Towards a mechanistic understanding of nanoparticle behavior using zebrafish

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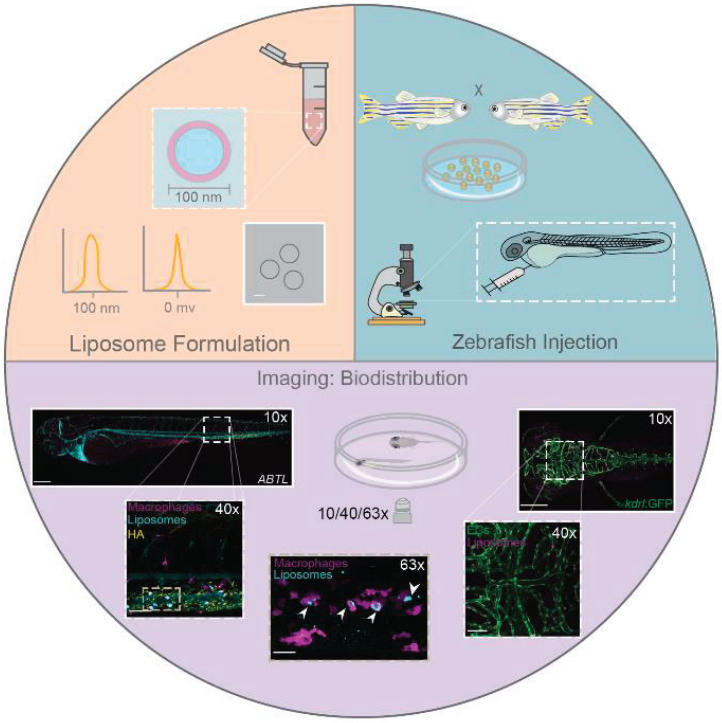
Chapter 7

Zebrafish embryos as a predictive animal model to study nanoparticle behavior *in vivo*



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7.1 Abstract

A failure to fully understand the complex *in vivo* behavior of systemically administered nanomedicines has stymied clinical translation. To bridge this knowledge gap, new *in vivo* tools are needed to rapidly and accurately assess the nearly infinite array of possible nanoparticle designs. Zebrafish embryos are small, transparent, and easily manipulated animals that allow for whole organism visualization of fluorescently labeled nanoparticles in real time and at cellular resolution using standard microscope setups. Furthermore, key nano-bio interactions present in higher vertebrates are fully conserved in zebrafish embryos, making these animal models a highly predictive and instructive addition to the nanomedicine design pipeline. Here, we present a step-by-step protocol to intravenously administer, image and analyze nanoparticle behavior in zebrafish embryos and highlight key nano-bio interactions within the embryonic zebrafish corresponding to those commonly found within the mammalian liver. In addition, we outline practical steps required to achieve light-triggered activation of nanoparticles within the transparent embryo.

7.2 Introduction

The embryonic zebrafish is a predictive, convenient and cost effective animal model to study the complex *in vivo* behaviour of systemically administered nanoparticles and to characterize key nano-bio interactions at a molecular level.[1-6] In contrast to conventional research animal models (*e.g.* mice and rats), zebrafish embryos are small (1-3 mm), optically transparent and readily available in large numbers. These features enable real-time visualization of nanoparticle injected doses using simple fluorescence microscopy setups, at cellular resolution and over large sample sets. In addition, the extensive range of fluorescent transgenic lines (*e.g. mpeg1*:GFP[7] -macrophages; *kdrl*:GFP[8] -endothelial cells), short generational timeframes (approximately 3 months), and the ease of genetic manipulation (*e.g.* CRISPR/Cas9 gene editing),[9, 10] facilitates mechanistic understanding of nanoparticle fate *in vivo*. Crucially, key biological mechanisms underpinning nanoparticle behaviour in higher order vertebrates (*e.g.* rodents and humans) are conserved and functional in zebrafish embryos. In particular, the embryonic zebrafish can accurately predict nanoparticle interactions within the mammalian liver.[3, 6] These interactions account for the (unwanted) clearance of up to 99% of systemically administered nanoparticles.

Here, we provide a step-by-step protocol for the intravenous administration, imaging and analysis of nanoparticles within zebrafish embryos. This protocol describes the use of liposomes but is appropriate for any nanoparticle. In addition, we detail how UV light can be applied to trigger the release of chemical photocages within the embryo *in situ* and *in vivo*. As an example, we have recently used UV light to switch the surface charge of liposomes (from neutral to cationic) within the embryonic zebrafish, revealing new insight into the behaviour of differently charged nanoparticles *in vivo.*[5] In practical terms, a skilled person can inject and mount several hundred zebrafish embryos in a day, and the throughput is generally limited by imaging timeframes. In our experience, confocal imaging (*i.e.* multi-colour whole embryo, 10x objective + 40x/63x ROI), as described below, takes approximately 1 h per embryo. Overall, the embryonic zebrafish is a uniquely powerful addition to the pre-clinical nanomedicine discovery pipeline.

7.3 Procedure

Formulation of fluorescently labelled nanoparticles.

1. Prepare liposomes (or other nanoparticles) using a preferred method (for example, extrusion, ethanol injection or microfluidic preparation[11, 12] for lipid nanoparticles). For fluorescently labelled nanoparticles, ensure that the incorporated dye does not adversely affect nanoparticle biophysical properties but can be easily detected above background autofluorescence within the embryo.

   Preparation of photoactive liposomes, as **Chapter 4** (containing 1 mol% fluorescent lipid probe, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl, DOPE-LR) by extrusion is briefly described. Any aqueous buffer can be used.

   i. Formulate liposomes in 10 mM HEPES buffer at a total lipid concentration of 1-5 mM. Generally, liposomes can be formulated by manual extrusion up to a lipid concentration of approx. 30 mM. Final liposome concentration is typically reported as a total lipid concentration.

   ii. Prepare individual lipid stock solutions (1-10 mM) in chloroform.

   iii. In a glass vial, combine lipids to the desired molar concentration and dry to a film, first under a stream of N_2 then > 1 h under vacuum using a bench-top vacuum desiccator. This is to ensure complete removal of residual chloroform. In the highlighted study, various molar ratios of lipids were used. For instance, to test the effect of increasing positive surface charge (**Figure 2**, **Chapter 4**),[5] liposomes were formulated at 1:1, 1:3 and 1:9 molar ratios of cationic lipids (1-3) and zwitterionic 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC).

   iv. Manually extrude above phase transition temperature (Tm) of all lipids to form large unilamellar vesicles.

   v. Hydrate lipids to create a suspension. Add aqueous buffer to the lipid film to achieve the desired final lipid concentration. **Note:** In the setup described, we recommend using 0.5-1 mL to avoid sample loss in the 'dead' volume (approx. 50 μL) within the mini-extruder block. In the highlighted example, 1 ml of a 10 mM storage solution was added to achieve a final lipid concentration of 1 mM.

   vi. Warm solution above Tm of all lipids. Vortex the solution vigorously to create a homogenous lipid suspension. There should be no visible film or visible aggregates remaining in the solution. Depending on the lipid mixture and concentration, a lipid suspension may appear cloudy or

transparent.

vii. Load suspension into syringe and fit to one side of extruder block. Fit a clean, empty syringe on other side of extruder block. Heat extruder block to desired temperature (*i.e.* 5-10 °C above Tm of all lipids).

viii. Pass 11 times through 2 x 400 nm polycarbonate (PC) membranes– always end in clean syringe to avoid particulate contamination (*e.g.* dust).

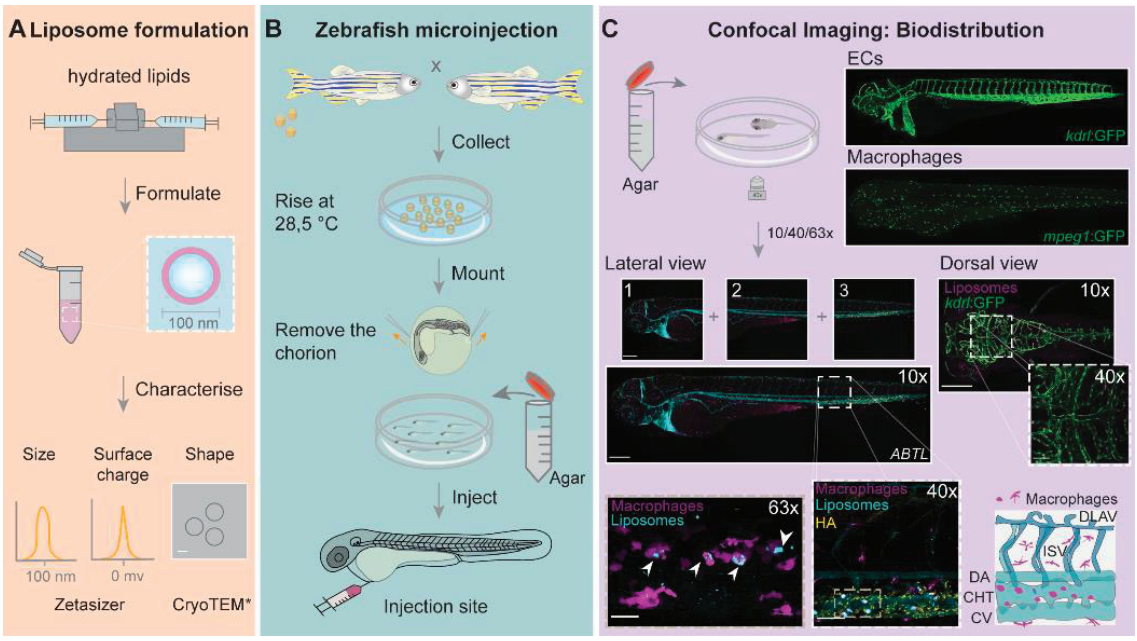

Figure 1. Protocol for intravenous nanoparticle administration, imaging and analysis in zebrafish embryos. (a). Nanoparticle formulation – *e.g.* extrusion of hydrated lipid solution. Following formulation and biophysical characterization, liposomes should be stored at 4 °C. *CryoTEM is optional but recommended. Immediately prior to injection, confirm, at the very least, nanoparticle size. Wherever possible, we recommend using freshly prepared nanoparticles. If this is not possible, confirm nanoparticle stability over time **(b). Zebrafish microinjection**. Cross adult zebrafish (male and female, specific transgenic line if required) to obtain zebrafish eggs by external fertilisation. Raise embryos at 28 °C until the desired stage (*i.e.* ~56 h post fertilisation, hpf). Remove the chorion if embryos have not yet hatched. Mount embryos with agarose gel (0,4% agarose + 0,01% tricaine). Intravenously inject (fluorescently labelled) nanoparticles within the sinus venosus/duct of Cuvier (at 2 dpf). For more details about the injections site see **Figure 2. (c). Confocal imaging nanoparticle biodistribution.** 10x objective to visualise whole body nanoparticle distribution (3-4 overlapping images), 40x objective to visualise tissue level distribution (example shows tail region containing scavenger endothelial cells of the CHT and CV as well as blood resident macrophages), 63x objective to visualise cellular level distribution (example shows liposomes accumulated within macrophages). Liposomes in cyan, macrophages in magenta and fluorescently labelled hyaluronic acid (as a marker for scavenger endothelial cells) in yellow. CV – caudal vein, CHT – caudal hematopoietic tissue, DA – dorsal aorta, DLAV – dorsal longitudinal anastomotic vessel and ISV – intersegmental vessels. Scale bars: 200 µm (10x), 50 µm (40x). Lateral view images are adapted from. [5]

ix. Pass 11 times through 2 x 100 nm PC membranes.
x. Collect the formulated liposomes in a clean glass vial and store in the dark at 4 °C until further use, time depends on liposome stability. We highly recommend using freshly prepared liposomes; however, if using stored liposomes, always confirm size (DLS measurement) immediately prior to use.
xi. (Optional) To analyze light activated liposomes *in vitro*, irradiate liposomes (370 ± 7 nm, 202 mW cm^{-2}) in quartz cuvettes with the LED mounted at a distance of 1 cm from the sample.

2. Characterize the physicochemical properties of the (freshly prepared) liposomes. Size and surface charge are measured using a Malvern Zetasizer Nano ZS. If using stored liposomes, always confirm size immediately prior to injection in embryonic zebrafish.
3. (Optional) CryoTEM analysis to confirm liposome morphology, size and structure [13-16] is strongly advised for any new nanoparticle, in particular, if unusual/unexpected biodistribution is observed in the zebrafish embryo.

Preparation of zebrafish embryos

Note: These steps require a specific license and corresponding approval by regulatory authorities (see *ethics* section below).

1. Breed adult zebrafish by setting up crosses in pairs in breeding tanks during the afternoon, separating male and female with a plastic and transparent divider and use a lid to prevent the zebrafish escaping from the tank.
2. The following morning, remove the divider at the beginning of the light period to allow and control breeding time.
3. Collect zebrafish eggs with a strainer and transfer to petri dishes containing egg water (see *recipes*) (~50-70 embryos per petri dish).
4. Remove unfertilized eggs (remain at a single stage and acquire an irregular shape over time) or dead embryos.
5. Raise eggs in an incubator at 28.5 °C. Replenish with fresh egg water every day.
6. (Optional) Add 25 µl PTU (see *recipe* below) to a petri dish containing 50 ml egg water and around 50-70 embryos, to prevent pigmentation at ~24 h post fertilization.

Microinjection of zebrafish embryos/larvae

1. Remove the chorion protecting zebrafish embryos, if the embryos have not hatched yet, at 56 h post fertilization (hpf). Under a microscope, use a pair of stainless steel tip tweezers to carefully remove the chorion by making a tear in the chorion and pulling opposite sides until the chorion is removed and the embryo is released. Avoid direct contact of tweezers with the embryo to avoid damage to the embryo. **Note:** Pronase treatment is a common alternative for dechorionation; however, we recommend the use of tweezers to avoid unnecessary exposure of the embryo to proteolytic enzymes. If high throughput is required, automated chorion removal using pronase can be performed.[17]
2. Prepare an agarose solution (0.4% agarose in egg water and add tricaine, 0.01% final concentration) to embed the zebrafish embryo. Submerge container in a water bath at 36-40 °C to prevent agarose gelation. Caution: be careful with the temperature control at this step, overheating can damage the zebrafish embryo during mounting steps.
3. Prepare injection microneedles by pulling glass needles with filament according to the instructions of the micropipette puller machine.
4. Transfer approx. 20 embryos, anesthetized in 0.01% tricaine, with a plastic transfer pipette and place them in a clean, empty plastic petri dish.
5. Remove any excess egg water and pipette in prepared agarose solution (~3 ml). Embryos should be evenly distributed and preferably on the bottom surface of the petri dish. Carefully use tweezers to manipulate embryos into the desired position (preferably in lateral view, see **Figure 2** for more detail). Allow agarose gel to cool for a few minutes to solidify.
6. Proceed with the microinjection.
    a. Load fluorescently labelled nanoparticles (~3 µl) in a glass microneedle with a pipette and insert needle into the arm of the injector.
    b. Calculate the injection volume (1 nl volume is recommended).
    c. Cut the length of the microneedle with stainless steel tip tweezers (removing a section of the thinner part of the pre-pulled microneedle tip).
    d. Press the foot pedal to expel one droplet.
    e. Measure the size of a droplet under the microscope. The incorporation of a scale into the ocular of the microscope (or a stage micrometer, *e.g.* agar scientific AGL4079, as an alternative) allows the measurement of a droplet equivalent to 1 nl (*i.e.* 100 µm at 40x).
    f. Adapt the volume accordingly by adjusting the micromanipulator

setting (*i.e.* pressure). **Note:** Repeat this step periodically during injections to ensure injection volume is consistent.

g. Position the microneedle towards the sinus venous/duct of Cuvier (**Figure 2**). **Note:** To reach the zebrafish embryo the needle must be carefully moved 'in and out' once inserted in the agar.

h. Penetrate the skin with the injection needle. **Note:** Be careful not to touch the yolk sac with the tip needle to avoid over-insertion of the microneedle that can lead to unsuccessful *i.v.* injection.

i. Once the needle is inside the duct of Cuvier, gently pull the needle back, creating a small pyramidal space and inject nanoparticle solution (1 nl). At the time of injection, a backward flow/spreading of circulating blood cells should be observed. (See videos in Notes section below, to differentiate between successful and unsuccessful injection).

7. Once injected, carefully remove the microneedle from the embryo.
8. Release the zebrafish embryo from the agarose gel. First, add egg water (~5 ml) on top of the agarose gel. Second, use tweezers to carefully separate the embryo from the agarose (without touching the embryo).
9. Once the embryo is released and able to swim, transfer the injected fish with a plastic transfer pipette to a clean petri dish containing fresh egg water and 0.01% tricaine to proceed with screening for successfully injected fish.

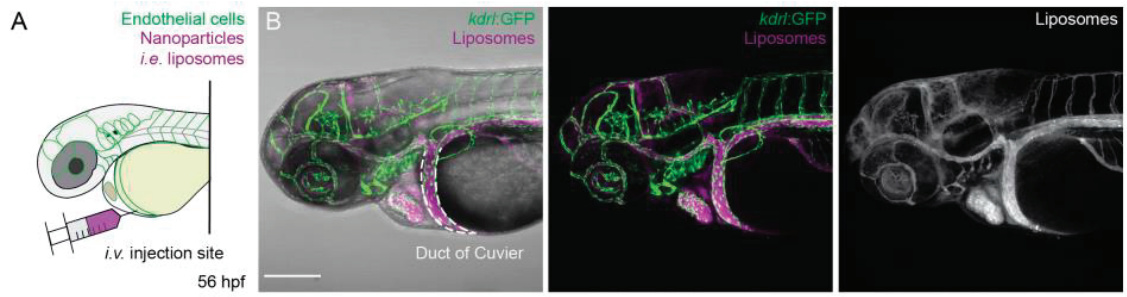

Figure 2. Microinjection site of zebrafish embryo/larvae. (a) Schematic of a zebrafish embryo showing intravenous injection site within the sinus venosus/duct of Cuvier at 56 hpf. **(b)** Zebrafish embryo Tg(*kdrl*:GFP) after injection of fluorescently labelled liposomes (in magenta/grey) at 56 hpf, 1 hpi. Volume of injection: 1 nl. Scale bar 200 μm (10x).

Screening of successfully injected embryos

1. Make a selection of well-injected zebrafish embryos under a fluorescent stereo microscope. Well-injected embryos show fluorescent nanoparticles in circulation (**Figure 3a**).

2. Discard any embryo that does not show nanoparticles in circulation, *i.e.* injected in the yolk sac, pericardial space, *etc.* (**Figure 3b-d**).
3. Ensure fish look healthy with no physical damage (*i.e.* from injection needle).

Imaging

1. Transfer the zebrafish embryo with a plastic transfer pipette to a petri dish containing fresh egg water (with no tricaine added) and keep the zebrafish embryo at 28.5 °C until further use (*i.e.* imaging).
2. (Optional step) Applying UV light after intravenous injection, as **Chapter 4**, of photoactive liposomes. In this case, a LED (375-nm) lamp driven by a custom-built LED driver (I = 350 mA), is used as UV light source.

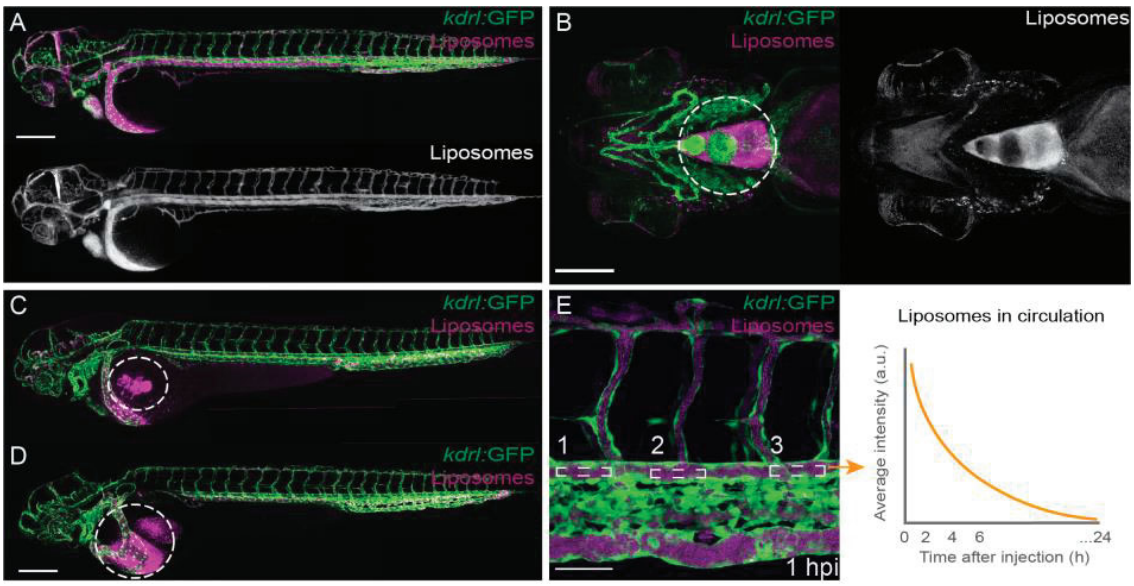

Figure 3. Selection of injected zebrafish embryos. (a). Successfully injected zebrafish embryos, nanoparticles are clearly in circulation, homogeneously distributed throughout the vasculature of the embryo. **(b-d). Unsuccessful injections**, nanoparticles accumulated in the pericardial space **(b),** zebrafish embryo in ventral view) or in the yolk sac **(c)**, within dashed circles) or a combination of both **(d)** and only partial volume of nanoparticles are in circulation. Do not use these embryos as representative of nanoparticle biodistribution. **Note:** For clarity, confocal images are shown here. Screening for successful injection should be done quickly under a stereo microscope. **(e) Nanoparticles in circulation** (in magenta, within boxes) in the dorsal aorta. Intensity values measured in this area can be used to calculate the liposome circulation life at different time points (*i.e.* 1-24 h post injection, hpi). We recommend to use n=6 for each point. All zebrafish embryo shown are 2 days post fertilisation, dpf; Tg(*kdrl*:GFP) 1 hpi. Scale bars: 200 µm (a-d), 50 µm (e).

   a. Determine the power density (in mW cm^{-2}) of the light source and calculate light doses (J per embryo) by multiplying the optical power density by the irradiation time.
   b. For embryo irradiation, the UV source (wavelength 370 ± 7 nm) was positioned approximately 1.5 cm above the agar-embedded embryo (~90 mW cm^{-2}).[5] A minimum irradiation dose of ~2.4 J per embryo was used.
3. Embedding of embryos/larvae in agarose solution (0.4% + 0.01% tricaine) for confocal imaging.
   a. Use a glass bottom dish suitable for confocal imaging.
   Note: This must be able to contain liquid agarose before gel formation, and therefore generic glass slides are not suitable.
   b. Randomly select at least six successfully injected zebrafish embryos to image and transfer them with a plastic transfer pipette into a glass bottom plate.
   c. Place ~3 ml of agarose solution into the glass bottom dish. Make sure the agarose covers the surface of the whole bottom dish and gently position the zebrafish embryos into the correct position for desired imaging (*i.e.* lateral, dorsal, *etc.*) with the help of the tweezers. Allow the gel to cool and solidify over a few minutes.
4. Mounted embryos (*i.e.* in dorsal or lateral view) can now be used for imaging. We recommend to add egg water on top of the agarose to prevent the sample drying out if the time of imaging is extensive (>3 h). After imaging, transfer the fish into petri dishes containing fresh egg water and allow to swim freely at 28.5 °C for at least ~24 h to monitor possible effects after injection and/or for further analysis.
5. Confocal imaging of nanoparticle biodistribution and identification of specific cellular interactions. For nanoparticle assessment, we recommend using transgenic zebrafish lines Tg(*kdrl*:GFP)[8] and Tg(*mpeg1*:GFP)[7] to identify endothelial cells and macrophages respectively. **Note:** the fluorescent nanoparticle probe must not overlap with transgenic fluorescence emission.
   a. Capture confocal z-stacks using a 10x air objective (HCX PL FLUOTAR), a 40x water-immersion objective (HCX APO L) or 63x water-immersion objective (HC PL APO CS).
   b. For whole-embryo views, maximum projections of three overlapping z-stacks (10x) were captured to cover the complete embryo.
   c. Figures 3, 4, 6 and 7c and Supplementary Figures 1-3, 7-9 in

Chapter 4[5] illustrate the use of confocal imaging after intravenous administration of liposomes in zebrafish embryo.

6. Identification of nanoparticle interactions with scavenger endothelial cells (SECs, *i.e.* analogous to liver sinusoidal endothelial cells in mammals), can be assessed using fluorescent hyaluronic acid (fluoHA) as a specific and non-competitive marker of SECs (**Figure 1c**)[3]. In addition, the involvement of the key scavenger receptors – Stabilin-2 and -1 –can be easily-assessed through co-injection of dextran sulfate within the embryonic zebrafish.[3, 6] Furthermore, *stabilin-2*, *stabilin-1,* and *stabilin-1* and *-2* knockout embryos have been generated, described in **Chapter 2 & 3**.[3, 6]

7. 4 Materials and Reagents

Zebrafish breeding tanks with divider (Tecniplast, Italy)
Stainless steel tip tweezers (IDEAL-TEK, catalog number: 3480641)
Borosilicate glass microneedles with filament, 10 cm (Science Products, Sutter Instruments, catalog number: BF100-78-10)
Microloader 20 µl (Eppendorf, catalog number: 5242956003)
Disposable petri dishes, 92 x 16 mm with cams (SAERSTEDT, catalog number: 82.1473.001)
Plastic transfer pipettes (SAERSTEDT, catalog number: 86.1171)
Glass bottom dishes (WillCo-dish, catalog number: GWST-5040)
Low-melting-point agarose (Sigma-Aldrich, catalog number: A9414)
Instant Ocean sea salt for aquariums (Instant Ocean, catalog number: SS15-10)
Fluorescent nanoparticles (*e.g.* liposomes as described below. Store in dark)
N-Phenylthiourea (PTU)* (Sigma Aldrich, catalog number: P7629. See Recipes. Store at room temperature)
Tricaine (ethyl 3-aminobenzoate methanesulfonate) (Sigma Aldrich, catalog number: A5040) (See recipe below, store at 4 °C and dark once diluted)
Adult zebrafish (wildtype (AB/TL) or transgenic line of interest)
Lipids (Avanti Polar Lipids, Lipoid GmbH and/or Sigma Aldrich)
Formulation buffer (*e.g.* HEPES* (10 mM), See Recipes, store at room temperature)
Glass vials, 5 ml (VWR international, catalog number: 548-0555)
Polycarbonate membranes, 400 and 100 nm pore size (Nucleopore Track-Etch membranes, Whatman, catalog numbers: 7065257, 6257028)
Chloroform (Sigma Aldrich, catalog number: 67-66-3)Egg water (See Recipes)
Ethanol* (Honeywell, catalog: 67-63-0)
Agarose gel (See Recipes) *Optional

Equipment

Mini-extruder (Avanti Polar Lipids, catalog number: 610000)
Syringes 1000 µl (Avanti Polar lipds, catalog number: 610017)
Vacuum desiccator (Fisher Scientific, model: Pirex 1594/02D)
Bench-top vortex (Scientific Industries, model: G-560E)
Nanosizer (Malvern Zetasizer Nano ZS)
LED-UV light source (wavelength 370 nm, FWHM = 13.4 nm; H2A1-H375-S, Roithner Lasertechnik)
Stereo microscope (Leica, model: MS5)
Micropipette puller (Sutter Instruments Co, model: P-97)
Injector (Eppendorf, FemtoJet, catalog number: 524702135) attached to a manual micromanipulator (World Precision Instruments, WPI model M3331R) on a steel base plate (WPI, code 5052) with a magnetic stand (WPI, code M10)
Incubator (Heraeus, model: B15)
Water bath (ELBANTON, Julabo, model: MWB)
Fluorescent stereomicroscope (Leica, model: M205 FA-2)
Confocal microscope (Leica Microsystems, model: SP8/SPE)
Fully approved zebrafish facility (See Ethics section below)

Software

Zetasizer Software Version 7.13.
Fiji distribution of ImageJ; Versions 1.51p and 1.52p[18, 19].
Confocal microscopy data were processed using Leica Software (Leica Application Suite X software, version 3.5.5.19976).

Data analysis

Process the images using the Fiji distribution of ImageJ[18, 19]. Adjust brightness and contrast, rotate and crop the image if needed. For the whole embryo view, stitch the maximum projection of 3 or 4 images at an appropriate overlapping point (**Figure 1c**). For comparison and/or quantification laser intensity, gain and offset settings must be identical between stacks and between experiments. Quantification of liposome circulation lifetime decay (n=3-6) shown in **Figure 4g** and **Supplementary Figures 7-8** in **Chapter 4**[5] was calculated using a previously described macro for ImageJ[3] using the measured nanoparticle fluorescent intensity within the dorsal aorta, as illustrated in **Figure 3e**.

Notes

These studies require knowledge, license and basic skills in zebrafish husbandry and handling. Good zebrafish husbandry practices are critical throughout this protocol to ensure the health and comfort of the zebrafish is maintained at all times. Reproducibility is essential. Repeat all experiments at least twice. Use different zebrafish lines, if possible, and always repeat with new batches of (freshly prepared) nanoparticles. In case of light application, do not irradiate excessive numbers of embryos simultaneously to avoid embryo-to-embryo variations in light dose. For experiments monitoring changes in liposome biodistribution following light activation, always image the same embryo before and after UV irradiation. Intravenous injections can be performed at any point between 2 and 4 dpf. Ethical approval is required for any experiment in zebrafish after 5 dpf.

Ethics

All animal experiments must be in accordance with institutional regulations. In this case, zebrafish (*Danio rerio*, strain *AB/TL*) were maintained and handled in accordance with guidelines from the European Convention on the protection of vertebrate animals used for experimental and other scientific purposes,[20] and in compliance with the directives of the local animal welfare committee of Leiden University.

Recipes

Egg water

Mix 60 µg of instant Ocean sea salts per ml. Store at room temperature.

N-Phenylthiourea (PTU)

Mix PTU powder in 85% ethanol solution to a final concentration of 3% (w/v). Store at room temperature and in the dark.

Agarose gel

In a clean glass container, mix low melting agar powder in egg water to a final concentration of 0.4%. Melt the agarose mixture in a microwave, shake until dissolved and keep it in a water bath at 36-40 °C. Add tricaine to a final concentration of 0.01%.

HEPES buffer

HEPES (10 mM) adjusted to pH 7.4 with 1 m aqueous NaOH. Ultrapure MilliQ® water, purified by a MilliQ Advantage A10 water purification system from MilliPore. Store at room temperature.

Tricaine

Mix 400 mg tricaine powder with 97.9 ml of deionized water, and adjust to pH 7 with 2.1 ml of 1 M Tris (pH 9). Store in the dark at 4 °C. Mix 1 ml of stock solution with 9 ml of egg water for the working solution.

7.5 Abbreviations

CRISPR/Cas9	clustered regularly interspaced short palindromic repeat/Cas9
CryoTEM	Cryo-transmission electron microscopy
CV	caudal vein
dpf	day(s) post fertilization
DOPE-LR	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)
DSPC	1,2-distearyl-*sn*-glycero-3-phosphocholine
fluoHA	fluorescent hyaluronic acid
hpi	hour(s) post injection
hpf	hour(s) post-fertilization
i.v.	Intravenously
SEC	scavenging endothelial cells
PC	polycarbonate
PTU	N-Phenylthiourea

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