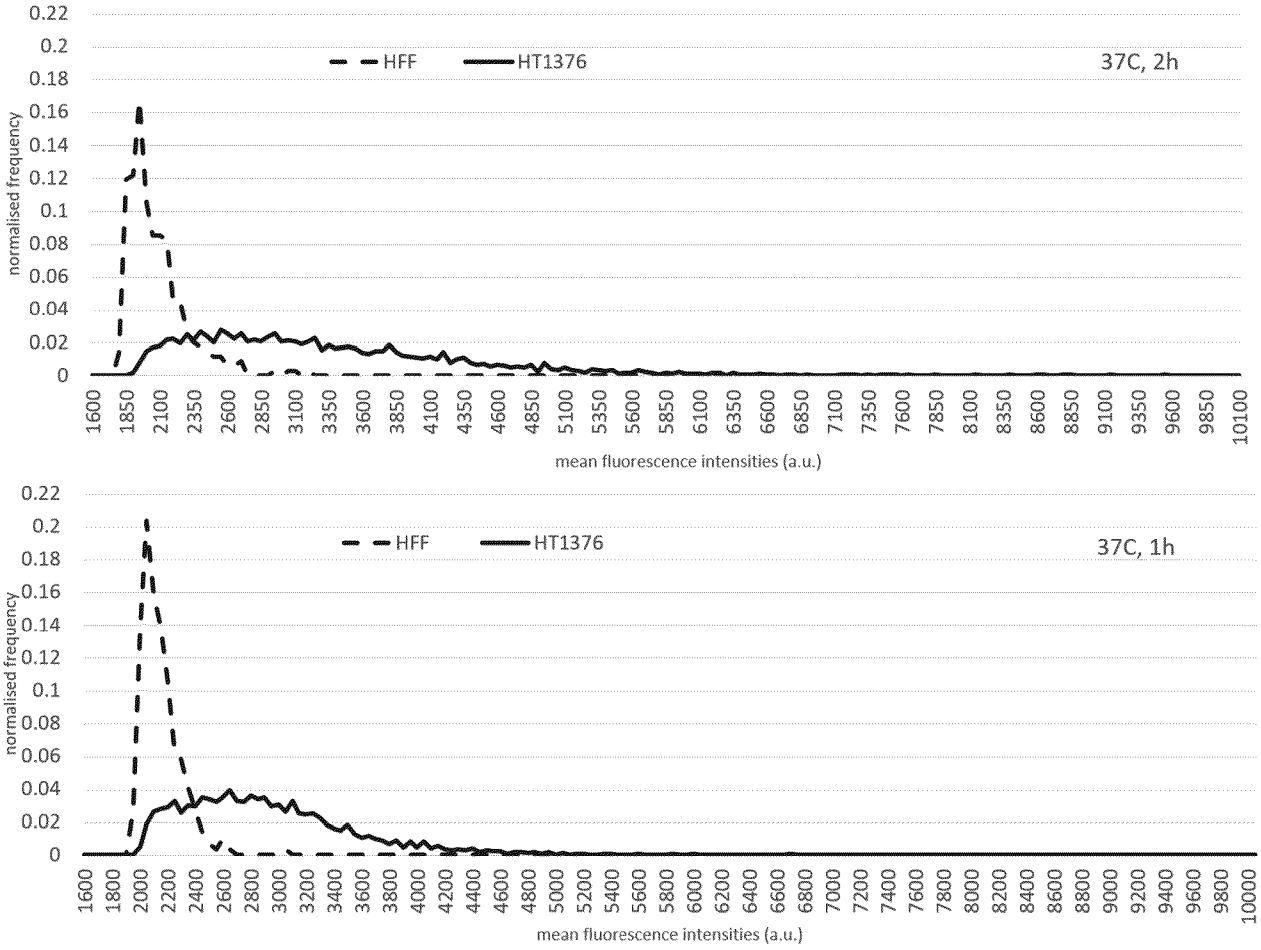


FIGURE 11

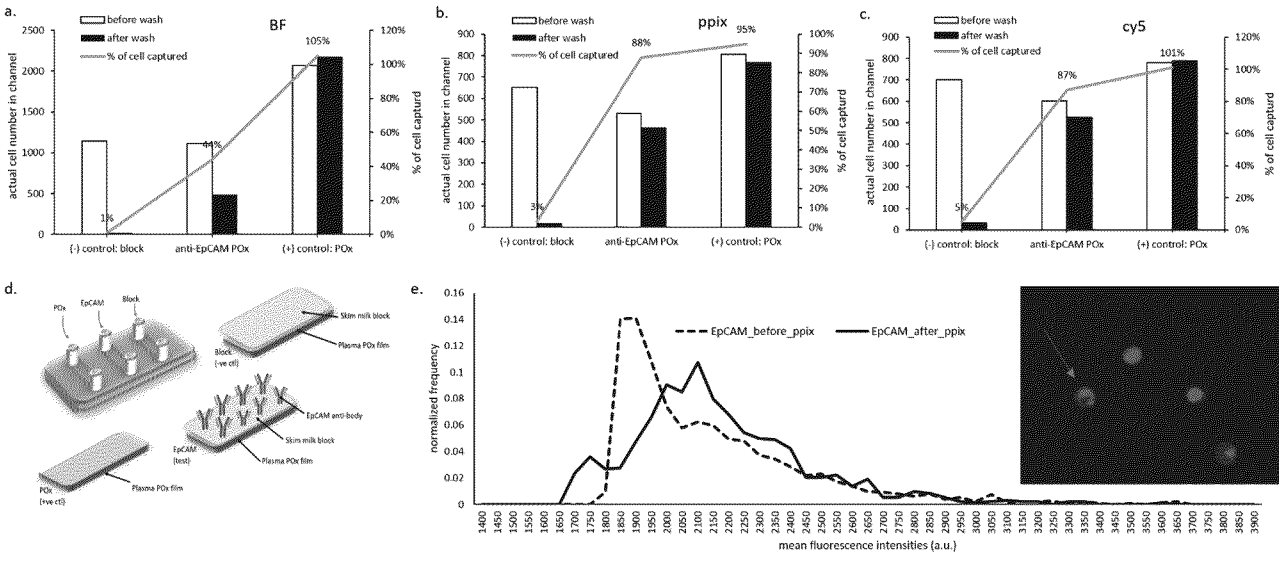


FIGURE 12

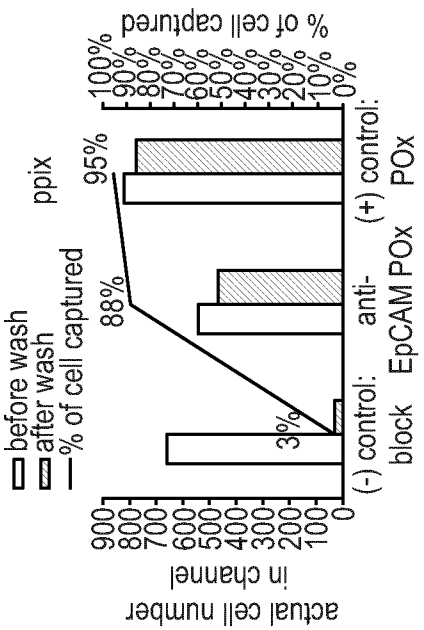


Fig. 12b

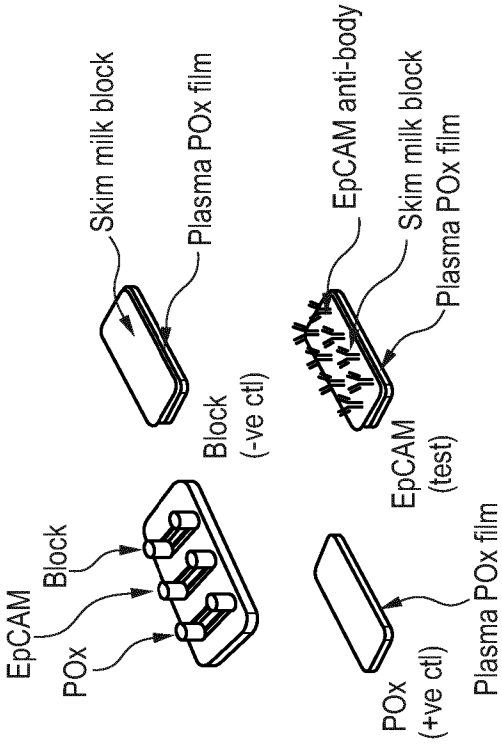


Fig. 12d

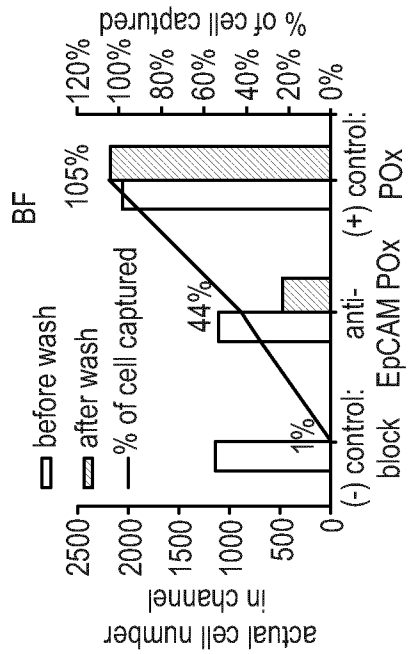


Fig. 12a

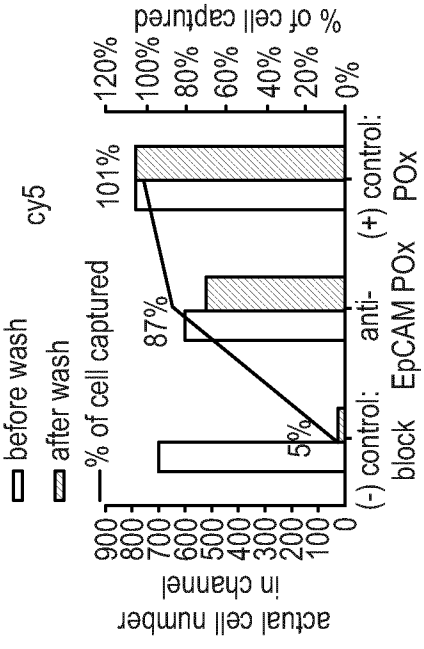


Fig. 12c

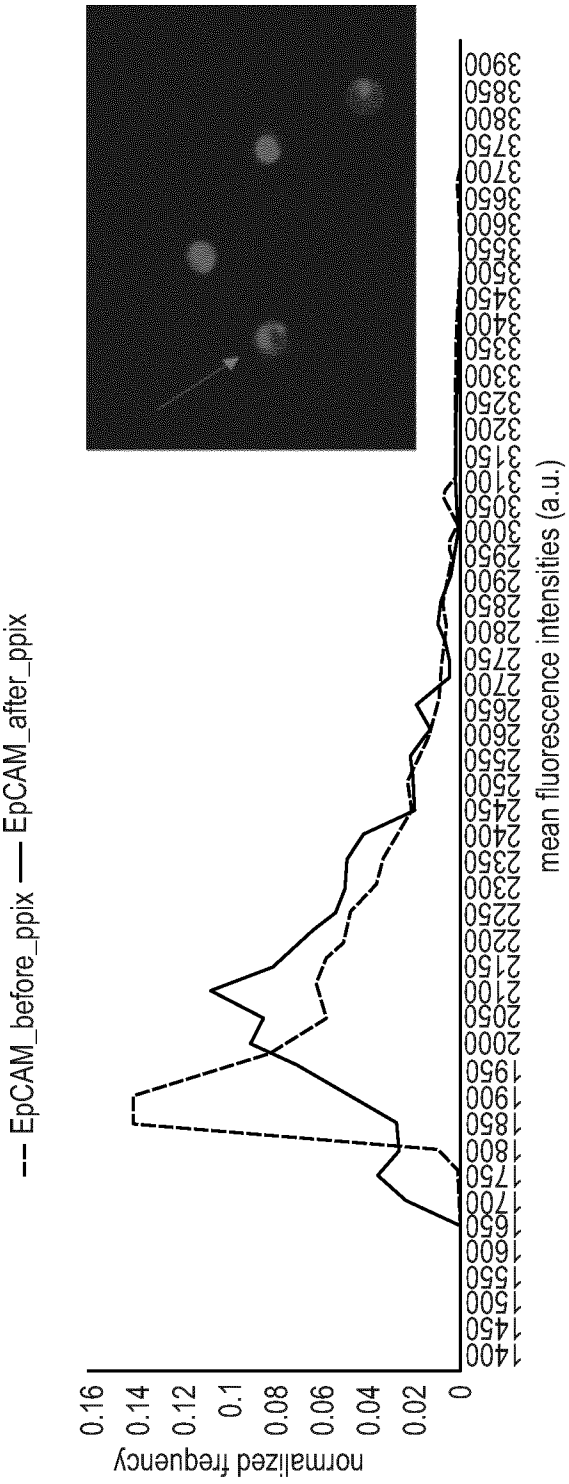

-- EpCAM_before_ppix — EpCAM_after_ppix
normalized frequency
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
mean fluorescence intensities (a.u.)
1400
1450
1500
1550
1600
1650
1700
1750
1800
1850
1900
1950
2000
2050
2100
2150
2200
2250
2300
2350
2400
2450
2500
2550
2600
2650
2700
2750
2800
2850
2900
2950
3000
3050
3100
3150
3200
3250
3300
3350
3400
3450
3500
3550
3600
3650
3700
3750
3800
3850
3900

Fig. 12e

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/066366

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/574 G01N33/58
ADD.

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)
G01N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TAUBER S: "Fluoreszenzzytologie der Harnblase nach Instillation von 5-Aminolävulinsäure", DER UROLOGE, AUSGABE A ; ZEITSCHRIFT FÜR KLINISCHE UND PRAKTISCHE UROLOGIE ORGAN DER DEUTSCHEN GESELLSCHAFT FÜR UROLOGIE, SPRINGER, BERLIN, DE, vol. 46, no. 9, 21 July 2007 (2007-07-21), pages 1123-1125, XP019535732, ISSN: 1433-0563, DOI: 10.1007/S00120-007-1456-9 the whole document In particular: Title; Abstract; Figures 1 and 2.	1-18

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"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	Date of mailing of the international search report
15 July 2020	23/07/2020

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	C.F. Angioni

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MAKITO MIYAKE ET AL: "Diagnostic approach for cancer cells in urine sediments by 5-aminolevulinic acid-based photodynamic detection in bladder cancer", CANCER SCIENCE, vol. 105, no. 5, 6 April 2014 (2014-04-06) , pages 616-622, XP055715074, JP ISSN: 1347-9032, DOI: 10.1111/cas.12393 the whole document In particular: Title; Abstract; Materials and Methods; Figures 1-5.	1-18
X	NAKAI YASUSHI ET AL: "Protoporphyrin IX induced by 5-aminolevulinic acid in bladder cancer cells in voided urine can be extracorporeally quantified using a spectrophotometer", PHOTODIAGNOSIS AND PHOTODYNAMIC THERAPY, vol. 12, no. 2, 1 June 2015 (2015-06-01), pages 282-288, XP029237245, ISSN: 1572-1000, DOI: 10.1016/J.PDPDT.2014.12.010 the whole document In particular: Title; Abstract; Materials and methods; Figures 1-5.	1-18
X	WO 2018/187830 A1 (UNIV SOUTH AUSTRALIA [AU]; FLINDERS UNIV OF SOUTH AUSTRALIA [AU]) 18 October 2018 (2018-10-18) the whole document In particular: Example 7 and figures 9-12.	1-18
X	US 2005/031541 A1 (GIERSKCKY KARL E [NO] ET AL) 10 February 2005 (2005-02-10) the whole document In particular: Example 18 and figures 28 and 29.	1-18

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2020/066366

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