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Focused Ultrasound Induced Hyperthermia Accelerates and Increases the Uptake of anti-Her2 Antibodies in a Xenograft Model

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Graphical Abstract **Abstract**

Image guided drug delivery has gained significant attention during the last few years. Labelling nanoparticles or macromolecules and monitoring their fate in the body provides information that can be used to modulate their biodistribution and improve their pharmacokinetics. In this study we label antibodies and monitor their distribution in the tumours post intravenous injection. Using Focused Ultrasound (FUS, a non-invasive method of hyperthermia) we increase the tumour temperature to 42°C for a short period of time (3-5 min) and we observe an increased accumulation of labelled antibody. Repetition of focused ultrasound induced hyperthermic treatment increased still further the accumulation of the antibodies in the tumour. This treatment also augmented the accumulation of other macromolecules non-specific in the tumour, such as IgG and albumin. These effects may be used to enhance the therapeutic efficiency of antibodies and/or targeted nanoparticles.

Abbreviations: DPBS, Dulbecco's Phosphate-Buffered Saline; DMSO, dimethyl sulfoxide; EGFR, Epidermal Growth Factor receptor; FDA, U S Food and Drug Administration; FUS,

Focused Ultrasound; HER-2, human epidermal Growth Factor Receptor 2; HIFU, High Intensity Ultrasound; IgG, Immunoglobulin G antibody; MRI, Magnetic Resonance Imaging; NIRF, Near Infrared Fluorescence, PET, Positron Emission Tomography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SHO, SCID Hairless Outbred; TIPS, Therapy and Imaging Probe System; XL750, XenoLight CF750 dye;

Keywords: focused ultrasound, Trastuzumab, Herceptin, *in vivo* imaging, antibody therapy, cancer

1. Introduction

Theranostic agents are diverse in nature, from single molecules to large complexes and nanoparticles which have the functions of diagnosis and treatment [1]. Theranostic agents are labelled with one or more probes (multimodal imaging), allowing real-time imaging of the therapeutic by a number of imaging modes such as MRI, PET, ultrasound or optical imaging [2-4].

Theranostic Nanoparticles have gained significant attention due to their properties and characteristic advantages for their use in cancer. Nano-theranostics have raised hopes for treating cancer using imaging and therapy combined in one nanoparticle [5] .

The data derived from imaging using theranostics provide information for a) drug biodistribution, b) monitoring of the therapeutic effect e.g. diminishing of tumour growth, c) interactions of the drug with receptors, d) mechanisms of action, and e) metabolism &

clearance. Overall, theranostics provide information how the *in vivo* biodistribution and clearance of the potential therapeutic agent develop. This in turn allows adjustment of treatment parameters (e.g. dosage, timings) to best enhance the therapeutic effects [6]. Labelled antibodies are an important group of theranostics as they can provide insight on the cancer target expression levels [7,8]. Labelled antibodies have been suggested for detecting and treating breast cancer[9].

Multifunctional nanoparticles that label tumours for additional imaging have been increasingly introduced in the field of oncology research [10]. These nanoparticles are usually coupled with targeting ligands such as antibodies for cancer biomarker targeting [11]. Therapeutic nanoparticles are coupled to antibodies for specific deliver of their cargo to disease cells [12]. Recently antibodies and or their versions are suggested for coupling on a variety of theranostic nanoparticles for better specificity and precision medicine [13-15]. Antibodies including Her-2 antibody coupled with radiolabels, MRI labels and or fluorescent labels have been suggested as theranostics [16-18].

Focused Ultrasound mediated drug delivery has recently raised great interest [19,20]. The method is suited to enhancing the delivery of nanoparticles in tumours for triggered release and targeted drug delivery [21]. It also allows for thermally sensitive liposomal drug delivery, enhancing delivery through sonoporation as well as improving drug delivery to the brain [20].

Along with chemotherapy, hyperthermia has experienced the development of techniques as safe and effective to treat certain forms of cancer [22]. In particular, the use of High Intensity Focused Ultrasound (HIFU or FUS) allows the non-invasive heating of internal tissue areas to coagulation temperatures, effectively destroying targeted tumour tissue [23,24]. Lower power settings may also be used to induce sub-lethal (normally < 43°C), highly localised

hyperthermia that does not damage tissues directly. There have been a number of recent reports discussing the effects of such mild hyperthermia on blood irrigation and the enhanced uptake of therapeutic agents [25]. The combination of mild hyperthermia and thermoresponsive chemotherapy agents has also emerged as a recent development in Thermodox clinical phase III [26].

The method of focused ultrasound mediated hyperthermia drug delivery has been harnessed for other carriers or macromolecules however to a much lesser extent. In a recent study, it has been used to deliver B3 mAb antibody in axenograft murine cancer model, suggesting that this method could be developed for the delivery of radio-immunotherapy in tumours [27]. Recently Cetuximab was also delivered in mice in a similar fashion, showing an improvement on anti-tumour effect [28].

Trastuzumab is a monoclonal antibody that binds to HER2/neu receptors, blocking human Epidermal Growth Factor Receptor 2 (HER-2) downstream signalling and inhibiting cancer cells proliferation. The HER2 gene encodes a transmembrane tyrosine kinase receptor that belongs to the Epidermal Growth Factor Receptor (EGFR) family. This family of receptors includes four members (EGFR/HER1, HER2, HER3 and HER4) that function by stimulating growth factor signalling pathways [29,30]. In 1998, Trastuzumab, under the brandname Herceptin[®], received FDA approval for the treatment of breast cancer [31]. At the same time FDA approved HercepTest (Dako, Denmark) for diagnosis of HER2 expression [31,32]. Preclinical and clinical studies have clearly demonstrated that the combination of Trastuzumab with small molecule chemotherapeutics (e.g. emtasine Kadcyła[®], antibody drug conjugates) significantly prolongs the survival of patients with HER2-positive metastatic breast cancer [33]. In a very recent study lapatinib, a tyrosine kinase inhibitor in clinical

development for cancer and a potent dual inhibitor of Epidermal Growth Factor Receptor was combined with Trastuzumab showing impressive effects on the disease free survival parameter of patients that underwent the treatment [34].

Since 2005, significant work has been done in the area of imaging, also including Trastuzumab as targeting molecule [35]. Dual labelled fluorescence and radio-labeled Trastuzumab has been tested to diagnose tumours in mice [36-38]. Labelling of Trastuzumab for MRI has been proved more challenging and more sophisticated approaches have been introduced [39].

With the aim to understand the potential effect of hyperthermia on antibody targeted theranostics (including nanotheranostics) this study investigates the biodistribution of labelled antibody in the tumour with and without Focused Ultrasound treatment. In this work, we report on the effects of FUS-induced mild hyperthermia on the uptake of Trastuzumab to murine xenograft tumours. The localisation of the antibodies (with a covalently attached dye) was tracked using a Near Infrared Fluorescence (NIRF) imaging system.

2. Materials and Methods

Unless otherwise stated, Trastuzumab/Herceptin was from Genentech (San Francisco CA, USA), XenoLight CF 750 NIRF dye and electrophoresis reagents from Perkin Elmer (Waltham MA, USA), buffers and cell reagents from GE Healthcare (UK) while other chemicals were from Sigma Aldrich (St. Louis MI, USA).

2.1 XL750-Trastuzumab conjugation

Trastuzumab (0.5 mL, 21 mg/mL) was buffer exchanged using a PD-10 column to Dulbecco's phosphate buffered saline (DPBS) with pH adjusted to 8.3 with redistilled triethylamine.

Antibody fractions were identified by absorbance at 280 nm. XenoLight CF 750 NHS (0.5 μ mole; 'XL750') previously dissolved in anhydrous DMSO (50 μ L) was then slowly added with vigorous vortexing. Then mixture was then left stirring at r.t. for 1 h before re-separating on the PD-10, run in DPBS alone. Conjugation appeared to be almost complete (> 90 %) with little dye retained on the column. The resulting deep blue solution was split into 200 μ L portions for storage at -20 °C. Estimated final concentration was 5 mg/mL antibody, 0.24 μ mol/mL XL750. Absorbance bands for a diluted sample were 280 nm (0.67 AU; protein) and 750 nm (> 2 AU; NIRF dye), fluorescence peaked at 785 nm on excitation at 750 nm. Samples of the labelled antibody were analysed by SDS-PAGE using 4-20 % tris-glycine non-reducing gels and highlighting protein bands with silver stain (SilverQuest, Sigma Aldrich). No significant differences were seen before and after incubation for 7 min at 42 °C, suggesting that the antibody should be stable to mild hyperthermia.

2.2 Cell culture and tumor generation

IGROV-1 (ovarian cancer moderately expressing Her-2 receptor), SKOV-3 (ovarian cancer highly expressing Her-2 receptor) and BT474 (breast cancer highly expressing Her-2 receptor) cells were routinely cultured in medium supplemented with fetal calf serum 10 % v/v. When cells reached 80-90 % confluence, they were harvested and prepared for implantation in mice. Post harvesting, cells were washed in saline and counted using a haemocytometer. Accordingly with the cell counting an equal volume of saline containing the cells was mixed with matrigel (Geltrex, Gibco). For the tumor generation, 5×10^6 cells contained in 50 % matrigel mixture were inoculated subcutaneously on both flanks of 8 weeks old SHO mice (Charles River, Germany). After about 2 weeks, the formed tumours on each flank had

reached an average diameter of 5-6 mm. All experiments were approved by the Home office UK. Imaging and treatments were performed in n=3 animals per group.

2.3 FUS-induced hyperthermia

Mice were treated with FUS-induced hyperthermia using a Therapy and Imaging Probe System (TIPS, Philips Research, Briarcliff NY, USA). Under isoflurane anesthesia they were placed on a warmed gel pad over an ultrasound absorbing mat. Two or three fine-wire thermocouples (T-type, 40 ga, Physitemp Instruments Inc, Cifton NJ, USA) were implanted above and below the target tumour and temperatures recorded (0.1 °C, 0.1 s resolutions) during the treatment. Thereafter, the target tumour was covered by ultrasound gel and the TIPS placed at a distance of 88 mm from the skin surface of the right-side tumour. Each FUS insonation was delivered at a frequency of 1.0 MHz, 99.9 % cycle duty and 12-15 W of acoustic power actively adjusted according to the attained and target (42 °C) temperatures. Once this was reached, insonation was continued for 3-5 min without further temperature increase.

2.4 Near Infrared Fluorescence Imaging

The tumour bearing mice were injected intravenously with XL750-Trastuzumab (200 µL of 1 mg/mL; ~ 8 mg/kg mouse body weight) in sterile mM HEPES pH 7.4 with 5% glucose (w/v). The injections were performed with anaesthetized mice using a syringe driver connected to a cannula inserted in the tail vein. The injection rate used was 400 µL/min. immediately post injection, each anaesthetised animal was placed into the Maestro EX (Perkin Elmer) for imaging. The MaestroEX (Perkin Elmer) settings were adjusted to Xenolight750 fluorescence: excitation filter 684-729 nm band-pass, emission filter 745 nm long-pass, and liquid crystal filter 740-950 nm in 10 nm steps. Image stacks were then collected at regular time points during the study. The resulting stacks underwent multispectral analysis compared to previously collected data for XL750-Trastuzumab using the supplied software (v. 3.0.1.2). The

processed grey scale images where then brightness balanced in groups and false coloured using ImageJ (v. 1.49f <http://imagej.nih.gov/ij/>, 1997-2015).

3.Results

The combination of a small animal FUS guided by real-time temperature measurements from implanted thermocouples allows the application of localised and repeatable hyperthermia without deviations from the target temperature (see Figure 1). In turn, NIRF imaging allows us to monitor the resulting changes in distribution and tumour uptake of a labelled material in real-time. Other methods of preclinical imaging such as MRI and PET (SPECT/CT) suffer from significantly longer setup and image acquisition times although resolution is substantially better [40]. The TIPS/NIRF combination we designed in our study allows the imaging of drug biodistribution 1-2 minutes after insonation and repeated imaging at ~ 2 min intervals following, for periods of several hours because the animal is allowed to regain consciousness [41]. This gives greater confidence in the drug biodistribution behaviours that are imaged.

NIRF optical imaging is non-hazardous (no radioactivity) preclinical and enables the tracking of the NIRF signal to ~ 1 cm deep inside the mouse body with high sensitivity. The short depth of the signal detection can be circumvented by rotating the mouse from ventral to dorsal position to provide information of the labelled drug accumulation in the RES (reticulo endothelial system, in particular liver and spleen) organs of the animal [42]. In our study we used NIRF imaging to detect the distribution of labelled Trastuzumab in the tumour (see supplementary video).

3.1 Effect of focused ultrasound treatment on labelled antibody biodistribution and tumoural uptake

Trastuzumab biodistribution was first studied without hyperthermia treatment. In Figure 2, mice were imaged from the dorsal side showing a steady and continuous accumulation in both IGROV-1 tumours, reaching its maximum at 24-48 h (Figure 2 upper panel and Sup. Figure S2 and S6) post injection. We also observed during the first hours an accumulation of the NIRF signal potentially coming from the kidneys which disappeared as soon as the animal urinated (Supplementary material Figure S2). On the ventral side, fluorescence accumulated principally in the liver and the bladder over the same period, as the consequence of kidney clearance previously described (Supplementary material Figure S3).

The effects of the application of FUS-induced mild hyperthermia on tumoural uptake were then examined (figure 2, middle panel). The temperature was monitored using thermocouples (Figure S5). The selected hyperthermia regimen was 41 °C for 5 min (this is brief compared to other Focused Ultrasound hyperthermia studies recently reviewed [43]). The change in apparent tumour uptake was noticed within 4 h, with a significant accumulation of NIRF signal in the treated by FUS area. But when the tumour was excised, only a small increase in accumulation was observed.

In order to improve the uptake, we repeated the FUS treatment at different time intervals post injection (see Figure 2 and 3). The effect of repetition of FUS induced hyperthermia led to a substantial increase in antibody accumulation in the area of the heated tumour. Excision of the tumour itself was performed at selected time points after the last application of the

FUS. After excision, the heated right-hand tumours presented a substantially more NIRF signal compared to the left-hand controls (NO-FUS treatment) but only for these mice that received repetition of the Focused Ultrasound treatments (Figure 3). This also shows that after one short treatment of hyperthermia there is an apparent increase in the fluorescent signal on the animal but a smaller increase of the concentration of Trastuzumab in the excised tumour. This might be due to the fact that tumour excision was immediate after application of hyperthermia indicating that the signal coming from the tumour on the living animal is from the blood vessels and tissues surrounding the tumour. This effect from hyperthermia application was reported by Khaibullina et al. who also described a large accumulation of the antibodies in the surrounding muscle and skin [44].

However the effect was evident when FUS-induced hyperthermia was repeated twice or thrice at different time points. The benefit of the use of FUS to improve the uptake of antibody therapeutics was already demonstrated [27] but in our study we observed a clear response in the antibody uptake upon repetition of thermal dosing that is dependent the number of repetition of FUS applications.

The NIRF signal coming from the label that is attached to Trastuzumab appeared to be retained in the tumours for several days either without or with FUS treatment (supplementary figures Fig S2 and Fig S4). This might be coming from Trastuzumab as the antibody has a very long half-life (28 days) [45]. Whether NIRF dye stays attached to the antibodies or not distributed in the tumours remains to be investigated. It is however likely that the signal comes from the antibody–label conjugate as the small NIRF molecule clears out of the animal. We also observed that the intensity of the signal appeared to be strong for at least 7 days (see

Supp. Material Figures S2 and S4). In a recent study the biological properties of Trastuzumab were not affected after the application of the effect of hyperthermia [46].

3.2 NIRF signal assessment in FUS treated tumours versus non treated tumours

As expected, without FUS treatment, the antibody uptake appeared to be the same in both tumours having similar sizes and development, and possibly the same vascularisation.

In Figure 4, we quantify the NIRF brightness from area matched regions (e.g. centre of the tumour) of left and right hand tumours, compared to a shoulder muscle considered as control.

In Figure 4, we quantify the NIRF brightness from area matched regions (e.g. centre of the tumour) of left and right hand tumours, in the absence of FUS, or in the presence of 1 or 3 rounds of FUS, in order to confirm the effect of treatment on tumour accumulation. Conscientious of the nature of the NIRF signal and the problems that arise from its absolute quantification, we propose here a relative way to assess the magnitude of the physiological events observed so far, using shoulder muscle brightness as control.

When the fluorescent antibody was injected it was distributed to the two tumours to the same extent (Figure 4a and 4b). Without FUS treatment uptake appeared to be the same in both tumours, as expected. Tumours presented similar sizes and development possibly having the same vascularisation. While injected fluorescent antibody distributed to the two tumours to the same extend (Figure 4a and 4b), after 1 round of FUS treatment the heated tumour (right tumour) presented clearly an increase of 2-3 fold (Figure 4c) in NIRF signal intensity. After 3 rounds of FUS treatment the gain in NIRF signal was estimated to be 3-4 fold (figure 4d) . This method

of assessment is considering the intensity of the signal and not the total phenomenon e.g. AUC of signal intensity versus time.

We performed the same quantification on tumours excised from mice, sacrificed at 5 h post treatment with 3xFUS immediately after the application of the last FUS hyperthermia (Figure 5). Figure 5c presents the NIRF signal analysis derived from the excised tumours coming from a small matched region (e.g. centre of the tumour), indicating that for that area a 2-fold increase in NIRF-antibody signal. These results demonstrate that 3 rounds of mild hyperthermia accelerate and increase the tumoral uptake of the antibody therefore changing the pharmacokinetics of the therapy. The tumours were excised immediately after the application of the last FUS induced hyperthermia.

3.5 Uptake comparison between several proteins and tumour models

In order to better understand the phenomenon of tumoral uptake induced by hyperthermia, two NIRF labelled non-tumour specific proteins: IgG and albumin, were also tested in mice bearing IGROV-1 tumours as before. Both presented similar uptake trends as the anti-Her2 antibodies in response to 3xFUS induced hyperthermia (see Figure 6 upper panel). This result suggests that the presence of the receptor in the tumour is not the limiting factor in the rate of uptake and the enhancement of antibody accumulation to the tumour when hyperthermia is applied and for a defined period (at least 4h as measured). It is possible that antibody specific interaction is occurring post this time point. Further studies will focus to investigate the effect of hyperthermia on specific antibody receptor interactions and their later intracellular fate.

The effects of the 3 rounds of FUS treatment (right tumour) on the XL750 Trastuzumab accumulation were assessed on two other different tumour models: the ovarian SKOV-3 and the breast cancer BT474 cell lines, which express higher level of HER2 receptor than IGROV-1. [47,48].

The level of Her-2 expression of the cancer cell lines appeared not to affect the biodistribution of the Trastuzumab in tumours after 3x FUS treatments (Figure 6, lower panel). The comparison between the cell lines suggest that tumoural uptake of antibodies under the effect of FUS hyperthermia and for the period of treatment is not restricted by the levels of HER-2 receptors but to wider phenomena. Here, we hypothesise that the vascularisation and its permeability of the tumour are key obstacles to overcome in order to increase the concentration of antibodies and or their conjugates in tumour tissue. However, post extravasation these nanosized structures need to diffuse and interact with receptors on the cell surface.

4. Discussion

In this study we investigated the effect of focused ultrasound induced hyperthermia on Trastuzumab concentration in the tumour after intravenous administration in xenograft mice.

The application of mild hyperthermia offers the possibility to accelerate and increase the accumulation in the tumours of Trastuzumab antibody in agreement with previous study [44].

In the study presented by Khaibullina et al. and Wang et al hyperthermia was applied for 8-15 min and only once [27,44]. In our studies the repetition of mild hyperthermia induced by FUS appears to have an additive effect on the uptake of the macromolecule by the tumour. Our results indicate that the hyperthermia effect on the tumour and its increased

extravasation is independent on the specificity of the macromolecule to a receptor overexpressed in the tumour at least during the first hours post injection as similar increase in concentration is appearing using Albumin or IgG or Trastuzumab in IGROV-1 cells. The concentration of the macromolecule in the heated tumour (right tumour) is always larger than the concentration on the unheated tumour (left tumour). Imaging of the labelled antibodies Trastuzumab and IgG as well as albumin indicate that hyperthermia affects significantly affect their uptake by tumoral tissue. For the case of Trastuzumab the uptake appears to be dependent on the repetitions of FUS induced treatments. In our study we performed the short treatments only thrice. As most antibodies have long half-lives these FUS treatments could be performed repeatedly during the blood circulation of the therapeutic. This methodology has definitely a clinical significance, but a better understanding and handling of the method are required to achieve improved specific tumour targeting. Despite good experimental and clinical observation on the positive effects of hyperthermia [22] more physiological studies need to be performed in order to understand what are the physiological changes and their magnitude upon the gradual raise of temperature from 37 °C to 43 °C. Hyperthermia increase locally the blood flow and the perfusion of the tumour and as described by Li et al. will also permeate the tumoural tissue for several hours [49]. It is possible that the enhanced vascular permeability could enhance even further the accumulation of the antibody after the last treatment.

This induced focused hyperthermia methodology could be compatible with a different imaging modality and not only with optical imaging but with MRI and PET which are widely present in clinical premises.

Nanotheranostics coupled to antibodies may have the potential to lead to a new era in treating tumours with a real time monitoring of the therapy. Targeted nanoparticles can provide an important tool for diagnosis and drug therapy and/or radiotherapy [50,51]. Nanoparticles provide a versatile tool that can accommodate the targeting ligand as well as the imaging probe. Their composition can provide additional imaging abilities (e.g. iron oxide nanoparticles)[52].

Antibody coupled nanotheranostics have the potential to provide personalisation of the treatment with a better adjustment to the patient therapeutic needs. Real time imaging of the treatment provides a great tool to the clinicians to make decisions for immediate treatments. Image guided focused ultrasound can enhance the accumulation of these nanotheranostics in the tumour and potentially improve the therapeutic effect. For instance if the nanotheranostic carries radiotherapy or highly toxic drugs. Nanotheranostics in combination with hyperthermia, can offer a better control of the therapy and can optimize the treatments with an improved targeting and reduce the dosage of administered drug. The entire procedure of image guided drug delivery has the potential to accelerate therapy and might reduce the cost and risks of treatment.

5 Conclusion

In this study we have shown that FUS-induced short duration hyperthermia applied non-invasively and locally in the tumour can increase the accumulation of macromolecular drugs such as antibodies specifically in the tumours. The effect is dependent on the repetition of focused ultrasound treatments.

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Figure 1: TIPS focused ultrasound (FUS); (left) overview of the equipment showing the water-filled transducer chamber, the thermocouple interface, and the control PC; **(right)** schematic of the *in vivo* configuration with the transducer **(a)** raised such that the ultrasound biconic **(b)** focuses just above the skin surface over the tumour **(c)**. The mouse is surrounded with warmed, degassed ultrasound gel **(d)** and placed on an ultrasound absorbing mat **(e)** to prevent reflections off the table. Temperature monitoring is via two or three fine-wire thermocouples **(f)** implanted around the tumour.

Figure 2: NIRF *in vivo* imaging of mice with bilateral implanted tumours (IGROV-1) at time point post-injection of XI750-herceptin (~ 8 mg/kg). FUS induced hypothermia treatment was either omitted (**top**) or applied at 1 h (**middle**) or 1 h, 2 h, and 3 h 30 (**bottom**) on the right tumour. The difference in labelled antibody uptake is clear and lasts for more than a week.

Figure 3: Comparisons of *in vivo* and excised IGROV-1 tumours labeled XL750-herceptin NIRF from mice treated with no, 1, 2, or 3 rounds of FUS hyperthermia to the right hand tumour. The animals were sacrificed at 1 h, 2 h 30 min, and 4 h post injection.

Figure 4: Assessed brightness from area matched regions of left and right hand tumours, compared to a muscle control for mice undergoing either **(a)** is an example of the areas of interest selected. **(b)** no FUS treatment; **(c)** 1 round of FUS treatment at 30 min or **(d)** 3 rounds of FUS treatment at 30 min, 2 h, and 3 h 30; all post injection of XL750-herceptin (~ 8 mg/kg).

Figure 5: NIRF imaging of representative left (no FUS) and right (3x FUS) sacrificed 5h post injection of XI750-herceptin. (a) false colour and (b) greyscale images taken from the excised tumours, with the latter showing the matched area regions used to calculate (c) mean and 1 SD pixel brightness (n=3).

Figure 6: Upper panel: Uptake of XL750-Albumin and XL750-IgG in IGROV-1 tumours after 3x FUS treatments. Lower Panel: Comparison 3x FUS treatments on XL750-Trastuzumab (XL750-T) uptake into IGROV-1, SKOV-3 and BT474 cell line tumours. NIRF *in vivo* imaging of mice at $t = 4$ h post-injection of XL750-herceptin (~ 8 mg/kg). FUS induced hypothermia treatment was carried out at 1h, 2h, and 3h30.