SUPPORTING INFORMATION

Surface design of photon-upconversion nanoparticles for high-contrast immunocytochemistry

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1 Materials and methods

1.1 Chemicals and reagents

Alendronate (pharmaceutical grade), neridronate, 5-azidopentanoic acid (APA), (1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-ylmethyl *N*-succinimidyl carbonate (BCN-NHS), 5(6)-carboxyfluorescein (FAM), 5-carboxyrhodamine *N*-succinimidyl ester (Rh-NHS), nitrosyl tetrafluoroborate ($NOBF_4$), bovine γ-globulin (BGG), bovine serum albumin (BSA), biotinylated BSA (BSA-biotin), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC), *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS), copper(II) sulfate pentahydrate, L-ascorbic acid sodium salt, DAPI, Tween 20, and 2-(*N*-morpholino)ethanesulfonic acid (MES) were purchased from Merck (Germany). Dry dimethylformamide (DMF), streptavidin, and SuperBlock TBS (SB) were obtained from Thermo Fisher Scientific (USA). Streptavidin-azide was obtained from 7 Bioscience (Germany). α-*N*-hydroxysuccinimide-ω-alkyne polyethylene glycol (*M*w 3000, Alkyne-PEG-NHS) was purchased from Iris Biotech (Germany). α-amino-ω-carboxy polyethylene glycol (*M*w 5000, NH_2-PEG-COOH) was purchased from Rapp Polymere (Germany). Poly(vinyl alcohol) (PVA; 6 kDa) was purchased from Polysciences (USA). The H&E staining kit was purchased from Carl Roth (Germany). All other common chemicals were obtained in the highest quality available from Merck or Carl Roth. Dialysis buffer consisted of 100 mM H_3BO_3, 80 mM Na_2CO_3, pH 9.4.

1.2 Synthesis and conjugation of UCNPs

1.2.1 Synthesis of UCNPs

$YCl_3 \cdot 6\ H_2O$ (728 mg, 2.1 mmol), $YbCl_3 \cdot 6\ H_2O$ (209 mg, 0.54 mmol), and $TmCl_3 \cdot 6\ H_2O$ (23 mg, 0.06 mmol) were dissolved in methanol (20 mL) and added into a 100-mL three-neck round-bottom flask containing oleic acid (9 g) and 1-octadecene (19.7 g). The solution was heated to 160 °C for 30 min under an N_2 atmosphere and then cooled to 50 °C. Then, the protective atmosphere was disconnected, and the solution of NH_4F (445 mg, 12.0 mmol) and NaOH (300 mg, 7.5 mmol) in methanol (20 mL) was added to the intensively stirred solution. The N_2 atmosphere was reconnected, and the solution was stirred for 30 min. The temperature was carefully increased up to 150 °C, avoiding extensive boiling to ensure the evaporation of methanol. After that, the solution was rapidly heated using the rate of ~10 °C per minute. At 300 °C, the heating was carefully regulated to 305 °C within two or three minutes. The flask was kept under N_2 flow at

305 °C for 150 min. The fluctuation of temperature was ± 3 °C during this time. Finally, the flask was placed on another stirrer and rapidly cooled to room temperature under air flux. The resulting nanoparticles were precipitated by adding isopropanol (30 mL) and collected by centrifugation (1,000 *g*, 10 min). The pellet was washed with methanol (30 mL), centrifuged (1,000 *g*, 10 min), and dispersed in cyclohexane (20 mL). By adding methanol (100 mL), the nanoparticles precipitated rapidly without the need for centrifugation. The precipitate was dispersed in cyclohexane (30 mL) and slowly centrifuged (50 *g*, 20 min) to separate coarse particles from the final product.[1]

1.2.2 Preparation of SA-PEG-Alen-UCNP conjugate

NH_2-PEG-COOH (M_W = 5000, 32 mg, 3.2 mM) was dissolved in 2 mL PBS (0.1 M, pH 7.4) followed by the addition of BCN-NHS in DMF (40 µL, 5.54 mg, 475 mM). The reaction mixture was incubated at 4 °C overnight. The resulting BCN-PEG-COOH was dialyzed for 48 h in a Float-A-Lyzer dialysis device (MWCO = 500–1000 Da) against ddH_2O (1.8 L, 6× exchanged) at 4 °C and 24 h against MES buffer (100 mM, 0.5 M NaCl, pH 6.0, 200 mL, 3× exchanged). The purified BCN-PEG-COOH was activated by the addition of EDC (12 mg, 8 mM) and sNHS (20 mg) for 15 min. Alendronate (5 mg) dissolved in NaOH (1 M, 50 µL) was added to the activated PEG linker, incubated 4 h at room temperature, and then overnight at 4 °C. The resulting BCN-PEG-Alen was dialyzed 72 h in a Float-A-Lyzer dialysis device (MWCO = 500–1000 Da) against ddH_2O (2× exchanged per day). The purified product was stored at 4 °C.[2]

For the preparation of SA-PEG-Alen-UCNPs, oleic acid capped UCNPs (10 mg) in cyclohexane were mixed with an equivalent amount of DMF, followed by the addition of $NOBF_4$ (approx. 1 mg per mg UCNPs). UCNPs were incubated for 20 min at 30 °C while shaking. The cyclohexane phase was removed, and the particles precipitated by adding an excess of chloroform. UCNPs were purified by centrifugation (1000 *g*, 5 min), redispersed in DMF, precipitated with chloroform and separated by centrifugation (1000 *g*, 5 min). An aqueous solution of BCN-PEG-Alen (750 µL, 16 mg/mL) was added to the UCNP pellet followed by 1 min sonication and 24 h incubation at room temperature. BCN-PEG-Alen-UCNPs were dialyzed for 48 h against Tris/borate (200 mL, 50 mM Tris, 50 mM borate, pH 8.6, 6× exchanged) in a Float-A-Lyzer G2 dialysis device (MWCO = 100 kDa).

For the preparation of SA-PEG-Alen-UCNPs, streptavidin azide (1 mg/mL. 100 µL) was added to a dispersion of BCN-PEG-Alen-UCNPs (10 mg, 750 µL) in Tris/borate buffer and the

mixture was dialyzed in a Float-A-Lyzer G2 dialysis device (MWCO = 500-1000 Da) overnight against MES (250 mL, 100 mM, pH 4.5, 3× exchanged). For purification, the mixture was dialyzed in Float-A-Lyzer G2 (MWCO = 100 kDa) for 48 h at 4 °C against 500 mL of TBS (1 mM KF, 0.05% NaN_3, 6× exchanged). The purified SA-PEG-Alen-UCNPs were stored at 4 °C.

1.2.3 Preparation of SA-BSA-UCNP conjugate

The SA-BSA-UCNP conjugates were prepared according to our previous work.[3] In the synthesis, click-reactive BSA-alkyne conjugate was first prepared and bound to carboxylated silica-coated UCNPs,[4] followed by copper-free click reaction with click-reactive streptavidin-azide.

To prepare the fluorescent click-reactive BSA-alkyne conjugate, 2.92 mg of BCN-NHS was dissolved in 20 µL of DMF and mixed with 1.72 mg of Rh-NHS dissolved in 100 µL of DMF. Afterward, the solution was mixed with 1880 µL of dialysis buffer with 132 mg of BSA for 4 h. The conjugate was dialyzed four times overnight against 500 mL of dialysis buffer.

For the preparation of click-reactive UCNPs, 1 mg of carboxylated UCNPs was centrifuged (1700 *g*, 10 min) and redispersed in 200 µL of 100 mM sodium MES, 30 mM Na_2CO_3, pH 6.0 with 0.4 mg of EDC and 0.2 mg of sulfo-NHS and the dispersion was sonicated for 10 min. Afterwards, the activated UCNPs were centrifuged (3300 *g*, 1 min) and immediately redispersed in 200 µL of 100 mM sodium MES, 30 mM Na_2CO_3, pH 6.0 containing 0.5 mg of BSA-alkyne. After 90 min of mixing at room temperature, the UCNP-BSA-alkyne conjugate was centrifuged and redispersed five times in 50 mM Tris with 50 mM H_3BO_3 pH 8.6 (1700 *g*, 15 min, 200 µL of buffer).

To prepare fluorescent click-reactive streptavidin-azide, free carboxyl groups of APA and FAM were first activated using EDC/sulfo-NHS chemistry. The 1.4 mg of APA was dissolved in 100 µL of DMF and mixed with 100 µL of 100 mM sodium MES, pH 6.1 containing 3.8 mg of EDC and 4.3 mg of sulfo-NHS and the solution was shaken for 1 h at room temperature. The 1.5 mg of FAM was dissolved in 100 µL of DMF and mixed with 400 µL of 100 mM sodium MES, pH 6.1 containing 15 mg of EDC and 3.5 mg of sulfo-NHS. After 1 h of reaction, 50 µL of activated FAM and 20 µL of activated APA solutions were mixed together with 5.2 mg of streptavidin dissolved in 60 µL of dialysis buffer. The solution was mixed for 4 h at room temperature, followed by five times overnight dialysis against 500 mL of dialysis buffer.[5]

To perform the copper-free click-conjugation reaction, 1 mg of click-reactive UCNP-BSA-alkyne was centrifuged (1700 *g*, 15 min), and the pellet was redispersed with 2.5 mg of click-

reactive streptavidin-azide in 500 µL of dialysis buffer. The solution was dialyzed four times overnight against 250 mL of 100 mM sodium MES, pH 4.5. The change of pH enabled electrostatic attraction of the UCNPs with streptavidin and allowed an efficient click-conjugation. The final SA-BSA-UCNP conjugates were centrifuged (1700 *g*, 15 min), redispersed in 50 mM Tris, 50 mM H_3BO_3, pH 8.6 to a final concentration of 5 mg/mL and stored at 4 °C.[6]

1.3 Characterization of UCNPs and their conjugates

1.3.1 Transmission electron microscopy

A 6 µL droplet of oleic acid-capped UCNPs dispersed in cyclohexane was placed on a 400-mesh copper EM grid coated with a continuous carbon layer and incubated at room temperature for 5 min. Afterwards, the droplet was removed, and the adsorbed particles on dried grids were imaged by transmission electron microscope Tecnai F20 (FEI, Czech Republic). The dimensions of individual particles were analyzed using ImageJ imaging software (National Institutes of Health, USA).[7]

1.3.2 Emission spectra measurement

Emission spectra were measured in an Aminco Bowman 2 spectrofluorometer (SLM Aminco, USA) using a 980-nm continuous-wave laser excitation (4 W) that was fiber-coupled into the device in a 90° angle to the detector. A UCNP dispersion (1 mg/mL, 500 µL) in cyclohexane was transferred into a fused silica cuvette, and the high voltage of the detector was adjusted to prevent detector saturation. The emission spectra were measured in 2 nm steps from 300 to 950 nm and a scan rate of 2 nm/s.

1.3.3 Dynamic light scattering and Nanoparticle tracking analysis

The hydrodynamic diameters of UCNPs and conjugates were determined by DLS using Zetasizer Nano ZS (Malvern, UK). The dispersion of 7 µg/mL of oleic acid capped UCNPs (in cyclohexane) and bioconjugate (in TBS) was used for the characterization.

The concentration of conjugate and hydrodynamic properties were also studied using NTA. The dispersion of UCNPs in TBS buffer was injected into the measurement cell of the NanoSight NS300 (Malvern, UK), and the tracking was performed at 25 °C as three cycles per 60 s.

1.3.4 Single-particle upconversion microscopy

Single-particle upconversion microscopy was used to evaluate the intensity distribution of the conjugates. No. 1 glass cover slips (1.5 × 1.5 cm^2) were cleaned by piranha solution (3:1 mixture

of concentrated sulfuric acid and 30% hydrogen peroxide; 20 min), washed with deionized water and modified by cationized bovine serum albumin (cBSA; 200 µL, 20 min). After washing with deionized water, the slide was incubated with UCNPs (0.7 µg/mL) for 20 min, washed again with deionized water, and dried with nitrogen.[8]

The cover slips were mounted with glycerol and imaged using an upconversion microscope with 40× objective. The images were taken with 5 s exposure time, and the mean intensities of the individual UCNPs were determined in the microscope software (NIS elements, Nikon) by placing regions of interest (ROIs) of identical sizes over the luminescent spots. Mean intensities of 400 randomly selected UCNPs were measured and background corrected, frequency count was performed, and the data were arranged in a histogram.

1.3.5 LC-MS/MS analysis

The successful conjugation of streptavidin to the surface of UCNPs was further verified by LC-MS/MS. The samples of conjugate (100 µL, 3.5 mg/mL) were washed twice by 200 µL of 50 mM ammonium bicarbonate buffer (AB), followed by resuspending in 15 µL of AB. The proteins were digested by trypsin (1 µg) for 2 h at 37 °C. Afterward, the UCNPs were removed by centrifugation (14,000 *g*, 10 min) and the resulting peptides were extracted using acetonitrile (addition of acetonitrile was followed by vortexing of the sample and evaporation of acetonitrile by rotary evaporator to a final volume of 15 µL).[3]

The LC-MS/MS analysis was performed using RSLCnano with a Q-TOF detector (Thermo Fisher Scientific, USA). The 65-min LC gradient was used for LC-MS analyses; MS and MS/MS spectra were recorded in a time of flight analyzer (TOF). The MS/MS data were processed using Proteome Discoverer software (version 1.4; Thermo Fisher Scientific, USA). The search engine Mascot (version 2.6; Matrix Science, USA) was used to search the cRAP contaminant database (version 181122; The Global Proteome Machine Organization), which contains 112 protein sequences, including the target sequence of streptavidin (P22629-cRAP). Peptide confidence was assessed based on Mascot expectation value, only peptides with high confidence value ($p < 0.01$) were considered for final data evaluation.

1.3.6 Testing of conjugate functionality in BSA-biotin assay

A 96-well microtiter plate (µClear, high binding, Greiner Bio-One, Austria) was coated with 100 µL of BSA-biotin in coating buffer (50 mM $NaHCO_3/Na_2CO_3$, 0.05% NaN_3, pH 9.6) at 4 °C overnight. Afterwards, the plate was washed four times with 250 µL of TBS-T and blocked for 1 h

with 200 μL of concentrated SuperBlock with 0.05% Tween 20. After four washing steps, 100 μL of the UCNP conjugate sample was added and incubated for 1 h. Finally, the plate was washed four times and allowed to dry.[9]

The readout was performed using Hidex Chameleon reader. Each well was scanned in a rectangular grid of 8 × 8 pixels with 1 s exposure time per pixel and a scanning step size of 0.1 mm. The truncated average of the luminescence intensities was calculated, excluding the 16 highest and the 16 lowest values. Averages and standard deviations were calculated from the truncated averages of three independent wells.[10]

The immunoassay for the testing of the SA-FAM was performed using the same experimental conditions, except switching the UCNP conjugate for SA-FAM conjugate. The readout was performed using Labrox reader. Each well was scanned in the grid of 8 × 8 pixels, with an exposure time of 500 ms and step size 0.1 mm. The data evaluation was performed in the same way as in the case of upconversion scans.

1.4 Preparation of fluorescent streptavidin

The 1.5 mg of FAM was dissolved in 100 μL of DMF and mixed with 400 μL of 100 mM sodium MES, pH 6.1 containing 15 mg of EDC and 3.5 mg of sulfo-NHS. After 1 h of reaction, 50 μL of activated FAM was mixed with 20 μL of MES buffer and 5.2 mg of streptavidin dissolved in 60 μL of dialysis buffer. The solution was mixed for 4 h at room temperature, followed by five times overnight dialysis against 500 mL of dialysis buffer.[3, 5]

2 Results and discussion

2.1 Characterization of labels

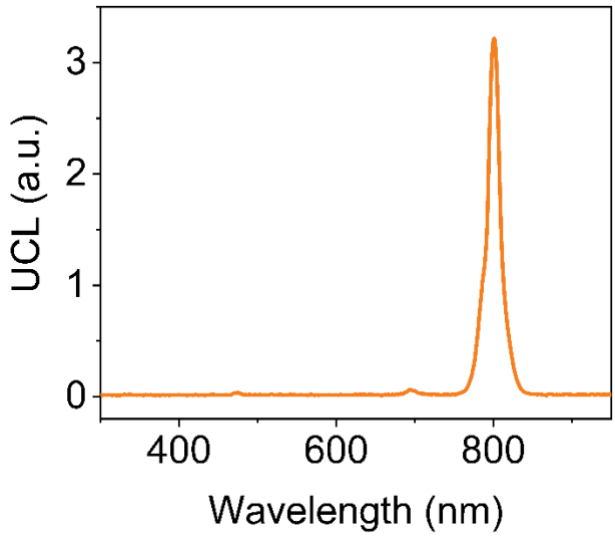

Figure S1: Upconversion luminescence spectrum of Tm^{3+}-doped UCNPs under 980-nm excitation.

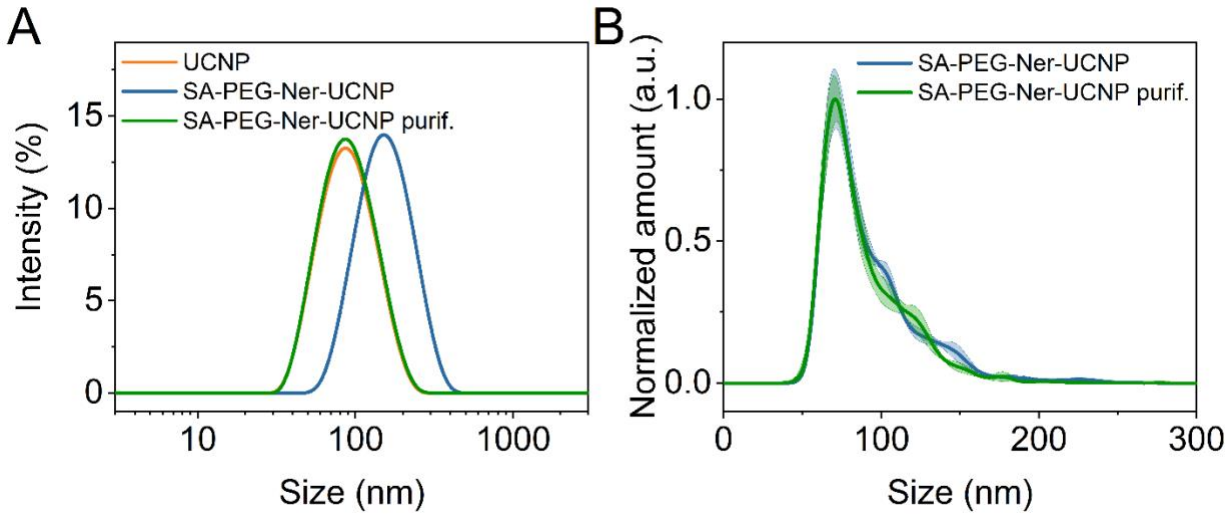

Figure S2: (A) DLS of oleic acid-capped UCNPs in cyclohexane, SA-PEG-Ner-UCNP conjugate in Tris buffer and purified SA-PEG-Ner-UCNP conjugate in Tris buffer. **(B)** NTA of non-purified and purified SA-PEG-Ner-UCNP conjugate.

Table S1: Proteins found in the SA-PEG-Ner-UCNP conjugate by LC-MS/MS.

Protein family	**Accession ID**	**Description**	**Score**	**Num. of sequences**	**emPAI**
1	P22629-cRAP	Streptavidin (cRAP) OS=*Streptomyces avidinii* PE=1 SV=1	4522	6 [a]	1.46
2	P04264-cRAP	Keratin, type II cytoskeletal 1 (cRAP) OS=*Homo sapiens* GN=KRT1 PE=1 SV=6	1321	19	1.30
3	P35527-cRAP	Keratin, type I cytoskeletal 9 (cRAP) OS=*Homo sapiens* GN=KRT9 PE=1 SV=3	840	18	1.32
4	iRT-fusion-cRAP	iRT Kit Fusion - real (cRAP)	501	9	4.88
5	P00761-cRAP	Trypsin (cRAP) OS=*Sus scrofa* PE=1 SV=1	464	4	0.6
6	Q8N1N4-cRAP	Keratin, type II cytoskeletal 78 (cRAP) OS=*Homo sapiens* GN=KRT78 PE=2 SV=2	76	3	0.17
7	P02662-cRAP-B6E	Alpha-S1-casein (cRAP-B6E) OS=*Bos taurus* GN=CSN1S1 PE=1 SV=2	54	1	0.12
8	P15252-cRAP	Rubber elongation factor protein (cRAP) OS=*Hevea brasiliensis* PE=1 SV=2	50	1	0.21
9	P02769-cRAP-B6E	Serum albumin (cRAP-B6E) OS=*Bos taurus* GN=ALB PE=1 SV=4	37	2	0.09
10	Q5D862-cRAP	Filaggrin-2 (cRAP) OS=*Homo sapiens* GN=FLG2 PE=1 SV=1	29	1	0.01
11	Q02413-cRAP	Desmoglein-1 (cRAP) OS=*Homo sapiens* GN=DSG1 PE=1 SV=2	22	1	0.03

[a] Protein sequence coverage: 44%.

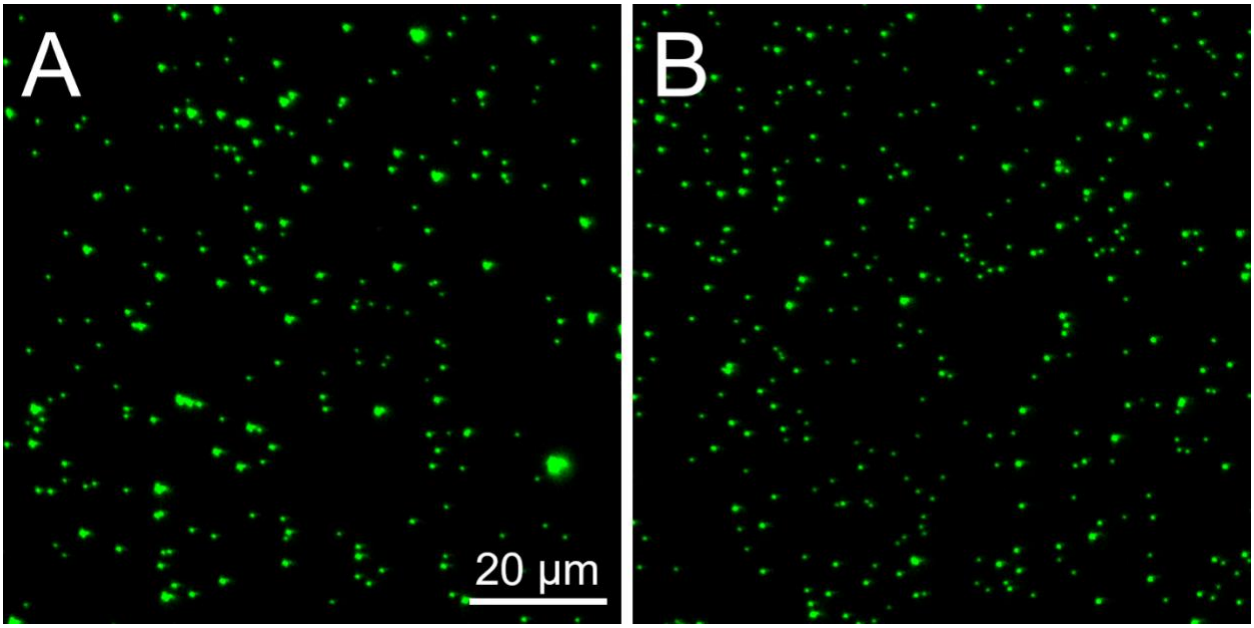

Figure S3: Upconversion microscopy images of **(A)** non-purified and **(B)** purified SA-PEG-Ner-UCNP conjugates.

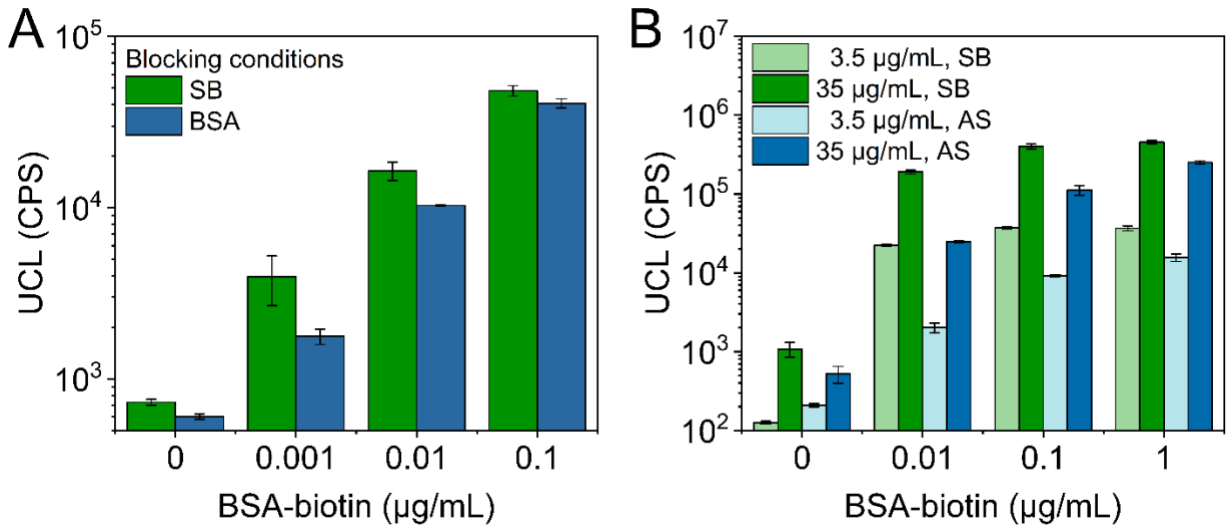

Figure S4: Functional characterization of the SA-PEG-Ner-UCNP conjugate in the BSA-biotin ULISA assay. **(A)** Optimization of blocking conditions with UCNP label in concentration of 35 µg/mL, SB – 10% SuperBlock in Tris buffer, BSA – 1% BSA in Tris buffer. **(B)** Optimization of SA-PEG-Ner-UCNP label dilution and buffer, SB – 10% SuperBlock in Tris, AS – assay buffer. The error bars correspond to the standard deviation of three wells.

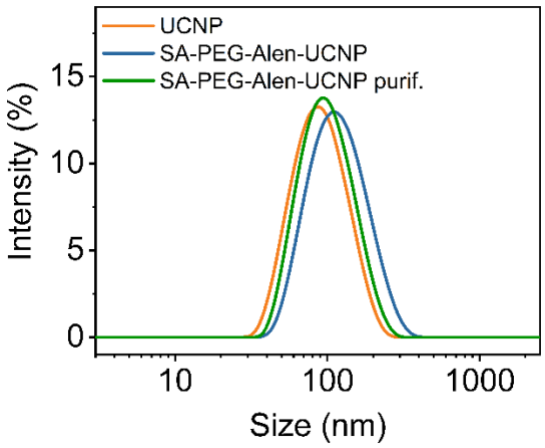

Figure S5: DLS of oleic acid-capped UCNPs in cyclohexane, SA-PEG-Alen-UCNP in Tris buffer, and purified SA-PEG-Alen-UCNP conjugate in Tris buffer.

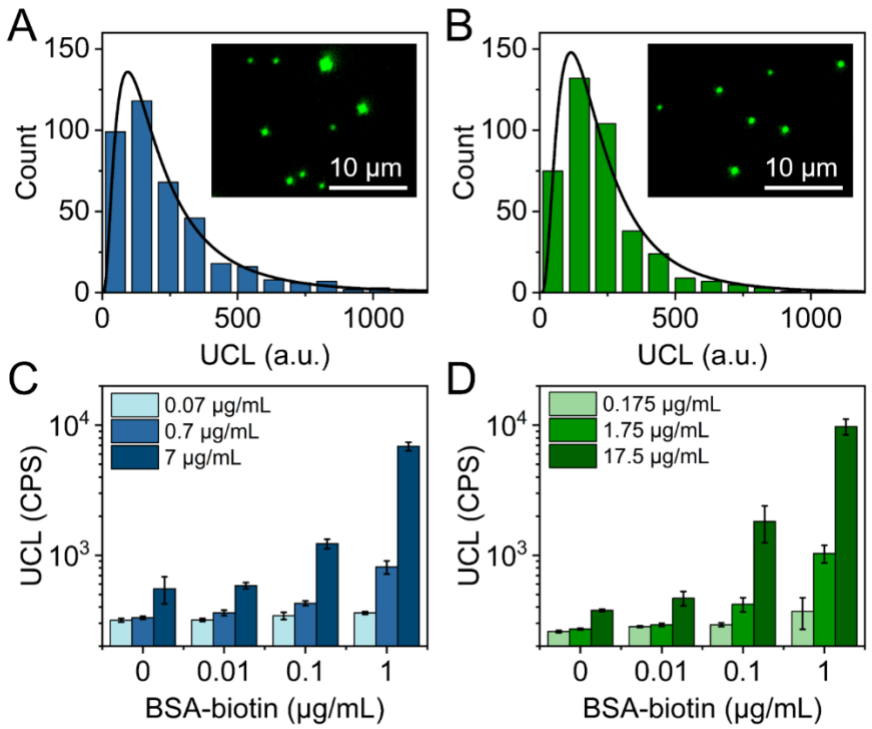

Figure S6: Characterization of the SA-PEG-Alen-UCNP conjugate. Brightness distribution of **(A)** non-purified and **(B)** purified conjugate. The insets show the upconversion microscopy images. Functional characterization of **(C)** non-purified and **(D)** purified conjugates in BSA-biotin assay. The error bars correspond to the standard deviation of three wells.

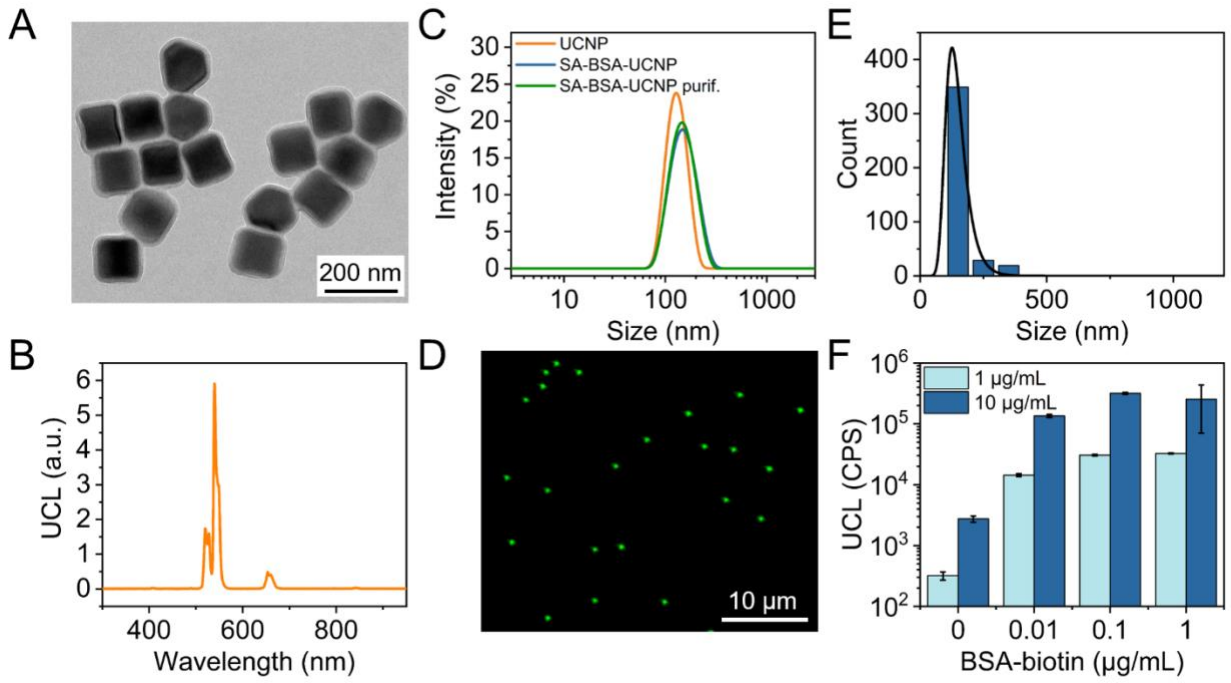

Figure S7: (A) TEM image of silica-coated Er^{3+}-doped UCNPs; **(B)** upconversion luminescence spectrum (980-nm excitation) of silica-coated UCNPs; **(C)** DLS of silica-coated UCNPs, SA-BSA-UCNP conjugate, and purified SA-BSA-UCNP conjugate. **(D)** Upconversion microscopy image of non-purified SA-BSA-UCNP conjugate, and **(E)** brightness intensity distribution. **(F)** Functional characterization of SA-BSA-UCNP conjugate in BSA-biotin assay. The error bars correspond to the standard deviation of three wells.

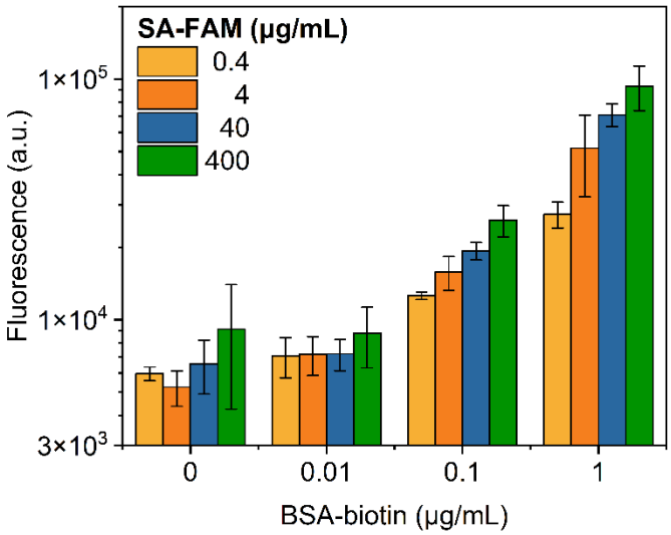

Figure S8: Binding of the streptavidin-carboxyfluorescein (SA-FAM) conjugate to BSA-biotin adsorbed on the surface of microtiter plate. The error bars correspond to the standard deviation of three wells.

2.2 ICC based on UCNP labels

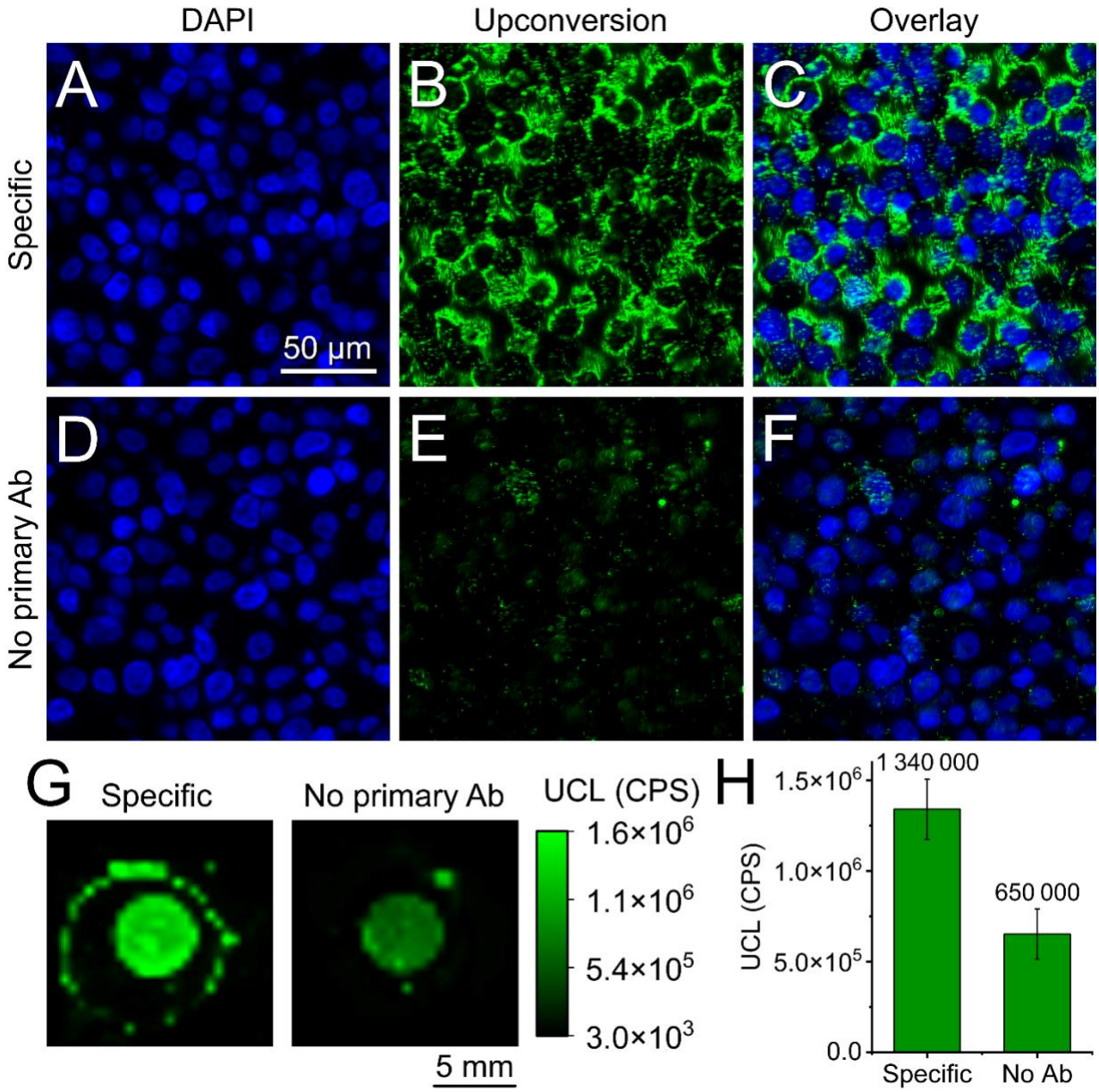

Figure S9: ICC staining of HER2-positive FFPE cells using SA-BSA-UCNP conjugate: **(A)** DAPI channel, **(B)** upconversion channel, **(C)** overlay. Negative control: **(D)** DAPI, **(E)** upconversion, **(F)** overlay. **(G)** Upconversion scan of the cell pellets; **(H)** average upconversion intensities. The error bars correspond to the standard deviations of intensities in the cell pellet.

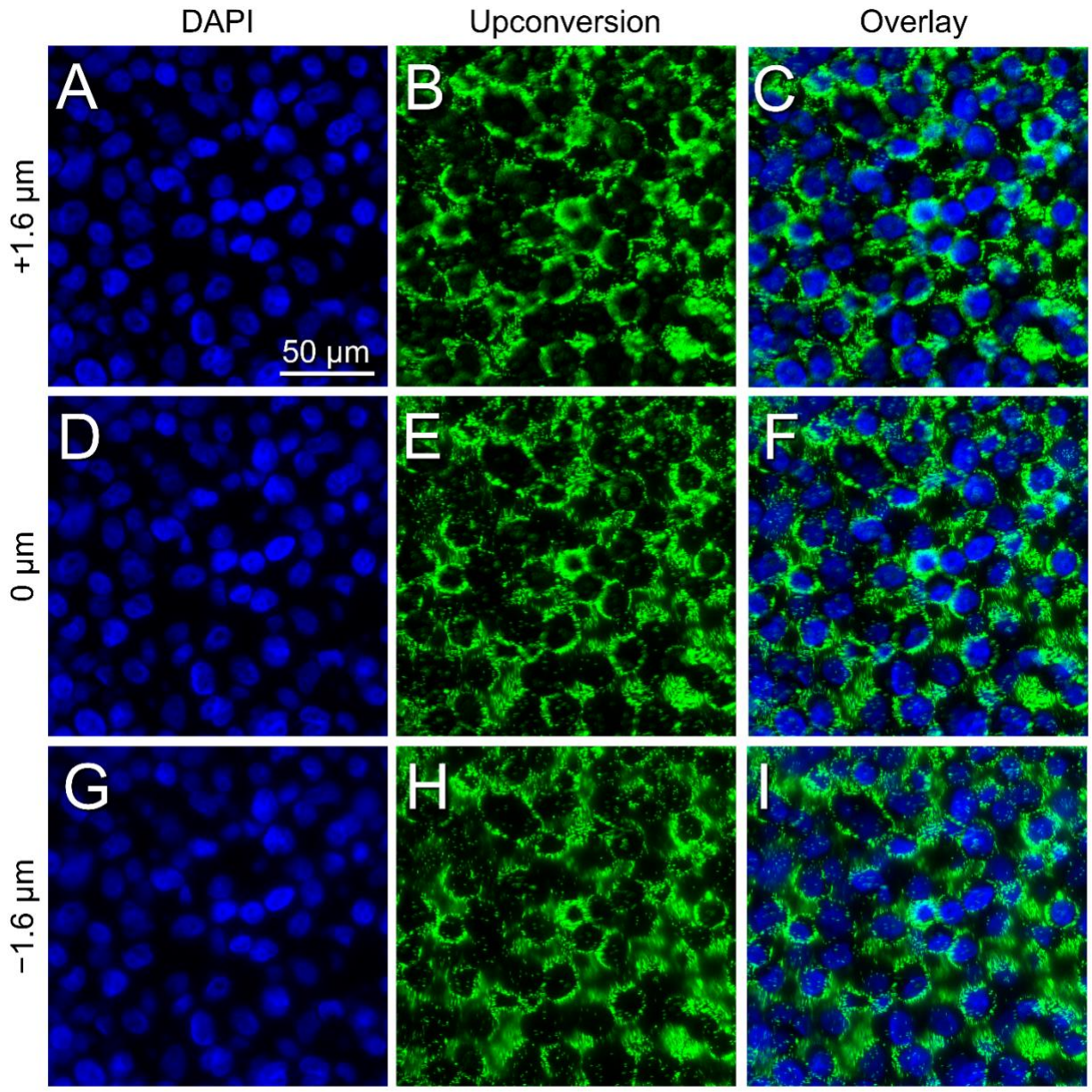

Figure S10: ICC staining of HER2-positive cells measured SA-BSA-UCNP label measured at three focus heights. Focus +1.6 µm: **(A)** DAPI, **(B)** upconversion, **(C)** overlay. Focus 0 µm: **(D)** DAPI, **(E)** upconversion, **(F)** overlay. Focus −1.6 µm: **(G)** DAPI, **(H)** upconversion, **(I)** overlay.

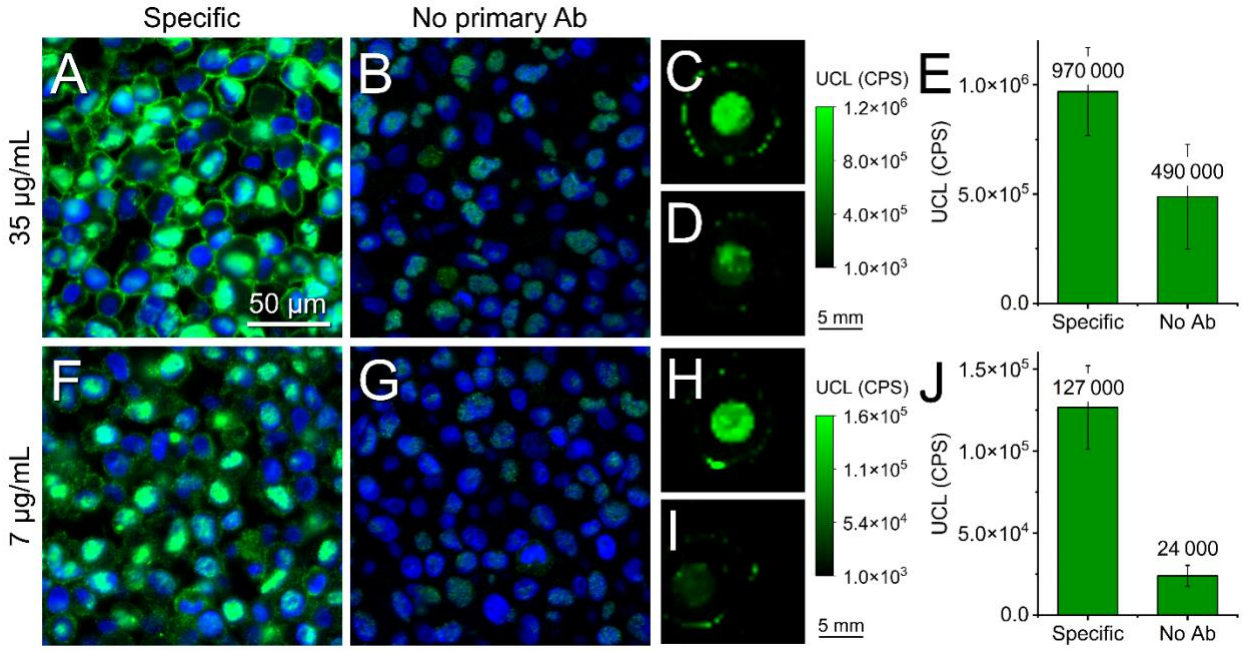

Figure S11: The effect of SA-PEG-Alen-UCNP label concentration on ICC staining of BT-474 cells. Overlay images (DAPI and upconversion channels) of label in the concentration of 35 µg/mL in **(A)** specific binding and **(B)** negative control. Upconversion scan of the cell pellet for **(C)** specific binding and **(D)** negative control; **(E)** average upconversion intensities. Overlay images of label in the concentration of 7 µg/mL in **(F)** specific binding and **(G)** negative control. Upconversion scan of the cell pellet for **(H)** specific binding and **(I)** negative control; **(J)** average upconversion intensities. The error bars correspond to the standard deviations of intensities in the cell pellet.

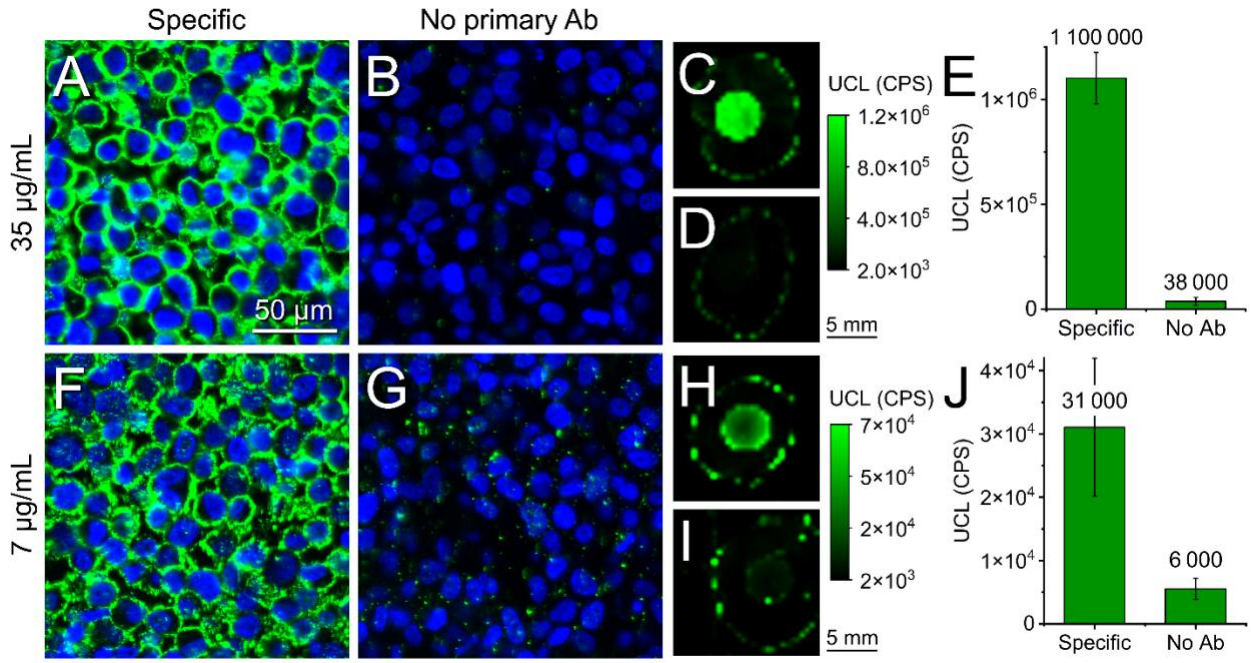

Figure S12: The effect of non-purified SA-PEG-Ner-UCNP label concentration on ICC staining of BT-474 cells. Overlay images (DAPI and upconversion channels) of label in the concentration of 35 µg/mL in **(A)** specific binding and **(B)** negative control. Upconversion scan of the cell pellet for **(C)** specific binding and **(D)** negative control; **(E)** average upconversion intensities. Overlay images of label in the concentration of 7 µg/mL in **(F)** specific binding and **(G)** negative control. Upconversion scan of the cell pellet for **(H)** specific binding and **(I)** negative control; **(J)** average upconversion intensities. The error bars correspond to the standard deviations of intensities in the cell pellet.

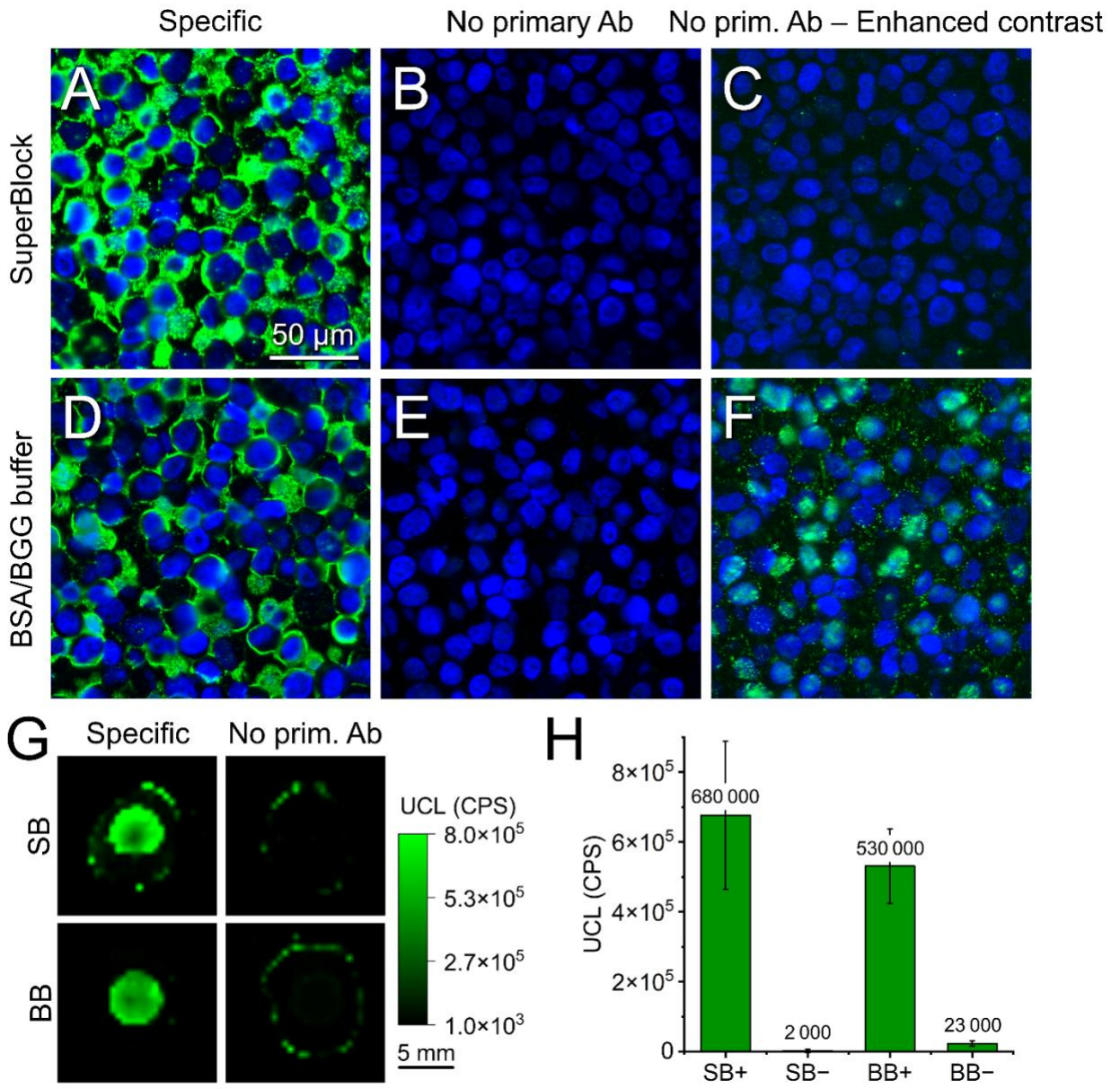

Figure S13: The effect of blocking conditions on ICC staining of BT-474 cells using SA-PEG-Ner-UCNPs. Overlay images (DAPI and upconversion) of cells blocked by SuperBlock: **(A)** specific binding, **(B)** negative control, **(C)** negative control with enhanced contrast; and by BSA/BGG assay buffer: **(D)** specific, **(E)** negative, **(F)** negative with enhanced contrast. **(G)** Upconversion scan of the cell pellets, SB – SuperBlock, AS – assay buffer; **(H)** average upconversion intensities. The error bars correspond to standard deviations of intensities in cell pellet.

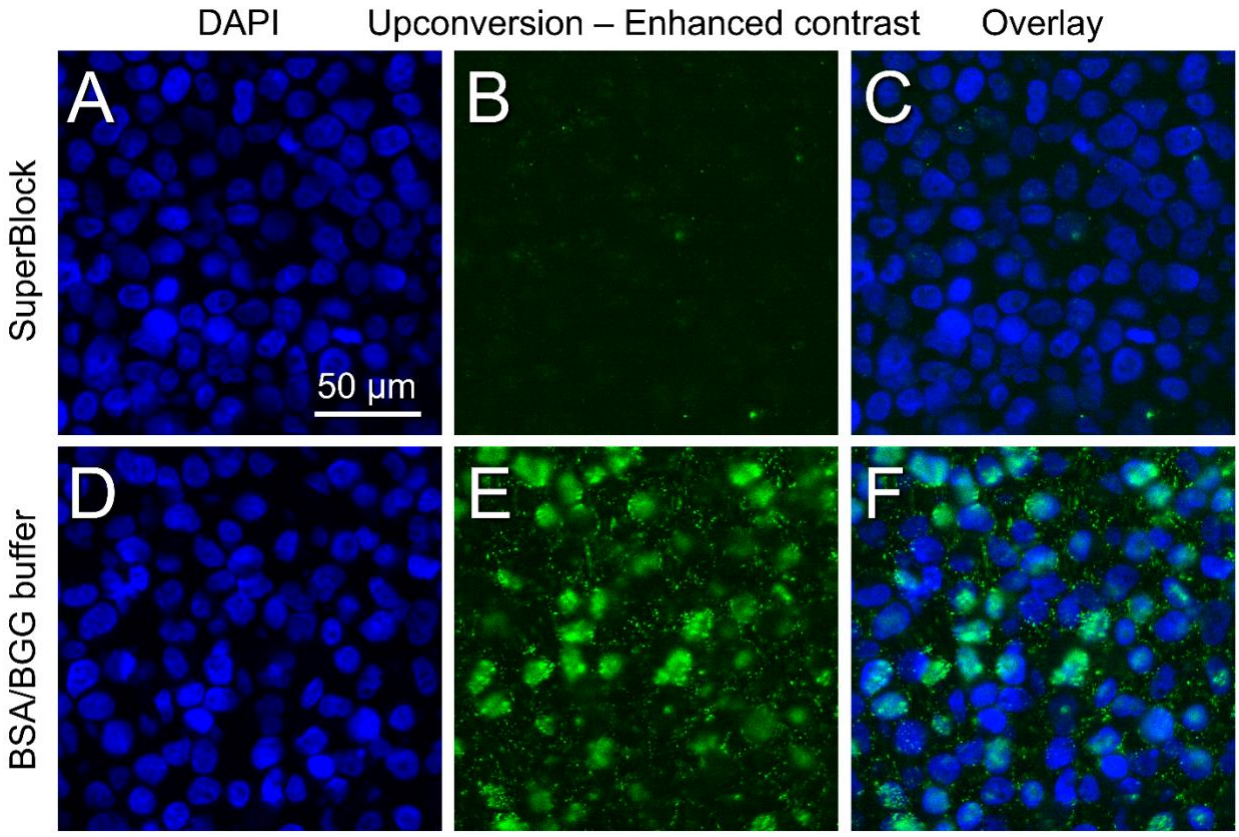

Figure S14: The effect of blocking conditions on the negative control (without primary antibody) in ICC staining of BT-474 cells blocked by SuperBlock: **(A)** DAPI channel, **(B)** upconversion channel with enhanced contrast, **(C)** Overlay; and BSA/BGG buffer **(D)** DAPI, **(E)** upconversion, **(F)** overlay.

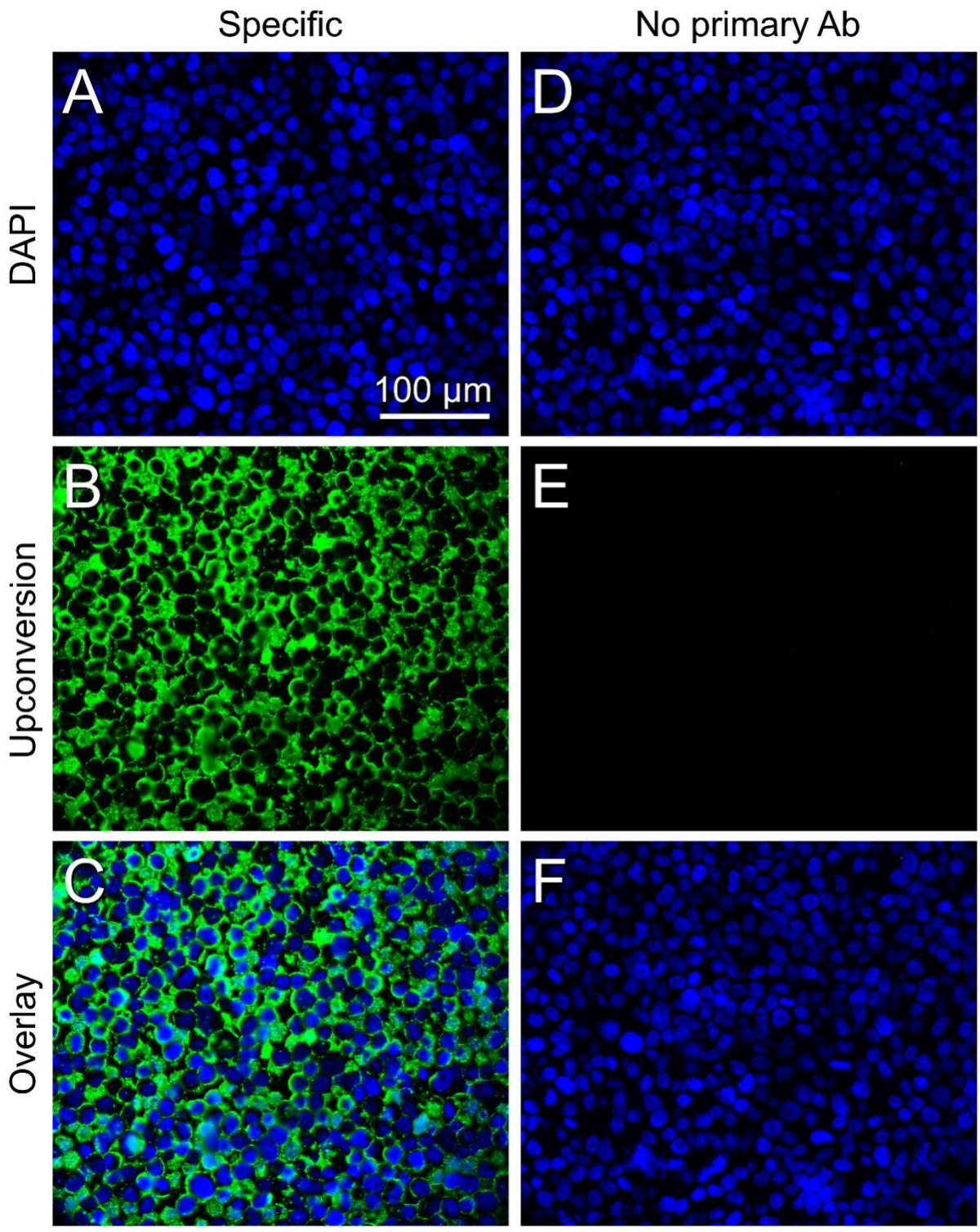

Figure S15: ICC staining of HER2-positive FFPE cells using SA-PEG-Ner-UCNPs: **(A)** DAPI channel, **(B)** upconversion channel, **(C)** overlay. Negative control: **(D)** DAPI, **(E)** upconversion, **(F)** overlay.

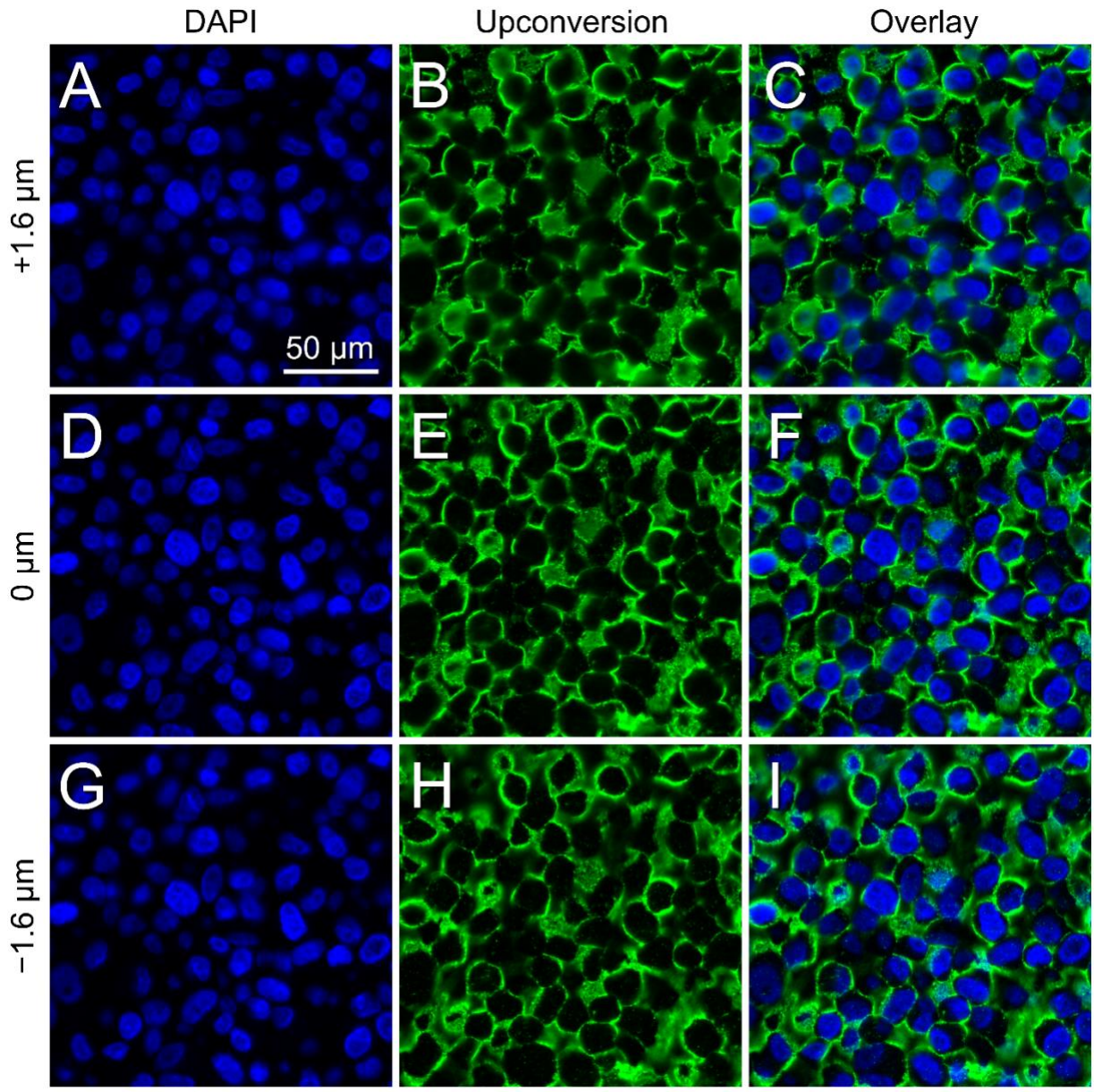

Figure S16: ICC staining of HER2-positive cells measured using SA-PEG-Ner-UCNPs measured at three focus heights. Focus +1.6 µm: **(A)** DAPI, **(B)** upconversion, **(C)** overlay. Focus 0 µm: **(D)** DAPI, **(E)** upconversion, **(F)** overlay. Focus −1.6 µm: **(G)** DAPI, **(H)** upconversion, **(I)** overlay.

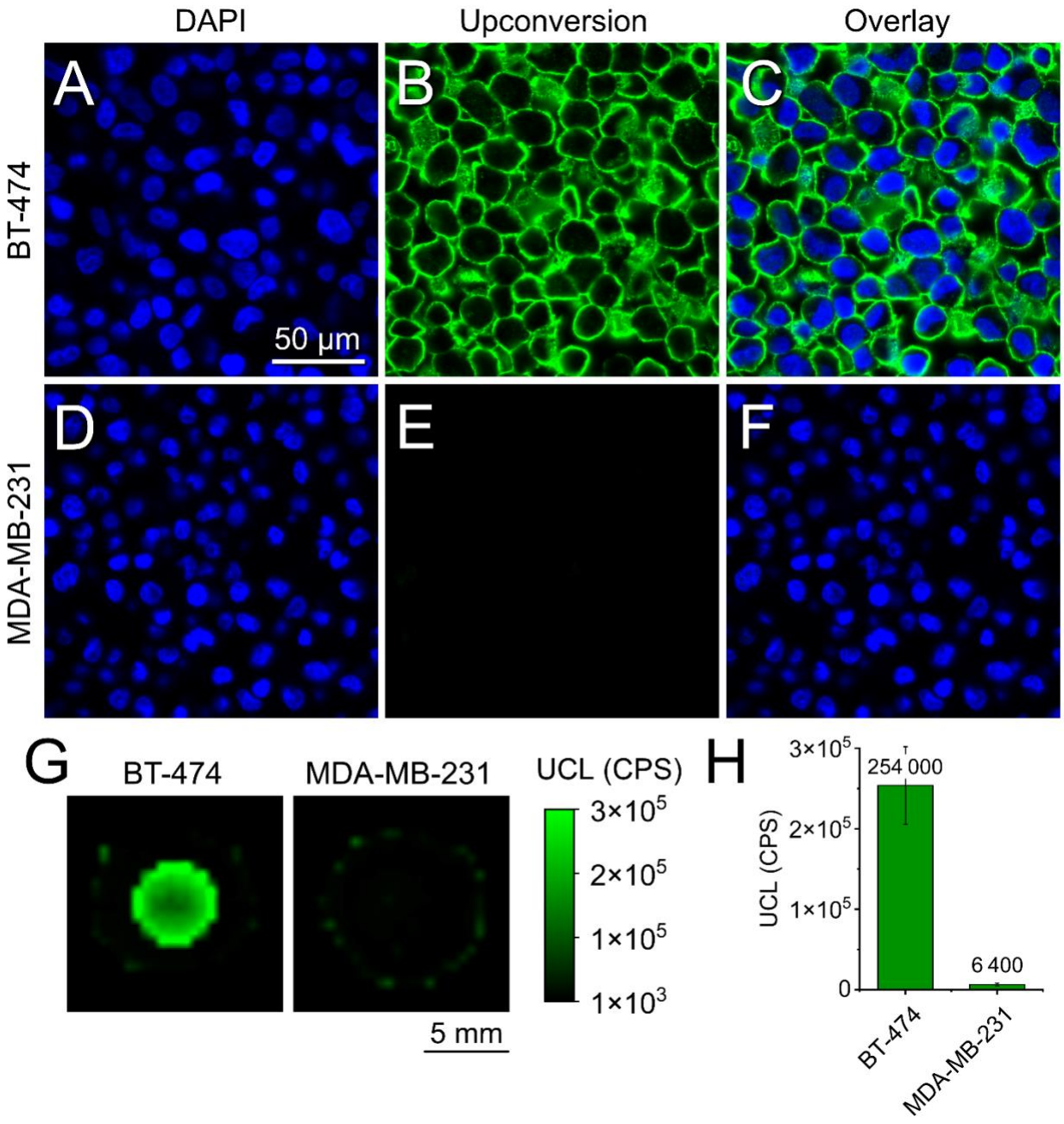

Figure S17: ICC staining of HER2-positive BT-474 cells and HER2-negative MDA-MB-231 cells using SA-PEG-Ner-UCNP label. BT-474 cells: **(A)** DAPI channel, **(B)** upconversion channel, **(C)** overlay. MDA-MB-231 cells: **(D)** DAPI channel, **(E)** upconversion channel, **(F)** overlay. **(G)** Upconversion scan of the cell pellets; **(H)** average upconversion intensities. The error bars correspond to the standard deviations of three independent cell pellets.

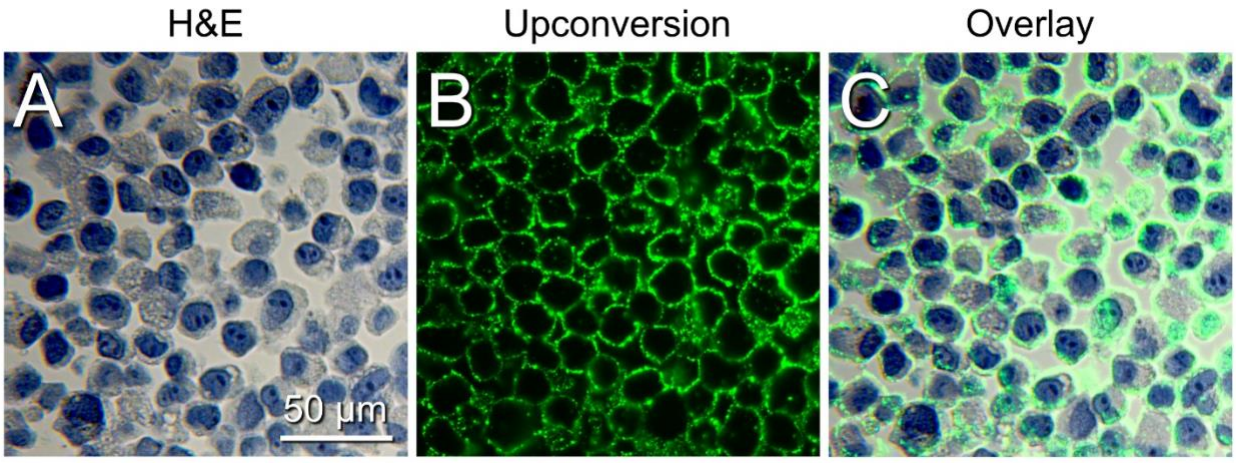

Figure S18: ICC of HER2-positive BT-474 cells using a combination of upconversion labeling and H&E staining. **(A)** Bright-field (H&E), **(B)** upconversion micorscopy, **(C)** overlay.

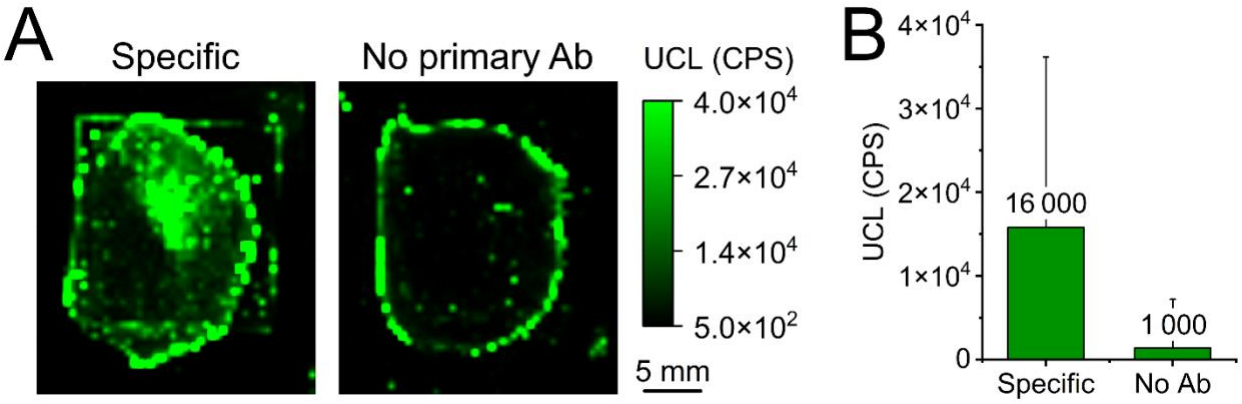

Figure S19: IHC of HER2 positive breast cancer tissue sections labeled with SA-PEG-Ner-UCNPs. **(A)** Upconversion intensity scans. **(B)** Average upconversion intensities. The error bars indicate the standard deviations of intensities in the cell pellet.

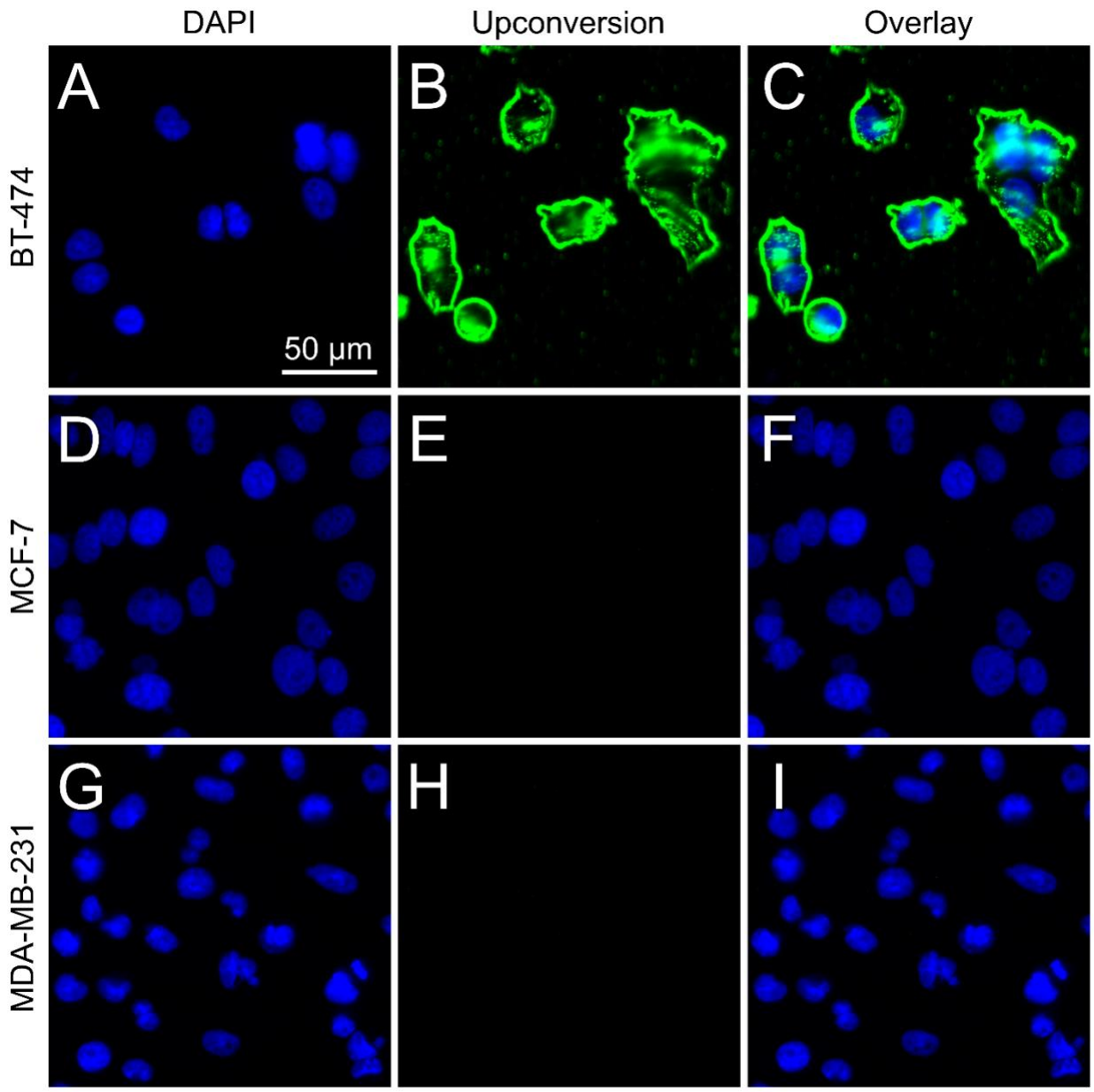

Figure S20: ICC staining of freshly cultivated cells using SA-PEG-Ner-UCNP of BT-474 cells: **(A)** DAPI channel, **(B)** upconversion channel, **(C)** overlay; MCF-7 cells **(D)** DAPI, **(E)** upconversion, **(F)** overlay; MDA-MB-231: **(G)** DAPI, **(H)** upconversion; **(I)** overlay.

2.3 ICC based on fluorescent labels

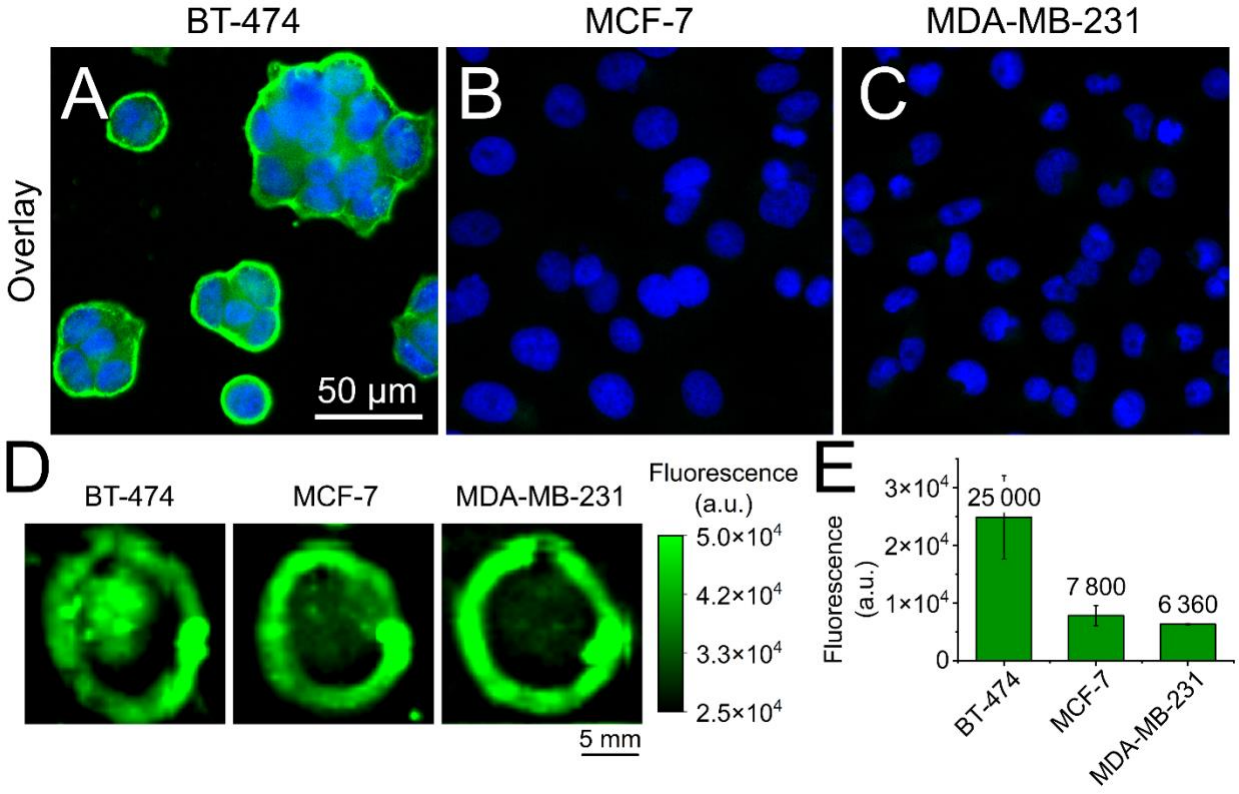

Figure S21: ICC staining of freshly cultivated cells using SA-FAM. Overlay images (DAPI and fluorescence) of cell lines: **(A)** BT-474, **(B)** MCF-7, **(C)** MDA-MB-231. **(D)** Fluorescence intensity scan, **(E)** average fluorescence intensities measured in the cell pellets. The error bars correspond to the standard deviation of three independent cell pellets.

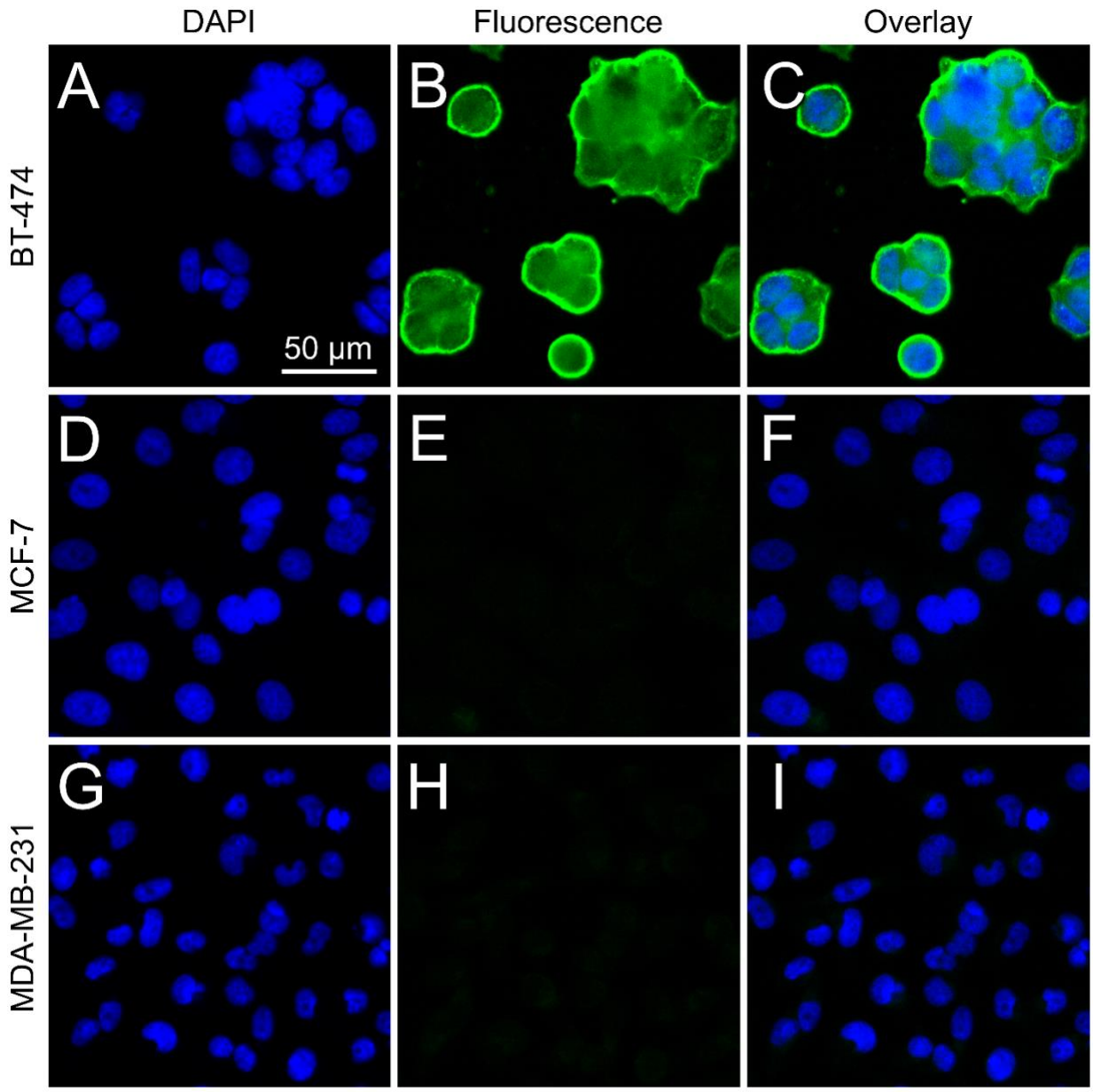

Figure S22: ICC staining of freshly cultivated of BT-474 cells using SA-FAM: **(A)** DAPI channel, **(B)** fluorescein channel, **(C)** overlay; MCF-7 cells **(D)** DAPI, **(E)** fluorescein, **(F)** overlay; MDA-MB-231: **(G)** DAPI, **(H)** fluorescein; **(I)** overlay.

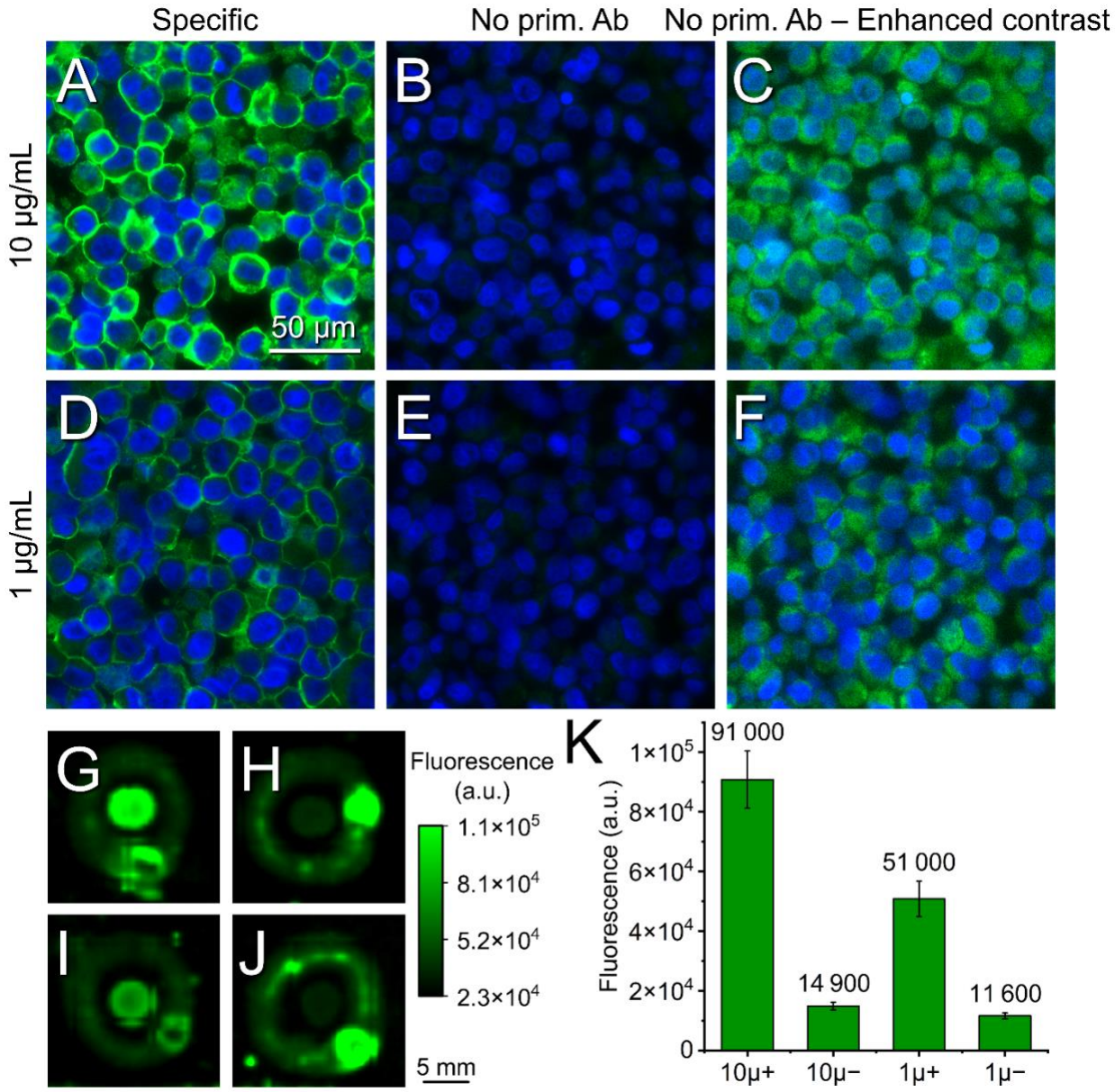

Figure S23: ICC staining of FFPE BT-474 cell pellets using SA-FAM in concentration of 10 µg/mL **(A)** specific, **(B)** negative control, **(C)** negative control with enhanced contrast and 1 µg/mL **(D)** specific, **(E)** negative control, **(F)** negative control. 2D fluorescence intensity scans (fluorescein channel) of: **(G)** 10 µg/mL specific, **(H)** 10 µg/mL negative; **(I)** 1 µg/mL specific; **(J)** 10 µg/mL negative. **(K)** Average fluorescence intensities measured in the cell pellets. The error bars correspond to standard deviations of intensities in cell pellet.

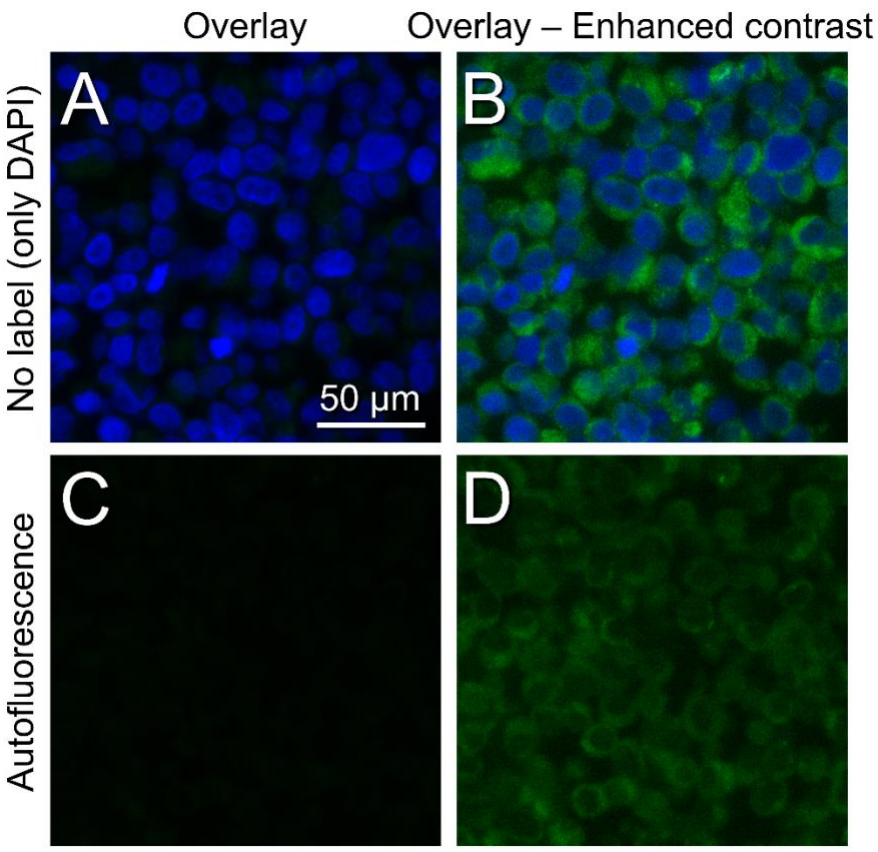

Figure S24: Fluorescence images (overlay signal of DAPI and fluorescein channels) of negative controls in ICC. No detection label, DAPI stained nuclei: **(A)** standard contrast settings, **(B)** enhanced contrast. No detection label, no DAPI: **(C)** standard contrast, **(D)** enhanced contrast.

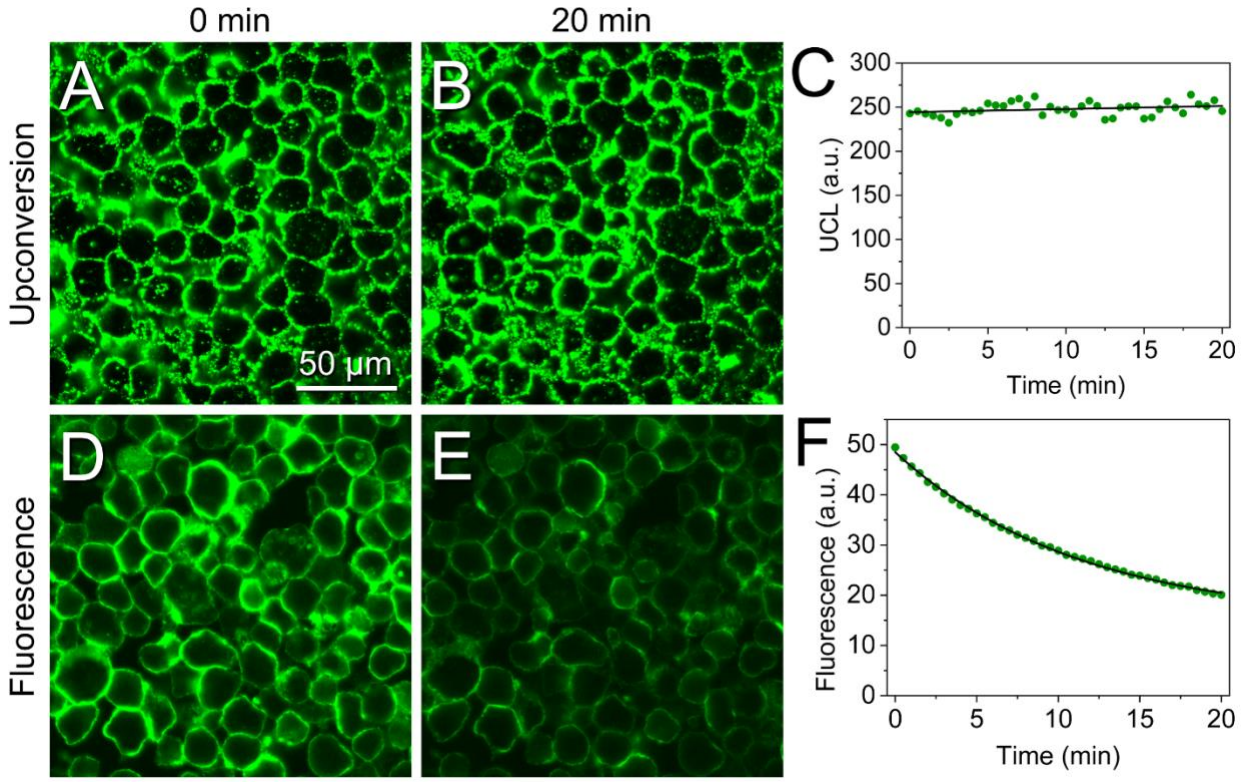

Figure S25: The photostability of ICC staining of the HER2-positive BT-474 FFPE cells. Labeling by SA-PEG-Ner-UCNPs after **(A)** 0 min, and **(B)** 20 min of 980-nm excitation. **(C)** Background corrected upconversion signals fitted with linear function. Staining by SA-FAM after **(D)** 0 min, and **(E)** 20 min of 480-nm excitation. **(F)** Background corrected fluorescence signals fitted with exponential function.

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