Virion-Mimicking Nanocapsules from pH-Controlled-Hierarchical Self-Assembly for Gene Delivery

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Reagents and Materials

Poly(ethylene glycol) methyl ether methacrylate macromonomer (mPEG, molecular weight (Mn) = 2.1KDa, polydispersity index (PDI) = 1.2) from Aldrich as 50% aqueous solution was dried at room temperature under high vacuum. Poly(ε-caprolactone) methacrylate-macromonomer (mPCL, Mn = 3KDa, PDI = 1.4) was synthesized according to literature.[1] PEI (branched, MW 25KDa) was purchased from Aldrich. pEGFP-C1 vector plasmids were purchased from CLONTECH (Mountain View, CA). The plasmids were propagated in Top 10 Escherichia coli. competent cells (Invitrogen Corp.) in Luria Bertani (LB) agar broth supplemented with 50 µg/mL kanamycin (Sigma) at 37 °C. The mixture was gently shaken overnight at 180 rpm. The plasmids were purified using QIAGEN plasmid Midi Kit (Valencia, CA) according to the manufacturer’s instruction. The DNA concentration was determined by measuring its UV-absorbance at 260 nm. The DNA purity was determined by measuring its optical density (OD) with BIO-RAD Smartspec™ 3000, and the DNA with an OD260/OD280 ratio greater than 1.8 was used. The pEGFP plasmids were verified by the restriction enzyme assay and followed by 0.9% agarose gel electrophoresis to confirm that there was no gene rearrangement during cloning and propagation. DNA aliquots were stored at -20 °C prior to use.
Instrumentation

Gel permeation chromatography (GPC) using a Waters SEC equipped with a Waters 2414 refractive index detector and a laser-light scattering detector (Precision Detector) and two 300-mm Solvent-Saving GPC Columns (molecular weight ranges: $5 \times 10^2$-$3 \times 10^4$, $5 \times 10^3$-$6 \times 10^5$) was used to determine polymer molecular weights and molecular weight distribution (PDI). The measurements were performed at a flow rate of 0.30 mL/min using THF as solvent at 30 °C. A SpectraMax 190 microplate UV spectrometer (Molecular devices, Union City, CA) was used to record UV absorbance at 570 nm for MTT assay.

Synthesis of poly(2-diethylaminoethyl methacrylate) (PDEA) macromonomer (mPDEA) (Scheme S1)

A typical synthesis procedure is as follows: A dried 100 ml round-bottomed flask was degassed by vacuum/nitrogen purging with heating for three cycles. Dried THF (20 ml) and 2-ethanolamine (0.121ml, 2 mmol) were added via dry syringes. Potassium naphthalene (2 mmol) in THF was then added dropwise. The solution was stirred at room temperature for several minutes to form the alcoholate initiator. Freshly dried DEA monomer (6 ml, 30 mmol) was added quickly to the solution. The polymerization was maintained at room temperature for 5 h before adding a small amount of methanol to terminate the reaction. The product was purified by repeated precipitation in cold n-hexane. The oily polymer (ω-amino-PDEA) was isolated in a yield of 93.4%. The resulting PDEA had a Mn of 2.5 KDa and a PDI of 1.3. $^1$H NMR (CDCl$_3$): δ 4.01 (br, 2H), 3.53 (t, 2H), 3.44 (t, 2H), 2.68 (br, 2H), 2.54 (br, 4H), 1.94 (br, 2H), 1.03 (br, 9H).

The ω-amino-PDEA (Mn: 2.5KDa) reacted with methacryloyl chloride to form PDEA macromonomer (mPDEA). The reaction was performed under dry nitrogen. Dried CH$_2$Cl$_2$ (60 ml) and triethylamine (1.95ml, 14 mmol) were added via dry syringes to a 100 ml round-bottomed flask containing ω-amino-PDEA (10 g, 4 mmol). Methacryloyl chloride (1.25 g, 12 mmol) in CH$_2$Cl$_2$ was added dropwise to the solution. The reaction was kept at 0° C for 2 h and then at room temperature overnight. The solution was filtered to remove the salt. The CH$_2$Cl$_2$ solution was washed with water for several times and finally precipitated in cold n-hexane. The oily product was isolated (yield: 57.8%) and dried under vacuum. $^1$H NMR (CDCl$_3$): δ 6.24 (s, weak), 5.83 (s, weak), 4.01 (br, 2H), 2.68 (br, 2H), 2.54 (br, 4H), 1.94 (br, 2H), 1.03 (br, 9H).
**Scheme S1.** Synthesis of PDEA macromonomer (mPDEA)

**Synthesis of the terpolymer brush by macromonomer copolymerization (Scheme S2)**

The brush terpolymer was synthesized by free radical copolymerization as we reported.\[^2\] mPCL (Mn = 3.0 KDa, 2.0 g, 0.6 mmol), mPEG (Mn = 2.1KDa, 1.25 g, 0.6 mmol), mPDEA (Mn = 2.5 KDa, 1.52g, 0.6 mmol), and AIBN (60 mg) were charged in a Schlenk tube. The tube was sealed and degassed by vacuum/nitrogen purging for three cycles. Degassed DMF (5 mL) was added via a syringe. The mixture was heated in an 80 °C oil bath. At timed intervals, the reaction solution (0.05 ml) was taken using degassed syringes. The progress of the polymerization and the resulting polymer molecular weight and PDI were measured by GPC.\[^2\] Finally, the reaction mixture was precipitated in 10-fold methanol and dialyzed against DI water (MWCO = 12 KDa) to remove the unreacted mPEG. The final product was isolated in a yield of 43.9% and dried under vacuum for 48 h. ¹H NMR (CDCl₃): 4.05 (br, COOCH₂CH₂N, COOCH₂(CH₂)₄), 3.62 (-OCH₂CH₂O), 2.71 (br, -CH₂COOCH₂CH₂), 2.58 (br, NCH₂CH₃), 2.30 (br, OOCH₂(CH₂)₄O), 1.94 (br, CH₂(CH₃)COO), 1.65 (br, OOCH₂CH₂(CH₂)₃O), 1.38 (br, OOC(CH₂)₂CH₂(CH₂)₂O), 1.25 (br, (CH₃)₂CHO), 1.03 (br, -C(CH₃)COO, NCH₂CH₃). The PCL/PDEA/PEG chain ratio determined with ¹HNMR was 0.90:1.0:1.9.

**Scheme S2.** Preparation of the terpolymer brush PCL/PDEA/PEG via macromonomer copolymerization
Agarose gel retardation assay. The complex solution (12 µL) mixed with 3 µL of 6 × loading buffer was loaded to agarose gel. Polymer/DNA complexes were electrophoresised on a 0.9% (w/v) agarose gel in 0.5×Tris-boric acid-EDTA buffer at 130 V for 90 min. A 1 Kb Plus DNA Ladder (Invitrogen, Carlsbad, CA) was used as a DNA size marker. DNA bands were visualized by staining with ethidium bromide excited by UV transillumination, and photographed by a Lumi-Imager.

Figure S1. Agarose gel retardation of the terpolymer brush/DNA polyplex at pH 5. N/P ratio of 1/1 (lane 1); 2/1(lane 2); 4/1(Lane 3); 10/1(lane 4); 20/1(lane 5); 50/1(lane 6); 100/1(lane 7); Naked DNA (lane 8); DNA ladder (lane 9)

Polyplex size and zeta-potential measurements and TEM observation: The sizes of the polyplexes were determined using a Nano-ZS Zetasizer (Malvern Instrument Ltd., UK) with a laser light wavelength of 632.8 nm and scattering angle at 173°. The polyplexes were prepared as described above. The Zetasizer was routinely calibrated with a 60-nm-nanosphere™ standard (Duke Scientific Corp. CA). Each measurement was performed in triplicate, and the results were processed with DTS software version 3.32.

The zeta-potentials were determined using phase analysis light scattering technology with the zetasizer, which was routinely calibrated with a -50-mV-zeta-potential standard (Malvern Instruments). The measurements were performed in disposable zeta capillary cells at 37 °C for 30 runs, and the results were processed with DTS software version 3.32.

Polymer/DNA complexes were further characterized with a transmission electron microscope (TEM). The polyplexes were prepared at the N/P ratio of 10. Samples were taken for observation at pH 5.0 and 7.2. Samples (8 µl) were applied onto 150-mesh carbon-
coated copper grids for 30 s. The excess solution was wicked off with filter paper. This process was repeated 5 times. Images were recorded using a Hitachi H-7000 transmission electron microscope operated at a voltage of 75 kV with an original magnification of 30,000× and 100,000×.

**Figure S2.** TEM images of PCL/PDEA/PEG/DNA nanocapsules at pH 7.2. N/P = 10. Bar = 200 nm.

**Figure S3.** Particle size and zeta potential of the nanocapsules at pH 7.2 as a function of N/P ratio.
**Cell culture:** SKOV-3 adenocarcinoma cells were obtained from American Type Culture Collection (Rockville, MD). Cells were propagated to confluence in T-75 flasks (Corning Costar, Cambridge, MA) at 37 °C under an atmosphere of 5% CO₂ in 15 ml of RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 10 µg/mL insulin, and 1% antibiotic/antimycotic solution (Sigma A9909). Cells were harvested from flasks with 0.25% trypsin/0.03% EDTA.

**Toxicity assay:** SKOV-3 cells were seeded in 96-well plates at an initial density of 15,000 cells/well in 200 µL of RPMI complete medium. The cells were allowed to grow for 24 h. The original media were replaced with 100 µL of fresh medium. The terpolymer, or PEI solution was added to the medium at polymer concentrations ranging from 0.5 µg/mL to 20 µg/mL. Each dosage was replicated in 3 wells. Treated cells were incubated at 37 °C under a humidified atmosphere of 95% air and 5% CO₂ for 8 h, and then the medium was replaced with 100 µL of fresh culture medium and further cultured for 40 h. MTT reagent (10 µL) was added to each well and the cells were incubated for another 2 h at 37 °C. Detergent reagent (100 µL) was added to each well and then the plates were placed in a 37 °C incubator for 2 h or until all the crystals dissolved. The absorbance at 570 nm of the solution in each well was recorded using a microplate reader and expressed as a percent relative to control cells.

![Figure S4. Cytotoxicity of the terpolymer (TC) and PEI (25KDa, branched) to SKOV-3 ovarian cancer cells estimated by MTT Cell Proliferation Assay; Data represent mean ± Std. E., n = 3.](image-url)
Ethidium bromide (EB) exclusion assay of the DNA in the nanocapsules using fluorescent microscopy: The nanocapsule samples were prepared as described above. The nanocapsule solution (200 μl) at pH 7.2 or 5 was transferred into the wells of an 8-well Chamber Slide system (Lab-Tek II, Balge Nunc International). EB solution (10 μl, 1 mg/mL) was added to each well. The naked DNA solution at the same concentration was used as the control. Samples were observed using an Epi-Fluorescent Microscope (Nikon TE 300) from the fluorescent channel (excitation at 500 nm, emission at 617 nm). Images were taken 30 min after adding EB.

Confocal microscopy observation of the nanocapsule membrane: The terpolymer (2 mg) was dissolved in pH 5.0 acetic acid buffer (1 ml) by vortexing and then PKH26 (10 μl, 1 μmol/ml) was added with vortexing. Fifty microliter of the sample was taken for confocal fluorescent microscope observation. DNA solution (40 μg/ml, 119 μl) (N/P ratio of 10) was added to a 50 μl of above polymer solution and mixed by gentle shaking. After 30 min, the pH of the solution was adjusted to 7.2. The sample (50 μl) was applied on a microscope slide. The PKH26-labelled polymer solution and nanocapsule solution were observed on a Leica TCS SP2 confocal microscope using a 543-nm laser for excitation. The emission wavelength was read from 560 to 610 nm and expressed as red.

**Figure S5.** Confocal microscopy images of PCL/PDEA/PEG polymer micelles labeled with PKH26. Original magnification is 2016 ×.

**DNA binding kinetics of ethidium bromide evaluated by fluorescent activity as a function of time:** Nanocapsule solution (200 μl) at pH 7.2 prepared as described above was added to wells of a 96-well assay plate (COSTAR, Corning, NY). EB solution (1 mg/mL, 10 μl/well) was added quickly to the wells. The fluorescent intensity in each well as a function
of time was recorded using a microplate spectrofluorometer (Gemini XS, Molecular devices, Union City, CA) at the excitation of 530 nm and the emission of 590 nm.

![Graph](image)

**Figure S6.** The fluorescent intensity as a function of time of the nanocapsules (■) and naked DNA (♦) after adding with ethidium bromide at pH 7.2.

**Cellular uptake observed by confocal microscopy:** SKOV-3 ovarian cells were plated into glass-bottom petri dishes (MatTek, Ashland, MA, no. P35G-1.0-14-C) at 80,000 cells per plate in 2 mL of RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 10 μg/ml insulin, and antibiotic/antimycotic solution. They were incubated for 24 h at 37 °C and 5% CO₂ before treatment. pEGFP plasmid (10 μg) was labelled with Label IT-Cy5™-nucleic acid labelling kit (Mirusbio, Madison, WI) according to the manufacturer’s protocol. The nanocapsules at an N/P ratio of 10 and the PEI/DNA polyplexes at an N/P ratio of 5 were prepared using the labelled plasmids. The Cy5-labeled nanocapsules and PEI/DNA complexes were added to the DMEM medium at a DNA concentration of 2 µg/mL. Control experiments using the plasmids or the polymers only were carried out at the same time. After 1 h incubation, the original medium in each dish was removed. Cells were washed twice with fresh medium. The Cy5-dye was excited using a 633-nm laser, and the emission wavelength was read from 660 to 810 nm and expressed as blue. Images were produced with a 63 × objective lens. Cells were kept at 37 °C and 5% CO₂ except being observed on the microscope. Images were processed with NIH ImageJ.
Figure S7. Cellular uptake of Cy5-labeled nanocapsules (A, B) and PEI/DNA complexes (C, D) observed by confocal laser scanning microscopy. 1 h incubated with SKOV-3 cells. Cy5 channel (left panel A, C); overlap of images from the Cy5 and transmittance channels (right panel, B,D). Original magnification is 63 ×.

Cellular internalization quantified by fluorescent activated cell sorting: SKOV-3 cells were seeded at a density of 60,000 cells/well (30,000 cells/mL in RPMI media) in 6-well tissue culture plates (Costar). The cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ until the cells reached about 70% confluence. The pEGFP DNA was first labelled with Label IT Cy™3 nucleic-acid-labelling-kit (Mirusbio, Madison, WI) according to the manufacturer’s protocol and used to make the nanocapsules at an N/P ratio of 10 or PEI-polyplexes at an N/P ratio of 5 as described above. The Cy3-labeled nanocapsules and PEI/DNA complexes were added to the DMEM medium at a DNA concentration of 2 µg/mL. Control experiments using the plasmids or the polymers only were carried out at the same time. After 1 h incubation, the medium in each dish was
removed. The cells were washed twice with PBS and harvested by trypsin-EDTA treatment into 1 mL of PBS (containing 3% FBS) in microcentrifuge tubes. They were centrifuged at 1000 rpm for 4 min at 4 °C. The supernatant was aspirated, and the cell pellets were washed with 1 mL of PBS. The cells were further washed twice and then resuspended in 1 mL of PBS. The cells having Cy3-fluorescence were enumerated by fluorescence-activated cell sorting (FACS, NPE Quanta™ system). Both forward and side-scatter (granularity measure) were measured in arbitrary units on a linear scale. Forward and side-scatter were "gated" to exclude debris and clumped cells; gating was used identically for all the analyses. Cells were excited using a 488 nm laser and the Cy3 signals were collected through a 570 nm band-pass filter in FL1 channel.

**Subcellular colocalization:** SKOV-3 cells were plated into glass-bottom petri dishes (MatTek, Ashland, MA, no. P35G-1.0-14-C) at 80,000 cells per dish in 2 mL of RPMI-1640 medium (Sigma-Aldrich) and incubated for 24 h at 37 °C and 5% CO₂. Treatments were prepared in the RPMI medium containing 10 mM HEPES (pH 7.4). The nanocapsules were prepared as described above at the N/P ratio of 10 except the presence of PKH26 (3 μl, 0.4 μM). The nanocapsule solution was added to the medium at a DNA concentration of 4 µg per well. After 3 h incubation, lysotracker (Molecular Probes, Carlsbad, CA) was added to the wells at a concentration of 150 nM. The images were taken 1 h later using a confocal microscope. Nuclear dye DRAQ5™ (5 μM, AXXORA LLC, San Diego, CA) was added right before the observation. Lysotracker was excited using a 488-nm laser, and its emission was read from 510 to 540 nm and expressed as green. The labelled nanocapsules were excited using a 543-nm laser, and its emission wavelength was read from 560 to 610 nm and expressed as red. The nuclear staining was excited using a 633-nm laser, and its emission wavelength was read from 660 to 810 nm and expressed as blue. Images were produced by using the lasers sequentially with a 63 × objective lens. Cells were kept at 37 °C and 5% CO₂ except being observed on the microscope. Images were processed with NIH ImageJ.
Figure S8. Subcellular colocalization of PCL/PDEA/PEG/DNA nanocapsules labeled with PKH26 after 4 h incubated with SKOV-3 cells observed by confocal scanning laser microscopy. Overlap of the images from the transmittance, LysoTracker, PKH26 and Draq 5 fluorescence channels. Original magnification is 63 ×. (black arrows point the yellow spots resulting from the nanocapsules in the lysosomes; white arrows point pink spots resulting from the nanocapsules in the nuclei).

In vitro gene transfection to SKOV-3 ovarian cancer cells: SKOV-3 cells were cultured as described above. The cells were transfected with pEGFP-encapsulated nanocapsules at N/P ratios ranging from 2:1 to 10:1 and PEI-polyplexes at an N/P of 5 prepared as described above. The polymers at the corresponding concentrations were employed as control. A typical example is as follows. The nanocapsules were prepared as described above and diluted with FBS-free DMEM medium. The cell original culture medium in each well was replaced with 500 μL transfection medium containing 4 μg of pEGFP per well. After 2 h incubation, 500 μL of RPMI-1640 medium containing 20% FBS was added and the cells were continuously incubated for additional 6 h. The transfection medium in each well was then replaced with 1 mL of RPMI normal culture medium. The cells were incubated for 40 h at 37 °C. The cells were harvested from each well by trypsin-
EDTA treatment, and suspended in 1 mL of PBS (containing 3% FBS) in a microcentrifuge tube. They were centrifuged at 1000 rpm for 4 min at 4 °C. The supernatant was aspirated, and the cell pellet was washed with 1 mL of PBS. The cells were further washed twice and then resuspended in 1 mL of PBS. The cells expressing GFP were enumerated by fluorescence-activated cell sorting (NPE Quanta™). Both forward and side-scatter (granularity measure) were measured in arbitrary units on a linear scale. Forward and side-scatter were "gated" to exclude debris and clumped cells; gating was used identically for the analyses. Cells were excited by a 488 nm laser and the GFP signal was collected through a 525 nm band-pass filter in FL1 channel.

**Cellular uptake and in vitro pALDH2 gene transfection of neonatal mouse cardiomyocytes (NMCMs) quantified by Western immunoblotting assay:** POPO-3 labeled DNA was used to monitor polyplex uptake rate. PEI/DNA polyplexes, naked DNA were employed as positive and negative controls, respectively. The NMCMs were isolated according to our reported procedure and transferred at a density of 15,000 cells/well into an 8-well Chamber Slide system (Lab-Tek II, Balge Nunc Interanitional) and cultured for 2 days before the treatment. The preparation of PEI-Polyplexes and nanocapsules, and the subsequent cell culture and observation procedures were similar to those described in the experiment using SKOV-3 ovarian cancer cells. A confocal microscope (Leica TCS SP2, Bannockburn, IL) was used to observe the cellular uptake. The excitation wavelength was 534 nm, and the emission wavelength was 570 nm.

**Figure S9.** Confocal microscopy of neonatal mouse cardiomyocytes cultured with the PEI/pALDH or the nanocapsules loaded with pALDH for 2 h. The DNA was labeled with POPO-3.
Western immunoblotting assay: NMCMs were seeded at a density of 60,000 cells/well in 6-well tissue culture plates (COSTAR). The cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ until the cells reached about 70% confluence. The cells were transfected as described above at 3 μg plasmids/well with the nanocapsules of pALDH2 at N/P of 10 or PEI-polyplexes at N/P of 5. The cells were incubated for 6 h at 37 °C and then the transfection medium in each well was replaced with fresh complete DMEM culture medium. After 72 h incubation, the cells were gently washed with 1× PBS and then harvested by scraping in lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1mM EDTA, 1 mM EGTA, 1% Triton, 0.1% SDS, and the protease inhibitor. After sonication, equal amounts of lysates (50 μg/lane) were separated on 10% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad) and transferred to nitrocellulose membranes (0.2 μm). The membranes were blocked in 5% (w/v) nonfat milk in TBS-T buffer, and then incubated with anti-pALDH2 (1:1000) antibody. Monoclonal antibody to ALDH2 was a generous gift from Dr. Henry Weiner, Purdue University, West Lafayette, IN. The antigens were detected by the luminescence method (ECL Western blotting detection kit, Amersham Biosciences) with peroxidase-linked anti-rabbit IgG (1:10000 dilution). The intensity of immunoblot band was detected with a Bio-Rad Calibrated Densitometer (model GS-800).

Figure S10. Comparison of the pALDH2 expression in neonatal mouse cardiomyocytes transfected by the nanocapsules (N/P of 10) and PEI-polyplexes (N/P of 5) quantified using western immunoblotting assay.
**In vivo gene delivery to subcutaneous SKOV-3 cancer tumors:** The following experiments were conducted with the approval of the University of Wyoming Animal Care and Use Committee. Athymic (nu/nu, 6-8 weeks old) mice (BALB/c strain) were inoculated by subcutaneous injection of SKOV-3 cells \((1\times10^7)\) suspended in 0.1 mL of PBS. Animals were maintained in a pathogen-free environment under controlled temperature \((24 \, ^\circ\text{C})\) and lighting \((12\text{L}:12\text{D})\) conditions. Sterilized rodent chow and water were supplied ad libitum. Targeted nanocapsules were fabricated from PCL/PDEA/PEG terpolymer \((1.67 \, \text{mg})\), pEGFP DNA \((80 \, \mu\text{g})\) and 5 mole% PEG2K-folate lipid \((\text{DSPE-PEG}(2\text{KDa})-\text{Folate}, \text{Avanti Polar Lipids, Inc})\) relative to the polymer as described above. After 6 weeks of tumor inoculation, four mice were injected with 150 \(\mu\text{L}\) PBS solution of the nanocapsules containing 15 \(\mu\text{g}\) of pEGFP DNA per mouse via the tail vein. Mice injected with PBS were the control. The mice were then sacrificed 48 h post-transfection. The tumor, liver, and skeleton muscle were isolated to perform histology evaluation for GFP expression. Tissues were excised and fixed by immersion in Histocoice (Amersco, Solon, OH). Samples were washed with PBS, dehydrated, cleared, infiltrated with paraffin wax, and sectioned at a thickness of 7 \(\mu\text{m}\). Sections were floated onto microscope slides, air-dried, deparaffinized in xylene, rehydrated, and examined by fluorescent microscopy. An Epi-fluorescence microscope (Nikon TE 300) was used to detect GFP in tissue sections from the transfection mice. GFP-expression was imaged using a digital Cascade F512 camera with fitted EGFP-specific filters. Identical exposure times were used for each image within the whole experiment. The tissues were also stained using a standard Hematoxylin and Eosin (H&E) staining procedure and observed using a light microscope (Figure S12).
**Figure S11.** GFP-expression in the tumor transfected with the nanocapsules imaged using fitted EGFP-specific filters. Original magnification is 100 ×.

**Figure S12.** The image of the tumor tissue after Hematoxylin and Eosin (H&E) staining in the mice transfected with the nanocapsules. Original magnification is 100 ×.
Figure S13. In vivo GFP-gene expression in muscle of athymic mice inoculated with SKOV-3 ovarian tumors injected with PBS only (a) and pEGFP-plasmid-loaded nanocapsules (b) containing 15 µg pEGFP plasmid via the tail vein. After 48 h, tissues were isolated. Original magnification is 100 ×. See Figure S12 for the Hematoxylin and Eosin staining image of the tumor.

References