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Surgery-Guided Removal Of Ovarian Cancer Using Up-Converting Nanoparticles

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Abstract

Ovarian cancer survival and recurrence rate are drastically affected by the amount of tumor that can be surgically removed prior to chemotherapy. Surgeons are currently limited to visual inspection, making smaller tumors difficult to remove surgically. Enhancing the surgeon's ability to selectively remove cancerous tissue would have a positive effect on a patient's prognosis. One approach to aid in surgical tumor removal involves using targeted fluorescent probes to selectively label cancerous tissue. To date, there has been a trade off in balancing two requirements for the surgeon: the ability to see maximal tumors and the ability to identify these tumors by eye while performing the surgery. The ability to see maximal tumors has been prioritized and this has led to the use of fluorophores activated by near-infrared (NIR) light as NIR penetrates most deeply in this surgical setting, but the light emitted by traditional NIR fluorophores is invisible to the naked eye. This has necessitated the use of specialty detectors and monitors that the surgeon must consult while performing the surgery. In this study, we develop nanoparticles that selectively label ovarian tumors and are activated by NIR light but emit visible light. This potentially allows for maximal tumor observation and real time detection by eye during surgery. We designed two generations of up-converting nanoparticles (UCNPs) that emit green light when illuminated with NIR light. These particles specifically label ovarian tumors most likely via tumor-associated macrophages (TAMs), which are prominent in the tumor microenvironment. Our results demonstrate that this approach is a viable means of visualizing tumors during surgery without the need for complicated,

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Author Contributions

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The authors declare no competing financial interest.

Supporting Information

The Supporting Information (SI) is available free of charge on the ACS Publications website as a PDF document.

Experimental method details (synthesis of TFA salt precursors; UCNP synthesis; silica coating procedure; Injection, Dissection, and Imaging); Characterization of UCNPs – TEM, SEM, emission spectra of UCNP derivatives (Figures S1–S4); Expanded Imaging Studies – experimental outline, additional tumor imaging of organs and resected human tissue, guided surgery images, and fluorescence imaging before and after surgical removal (Figures S5–S9); Additional discussion/conclusions; additional references (PDF)

Video of surgery guided example (Supplemental Video) (MP4)

expensive, and bulky detection equipment. Continued improvement and experimentation could expand our approach into a much needed surgical technique to aid ovarian tumor removal.

Graphical Abstract

Keywords

Ovarian Cancer; Silica Coating; Up-Converting Nanoparticles; Near-IR; Guided Surgery; Tumor Removal

INTRODUCTION

Ovarian cancer is a deadly disease that afflicts approximately 22,000 women per year in the United States. Once it has reached stage III and metastasized to the abdominal cavity, there is a 5-year survival rate of only ~30%.¹⁻⁴ Surgery is the frontline therapy for ovarian cancer and has two purposes. The first is to stage the cancer - to see how far the cancer has spread from the ovary. The second is to remove as much of the disease as possible - this is called debulking. Surgery is critical to patient outcomes with survival linked to the degree of tumor removed from the abdomen.⁵⁻⁹ Currently, surgeons remove tumors by direct visual inspection and can generally remove tumors >0.5 cm in diameter; however smaller tumors can be very challenging to detect and remove. Therefore, there is a clinical need for more precise, real-time detection techniques during surgery that enable surgeons to identify and remove smaller tumors they would otherwise miss.

One approach to aiding surgical tumor removal focuses on targeting fluorescent probes specifically to ovarian cancer. The most clinically advanced strategy for fluorescent detection of ovarian tumors (but not yet approved) is conjugating a fluorescent dye to folate which binds to folate receptors that are highly expressed in ovarian cancer cells.^{8,10,11} However, over-expression of this receptor varies widely between different tumors, and it is present in lymph nodes and other normal tissue, leading to false-positive signals.¹¹⁻¹⁵ Some efforts to lower false-positive signals involve using near-infrared (NIR) fluorophores. Although they produce better tumor-to-background ratios, they involve multi-camera setups that must be processed in parallel in order to achieve real-time detection.¹⁴⁻¹⁶

Tumor-associated macrophages (TAMs) are a focus of alternative labeling strategies for several types of cancers due to their large presence in the microenvironment of tumor cells and their close association with the cells.¹⁷⁻²¹ In addition, TAMs play a pivotal role in tumor angiogenesis and are thought to aid in cancer metastasis.²²⁻²⁵ Some studies suggest that removal, suppression, or conversion of TAMs could provide beneficial therapy by slowing or inhibiting metastatic growth.²⁶⁻²⁹ One focus of specific targeting to TAMs involves nanoparticle uptake to the macrophages. The particles are physically engulfed into the

cytoplasm and the payload (fluorescent marker or drug therapy) is released.^{30–34} These properties make targeting TAMs an intriguing option for ovarian cancer detection.

We recently discovered that when fluorescent silica nanoparticles (SiNP) with a diameter range of 200–1000 nm and a hydroxyl surface are injected intraperitoneally (IP), they selectively accumulate in the TAMs of abdominal metastatic ovarian cancer in mouse models.³⁵ Moreover, this phenomenon was also observed when freshly resected patient samples were tested – tumor tissue was labeled by the SiNP while normal tissue was not. This selective tumor labeling offers significant potential for surgical applications; however, red fluorescent particles are not ideal for use during surgery as they are difficult to distinguish from blood and tissue. One promising approach is to move from visible fluorophores to NIR fluorophores for proposed clinical use. NIR light has the greatest depth of penetration in biological tissue and there is minimal background fluorescence when biological tissue is excited at these wavelengths.^{36–38} In the case of ovarian surgery, current research towards alternative clinical imaging focuses on labeling tumors with NIR dyes via directing polymers, micelles, or nanoparticles.^{32,39–42} However, NIR light is invisible to the human eye, thus complicating imaging in the surgical setting. A detector must be mounted over the cavity in order to display the NIR signals – making tumor exploration slow, laborious, and challenging. In addition, the detector and imaging equipment's size and cost limit its utility to specialized centers.

We sought to test an alternative tumor labeling/imaging for surgical use that combines the unique tumor-targeting behavior of our previous SiNP with another specialized nanomaterial: up-converting nanoparticles (UCNPs). UCNPs are inorganic nanoparticles that absorb and combine low energy light (NIR) to emit higher energy light (UV and visible).^{43,44} They are typically lanthanide-doped NaYF₄ nanocrystals with cubic or hexagonal morphology.⁴⁵ Lanthanides have long lived excited states, which allows for multi-photon addition to occur and produces the anti-Stokes property observed in the fluorescence of the particles.⁴⁶ Spectral tuning and relative emission intensity can be controlled during synthesis by varying specific particle properties: size, morphology, elemental composition, and layering construction.^{47–53} UCNPs are ideal for biological applications because they are activated by NIR light, which penetrates further into tissue than UV or visible light.^{36–38} This opens the possibility of using a low energy, minimally invasive light source to illuminate localized nanoparticles. Although nanoparticle toxicity is an active field of research, lanthanide containing particles show decent biocompatibility and can be enhanced with specific coatings, like silica.^{54–58} These materials have been a focus for therapeutic applications such as photodynamic therapy, photopolymerization, and biological imaging.^{50,51,53,59–61} Our approach could provide a unique alternative to current tumor labeling strategies by providing a distinct visible light signal while maintaining the deeper biological penetration from using NIR light excitation.

In this study, we synthesized large (>150 nm diameter), green emitting (545 nm), silica-coated UCNPs to be used as a visual indicator to enhance guided surgical tumor removal (Figure 1). Two generations of these particles were made, where the second generation exhibited improved emission for tumor identification and removal. These materials were injected IP to the mouse abdomen cavity and incubated for sufficient labeling. We used

a combination of NIR light, eGFP expressing tumor cells, and light microscopy for tumor identification for delivery to malignant tissue over non-malignant tissue. To test the viability and ease-of-use for our approach, we performed a mock surgery where we compared the tumor removal through our UCNP-guided approach to that of unlabeled, visual identification. Our material aims to capture the distinct advantages of both the tumor targeting and NIR imaging to provide a tool to enhance tumor imaging/detection in real-time surgical removal. The visible, green emission from our nanomaterials could simplify surgical procedures by minimizing the need for specialized imaging detectors and maintaining the familiar visual exploration currently done. With further investigation, this technique could potentially aid in identification and removal of smaller tumors. Enhancing surgical removal of ovarian tumors will have profound short and long term effects on patient prognoses and remission rates.^{5–9}

RESULTS AND DISCUSSIONS

All UCNPs were synthesized using modified trifluoroacetate (TFA) salt precursors and thermolysis approach.^{47,48,62,63} A core was grown and layers of varying compositions were sequentially added. The final layer was an inert shell that enhances up-conversion by protecting from external quenchers.^{49,52,64,65} Individual layer composition and construction were labeled with the following format: Core@1st Shell@ 2nd Shell@...@nth Shell. The “@” symbol indicates a distinct, new layer where the composition is unique. UCNPs were characterized by TEM, SEM, and fluorescence from 980 nm irradiation and are shown in Supplemental Figures S1 and S3. Collected UCNPs were coated in oleic acid (by virtue of the synthesis) and subjected to a reverse microemulsion protocol in order to replace the oleic acid with a deposited silica coating.^{66,67} Silica coated UCNPs were characterized by SEM, with SEM-EDS analysis to identify surface silica coating (Supplemental Figures S2 and S4). Our first generation particles were NaYF₄@NaYbF₄:Ho(1%)@NaYF₄ (**Yb-Ho-1**), which showed strong emission at 545 nm (green) with 980 nm irradiation (Figure 2). These were converted to a silica-coated version (NaYF₄@NaYbF₄:Ho(1%)@NaYF₄@SiO₂; **Yb-Ho-SiO₂-1**) that were between 250 and 320 nm in diameter (Figure 2). The emission spectra remained unchanged from the silica coating, however a decrease in luminescence was visually observed (data not shown).

Yb-Ho-SiO₂-1 were suspended in water for IP injections into mice inoculated with eGFP-ovarian human cancer cells (OVCAR8). Previously, maximal fluorescence signal of embedded SiNPs was observed four days after injection.³⁵ Using this same procedure, the mice were euthanized for tissue imaging four days after **Yb-Ho-SiO₂-1** injection (Supplemental Figure S5). Figure 3 and Supplemental Figure S6 show overlap in emission signals from both the tumors and **Yb-Ho-SiO₂-1**. We also tested this incubation/imaging protocol for resected human samples. When tumor tissue and non-malignant tissue from the same patient were exposed to **Yb-Ho-SiO₂-1**, the tumor tissue exhibit UCNP uptake and fluorescence while the normal tissue had minimal signal (Supplemental Figure S7).

The initial success of these UCNP labeling studies led us to try to further improve imaging modality. We added a fiber optic coupler and collimator in an attempt to expand the imaging field for our UCNPs. The new setup allowed the 980 nm laser (1 W) to occupy

wider illumination areas; however, this also caused a corresponding power density decrease from 2.5 W/cm² (0.4 cm² illumination area) to ~0.3 W/cm² (3.1 cm² illumination area). Unfortunately, the corresponding drop in power density caused a significant decrease in luminescence intensity of **Yb-Ho-SiO₂-1**, which complicated the visual identification of localized UCNPs.

To address this new issue, we aimed to make second-generation UCNPs that would produce visible green emission at these reduced excitation power densities. Lowering the excitation power has additional advantages that include reduced costs associated with high power lasers and improved patient safety from irradiation exposure. For reference, skin exposure limits for 980 nm light is 0.72 W/cm² for pulsed durations greater than 10 seconds.⁶⁸ We targeted a layering scheme of “Yb,Ho@Yb@Nd@Y” for our second generation particles in order to achieve better emission intensity with lower excitation powers (see SI for further details). The second generation particles were synthesized using the same procedure as the first generation, **Yb-Ho-1**, with the introduction of an additional Nd layer. Our second generation NaY:Yb:Ho(10:89:1)F₄@NaYb:Y(9:1)F₄@NaNd:Y(9:1)F₄ (**Yb-Ho-2**) particles also showed strong emission at 545 nm (green) with 980 nm excitation (Figure 4). These were subsequently coated with a silica layer using the reverse micro-emulsion process to provide NaY:Yb:Ho(10:89:1)F₄@NaYb:Y(9:1)F₄@NaNd:Y(9:1)F₄@NaYF₄@SiO₂ (**Yb-Ho-SiO₂-2**). Representative emission spectra, SEM, and SEM-EDS images for the oleic acid and silica coated variants are shown in Figure 4 with full characterization in Supplemental Figures S3 and S4. The most important observation, however, comes from the stronger luminescence for **Yb-Ho-SiO₂-2** at the lower 980 nm power densities compared to **Yb-Ho-SiO₂-1**. With the new fiber optic coupler and collimator attachments, we were able to successfully identify the green emission from **Yb-Ho-SiO₂-2** by simple visual inspection. We believe this effect comes from a blend of new aspects from the particle construction. The addition of Y³⁺ through all the layers creates a more uniform crystal structure that promotes energy transfer and additional layers provide blocking from external quenchers.^{69,70}

We repeated the human biopsy labeling and imaging test to ensure reproducibility in tumor labeling. The results demonstrated that **Yb-Ho-SiO₂-2** were also selectively localized to tumor sites while avoiding non-cancerous tissues (Supplemental Figure S7). In order to quantify these results the tumor-to-background ratio was measured. The average tumor-to-background ratio is 397 while the healthy tissue ratio to background is 4. This further confirms that the UCNPs selectively localized and labeled the metastatic tumor samples with minimal labeling of matched healthy tissue samples.

Based on the success of our UCNPs to selectively distribute to tumors in the IP cavity, we wanted to evaluate **Yb-Ho-SiO₂-2** as a potential intraoperative fluorescent probe for guided-surgical resection of ovarian tumors in mice. Our group performed these mock surgeries to the best of our abilities with the intent to mimic real-life surgeries by trained medical professionals. For these studies, we compared surgical tumor removal from UCNP (**Yb-Ho-SiO₂-2**) injected and PBS (control) injected mice. Both conditions were injected IP to tumor-bearing mice, and their IP cavity organs were harvested after 4 days. In order to evaluate the potential benefit of the UCNPs during surgery, we followed a three-step protocol as a way to measure removal efficacy.

First, all abdominal organs (organ block) were removed and imaged using a dissection microscope. For the UCNP treated organs and controls, both tumors (eGFP signal) and UCNP (980 nm excitation) were imaged (Figure 5a). The presence of UCNP uptake was confirmed by green emission (false colored as red) when the samples were exposed only to a 980 nm light source.

Second, we surgically resected the tumors from each organ. For the UCNP treated organs, we used the NIR illumination to guide removal. The fiber-coupled laser was kept between an imaging area between 1 cm ($\sim 1.3 \text{ W/cm}^2$) and 1.5 cm ($\sim 0.57 \text{ W/cm}^2$) to produce a green emission from **Yb-Ho-SiO₂-2**. A pre-organ resection example of this effect like can be seen in Supplemental Figure S8 and the Supplemental Video. For the control (PBS) organs, we surgically resected all tumors that could be seen by unlabeled, visual identification.

Third, all abdominal organs were imaged post-surgical removal using a dissection microscope. The UCNP treated organs and controls were imaged for both tumors (eGFP signal) and UCNP (980 nm excitation). We compared the residual tumor (eGFP signal) from the before and after surgery images for both conditions to calculate the tumor area left over (Figure 5b).

The imaging analysis shows a substantial reduction in residual tumor mass after the image-guided surgery compared to the surgical resection based on eyesight (Figure 5, with additional images in Supplemental Figure S9). It was found that $\sim 10.7\%$ of the original tumor signal remained after resection by eye while only 0.2% of the original tumor signal remained after using UCNPs-guided detection (Figure 5b). These results demonstrate IP injection of UCNPs can aid in increased tumor removal during surgery compared to traditional (visual) removal.

This successful proof-of-principle test shows that our approach warrants continued investigation and more thorough investigation as a potential alternative to targeted ovarian cancer imaging. Further analysis to identify TAMs as the main source of UCNP uptake and additional guided-surgical studies would provide further support for this technique. In addition, continued UCNP modifications that enhance activation at lower irradiation powers could improve tumor identification. A more refined version of our technique could enable rapid detection and reliable removal of small ovarian tumors not visible to the naked eye, offering the potential for a significant improvement in the standard of care for surgical treatment.

CONCLUSIONS

We have successfully synthesized and tested UCNPs that can be used to guide surgical removal of ovarian cancer from mice. Large, silica-coated UCNPs accumulated selectively to tumors in the body cavity and produced a bright, green (545 nm) emission when excited with a NIR light source (980 nm). This approach provides a simple, real-time visualization with a marked improvement in tumor resection compared to unlabeled, visual removal. Further investigation is needed to make this a viable surgical alternative but the initial success of our approach is encouraging. With continued UCNP development and more

extensive animal testing, we can improve our surgical tool that would translate to a potential increase in survival rates and a decrease in rates for patients suffering from ovarian cancer.

MATERIALS AND METHODS

Full details of the methods can be found in the Supporting Information document, while a generalized outline is provided here.

Chemicals.

Trifluoroacetic acid (99%, purified by re-distillation), oleic acid (90%), 1-octadecene (90%), ytterbium (III) oxide (99.9%), neodymium (III) oxide (99.9%), Holmium (III) oxide (99.9%), yttrium (III) trifluoroacetate hydrate, sodium trifluoroacetate (98%), 1-hexanol (98%), Triton X-100, tetraethyl orthosilicate (98%), and ammonium hydroxide solution (28% in water) were purchased from Millipore Sigma. All chemicals were used as received.

UCNP Synthesis.

Hexagonal shaped, Ho-doped UCNP were synthesized following previously reported thermolysis procedures with some modifications.^{47,48,62,63} The particle “core” is synthesized in the main reaction flask while the additional “shell” layers are prepared in separate flasks that are added directly to the main reaction flask during the synthesis. Each core and shell mixture has a 1:4 molar ratio of lanthanide:sodium TFA and a 1:1 volume ratio of oleic acid:1-octadecene. Each mixture is heated to 100 °C under vacuum for 30–60 min and back filled with argon to remove residual water and oxygen from the flasks. Shells are maintained at a 50 °C for later use. The core is heated to 320 °C at a rate of ~10 °C/min and maintained at that temperature for 70 min. While at the elevated temperature, all of a single shell mixture is added slowly to the main flask ensuring the temperature of the reaction vessel does not drop below 300 °C. This is left to incubate at 320 °C for 45 min. Subsequent shells are applied in the same manner. Once all shells have been added sequentially and incubated, the reaction vessel is cooled to room temperature naturally. UCNP are precipitated with ethanol (1:1 volume of ethanol to the final reaction volume) and centrifuged for 15 min to pellet out the particles. They are subsequently dispersed in 25 mL ethanol and centrifuged two more times to clean and recover the UCNP. Argon is blown over the particles for 30 min, which are then dispersed in chloroform or cyclohexane at a concentration of ~20 mg/mL. The final UCNPs product has oleic acid as a ligand attached to the surface as a result of the synthesis. They can be stored as a stock solution or as the dried pellet.

Silica Coating UCNP.

Cyclohexane, 1-hexanol, water, and Triton X-100 are vigorously mixed for 15 min. UCNP@OA in cyclohexane are added and stirred for an additional 5 min. The stir rate was set to 600 rpm and tetraethyl orthosilicate (TEOS) was added and stirred for 2 hours. Ammonium hydroxide was added and the reaction was left to stir for 48 hours. Silica coated UCNP were precipitated with an acetone/ethanol mixture and collected via centrifuge. The particles were washed twice with water (15 mL each) and then once with ethanol (15 mL). Particles were collected by centrifuge at each washing step. UCNP@silica were

dried for 30 min under an argon stream and then dispersed in DI water as a stock solution (concentration ~10 mg/mL).

Imaging.

Leica Z16 dissection Macroscope was used to image the tissues that removed from the IP cavity and mice intact IP cavity with GFP-green filter cube (BP 527/30). A 1 W, 980 nm laser (MDL-III-980-1W from Changchun New Industries (CNI laser) Optoelectronics Tech. Co., Ltd) was used as the excitation source for UCNPs emission imaging. A fiber coupler/collimator (Laserglow Technologies), 1000 mm fiber patch cable (Thorlabs), and fiber collimator (Thorlabs) were added to the 980 nm laser to increase the illumination area.

Imaging analysis and tumor to background ratio measurement.

The tumor to background ratio for each tumor and non-malignant tissue was measured by using Image J software and a circular region of interest (ROI) was used to select the target tumor/non-malignant tissue. The maximum pixel value of ROI was measured as the UCNPs localization around the tumor/non-malignant tissue. The background UCNPs labeling was measured as follows: the average mean pixel value of 3 ROIs was used to determine the background UCNPs labeling. Tumor-to-background was calculated as the maximum pixel count of the tumor or non-malignant tissue divided by the average mean pixel count of the background.

Animal Experiments.

All animals were maintained under specific pathogen-free conditions at the City of Hope Animal Resource Center, and all procedures were reviewed and approved by the City of Hope Animal Care and Use Committee.

Female, athymic nude mice (Charles River) that were 7 weeks old were inoculated with 2 million OVCAR8-GFP cells via intraperitoneal injection. After 21 days, mice (n = 8 per group) were IP injected with: 1.37×10^{10} green emitting UCNPs in 1 mL PBS. Control mice received 1 mL PBS injection. After 4 days the mice were euthanized, and were imaged (Leica Z16 dissection Macroscope).

Human Tissue Procurement and Processing.

Fresh tumors and non-malignant tissues were obtained from patients who gave institutional review board (IRB)-approved informed consent [City of Hope (COH) IRB 15280] before tissue collection at the City of Hope Medical Center, the fresh tumors and non-malignant tissues were incubated in a solution of green emitting UCNPs in 4 mL DMEM media. 4 days later the tissues were washed in PBS 3 times, were placed in a new plate and imaged with the Leica Z16 dissection Macroscope.

Statistical Analysis.

Results are presented as mean $\pm$ SD unless otherwise stated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

UCNP	Up-converting nanoparticle
NIR	near-infrared
TAMs	tumor associated macrophages
SiNP	silica nanoparticles
IP	intraperitoneally
TFA	trifluoroacetate
TEOS	tetraethyl orthosilicate
SI	supporting information

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Guided Tumor Removal by Naked Eye

Figure 1:

Treatment scheme using UCNPs for surgery guided removal. An IP injection of silica-coated UCNPs incubates for 4 days to allow sufficient nanoparticle uptake. NIR light (980 nm) is used to excite the particles, which emit green light (~545 nm). This provides a visual indicator to guide surgical removal of ovarian cancer. "Particles for Injection" image was obtained using an SEM.

Figure 2:

First generation UCNPs. *Top row* is the emission spectrum of $\text{NaYF}_4@\text{NaYbF}_4:\text{Ho}(1\%)@\text{NaYF}_4$ (**Yb-Ho-1**) UCNPs with an inlay of a TEM image of the oleic acid coated particle. 980 nm light was used for excitation. The scale bar is 50 nm in the TEM image. The *bottom row* contains the SEM and SEM-EDS elemental analysis (Si signal) for the silica coated variant (**Yb-Ho-SiO₂-1**). Presence of the Si x-ray fluorescence signal indicates coverage of the UCNPs. The left image has a scale bar of 300 nm while the right image is 1 μm .

Figure 3:
Yb-Ho-SiO₂-1 demonstrate selective tumor targeting when injected IP into a metastatic ovarian cancer mouse model. Leica Z16 dissection macroscope images of IP cavity organ block 4 days after the IP injection of UCNPs. Tumors green, UCNPs red (false colored red for clarity), and merge.

NaY:Yb:Ho(10:89:1)F₄@NaYb:Y(9:1)F₄@NaNd:Y(9:1)F₄@NaYF₄@SiO₂

Si K α 1

Figure 4:

Second generation UCNP. *Top row* is the emission spectrum of NaY:Yb:Ho(10:89:1)F₄@NaYb:Y(9:1)F₄@NaNd:Y(9:1)F₄@NaYF₄ (**Yb-Ho-2**) UCNP with an inlay of a SEM image of the oleic acid coated particles. 980 nm was used for excitation. The scale bar is 200 nm in the SEM image. The *bottom row* contains the SEM and SEM-EDS elemental analysis (Si signal) for the silica coated variant (**Yb-Ho-SiO₂-2a**). Presence of the Si x-ray fluorescence signal indicates coverage of the UCNP. The left image has a scale bar of 1 μ m while the right image is 2.5 μ m.

Figure 5:

Image guided surgery based on use of the new UCNP: **Yb-Ho-SiO₂-2**. (A) Leica Z16 dissection microscope images of the IP cavity organs 4 days after IP injection of UCNP (EGFP tumors green, UCNP false colored red) before surgery and after image guided surgery. Scale bar = 1.0 cm (B) Quantification of percent (%) tumor area comparing surgical resection of tumors by the naked eye versus an image guided surgery with UCNP. Error bars are presented as mean $\pm$ standard deviation, $P < 0.05$ (two-tailed Student's t-test), (using Image J software, NIH, USA).