Chapter 14

Non-Enzymatic Tissue Homogenization for Biodistribution Analysis

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Abstract

Biodistribution is a valuable technique used to determine payload delivery from nanocarrier to organs of interest in preclinical models. Fluorescent probes can be used as drug surrogates, providing indirect but relevant measurement of tissue exposure to the carrier. This may be useful, for example, to perform a first-pass evaluation of how targeting affects delivery of encapsulated compounds to target organs. This protocol is designed for non-enzymatic tissue homogenization of a variety of organ types allowing tracking of small molecule fluorophores injected freely or encapsulated in nanoparticles.

Key words Biodistribution, Tissue homogenization, Mechanical homogenization, Nanoparticles, Drug delivery, Small molecule fluorophores

1 Introduction

Preclinical evaluation of drug carriers often involves analysis of the biodistribution of their delivery to various tissue sites. Measurement of either payload or carrier concentration in target tissue is an important aspect of evaluating whether targeting was achieved; this initial evaluation can be an essential step toward more detailed pharmacokinetic analysis of specific drug payloads [1]. Biodistribution assessment can be performed in a variety of ways, including in intact tissues (for example, with magnetic resonance imaging, positron emission tomography, or fluorescent imaging [2–4]). Here, we will focus on extraction of drug or label from tissue homogenates, which is a simple and easily implemented technique requiring enzymatic or mechanical homogenization of tissue. Enzymatic methods of homogenization are often time-intensive due to long incubations and introduce chemical variables into the samples. The use of detergents, lytic enzymes, and choatropes can alter fluorescence signal by denaturing proteins and altering the

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fluorophore-tissue environment. While there are several methods of non-enzymatic homogenization available, we have observed significant variation in the degree of homogenization achieved in different organs, which complicates comparison of extraction from different organs. Here, we demonstrate a non-enzymatic method for mechanically homogenizing a variety of organs (brain, heart, lung, liver, spleen, blood, uterine horn, spinal cord, kidney, and muscle) using a single protocol. We provide an example of utilizing this approach to detect fluorescence in mouse organs.

2 Materials

2.1 Tissue Collection

1. Peristaltic pump.
2. GP tubing 1.6 mm.
3. Leur lock syringe needles (25G × 1 ½).
4. 1 mL insulin syringes with needle (29 G × 1/2 in) attached.
5. 2 mL microcentrifuge tubes.
6. Tubes for blood collection (K2 ethylenediaminetetraacetic acid [EDTA] for whole blood or sodium heparin coated for plasma).
7. Heparinized Saline: 10 units of heparin to 1 mL of 1× saline (*see* **Note 1**).
8. Ketamine/Xylazine: A standard solution contains 16/1.6 mg katamine/xylazine per mL sterile saline (0.9% sodium chloride), which is equivalent to 100/10 mg/kg when administering 100 μL to a 16 g mouse (*see* **Note 2**).
9. Alcohol Prep pads.

2.2 Tissue Mincing, Homogenization, and Probe Sonication

1. Probe sonicator.
2. Bead homogenizer.
3. Small diameter (e.g., 0.156″) stainless steel homogenization beads.
4. Polystyrene weighing dishes.
5. Razor blade.
6. Surgical tweezers, forceps, and fine scissors for dissection.
7. Large magnet, suitable for sliding along the outside of tubes to retrieve beads.
8. Microcentrifuge tubes (2 mL) with locking lid.
9. 15 mL sterile conical tubes.

2.3 Fluorophore Detection

1. 96-well bottom plates (black, flat bottom).
2. Dimethyl sulfoxide (DMSO).

3 Methods

Personal protective equipment should be worn according to institutional policies and MSDS guidelines (*see* **Note 3**). Keep samples on ice and protected from light at all times (*see* **Note 4**). The following protocol has been optimized for measuring biodistribution of fluorescent small molecules in mice and assumes the agent has already been administered to the mouse and allowed to distribute for an appropriate amount of time. Typical measurements might be made at 0.5, 2, 6, 12, and 24 h, although the exact time points should be chosen to span the expected kinetics of the agent being tested.

3.1 Tissue Collection

1. Set up the perfusion pump, dissection board, clean surgical instruments, a beaker of diH_2O, and pre-labeled sample tubes on top of an absorbent laboratory pad.
2. Attach a 25G needle to the free end of the perfusion pump tubing.
3. Prime tubing with heparinized saline to ensure no air bubbles are present (*see* **Note 5**).
4. Approximately 10 min prior to the planned collection time point, anesthetize mouse with an overdose of ketamine/xylazine or according to the institution's IACUC protocols (*see* **Note 6**).
5. Once deeply anesthetized and unresponsive to toe pinch, place mouse supine on dissection board and secure limbs with dissection needles. The abdomen should be taut.
6. Firmly wipe the abdomen with an ethanol pad to dampen the fur.
7. Using surgical scissors, make a vertical incision at the level of the hind limbs. Carefully cut toward the diaphragm, using tweezers in the non-dominant hand to pull skin upward, which will help to avoid nicking internal organs.
8. Grasp the sternum with a pair of tweezers and pierce the diaphragm with the scissors (pneumothorax). Once the diaphragm is pierced, the mouse will begin to gasp and dissection must proceed quickly. Rotate the scissors and continue cutting to one side along the bottom of the ribcage. Rotate the scissors a second time to cut up through the rib cage toward the collarbone. Repeat this procedure along the other side to fully release the ribcage.
9. Lift the entire ribcage by grasping the sternum with tweezers. Pin securely in the open position to expose the heart and lungs.
10. A blood sample can be collected by cardiac puncture. Hold a pair of tweezers in the non-dominant hand and use them to grasp the apex of the right ventricle. Use the dominant hand to hold an insulin syringe, resting the back of the thumbnail on

the bottom of the plunger to enable one-handed manipulation. Holding the heart steady with the non-dominant hand, use the dominant hand to insert the syringe needle carefully into the right ventricle, taking care not to pierce through the septum or into the atrium. Slowly withdraw 100–500 μL of blood. This may take anywhere from 3 to 15 s. If the ventricle collapses, pause to allow blood to refill and proceed at a slower rate.

(a) If analysis is to be performed on whole blood, immediately eject the sample into a K2 EDTA coated tube.

(b) If analysis is to be performed on blood plasma, collect a minimum of 500 μL of blood and immediately eject into a sodium heparin coated tube. After collection, place blood vials on ice. Blood samples should be spun down and processed within 1 h of being collected (*see* **Notes 7** and **8**). Tubes should be processed according to the manufacturer's instructions for optimal blood plasma separation. After centrifugation, collect 60 μL of plasma without disturbing the blood pellet. Place the plasma aliquot in a 2 mL microcentrifuge tube (*see* **Note 9**).

11. The right atrium can be identified by its dark color relative to the ventricle. Use scissors to snip the top. Blood should begin flowing freely into the exposed cavity.
12. Use tweezers in the non-dominant hand to again grasp the heart by the apex of the right ventricle. Insert the perfusion needle into the left ventricle, directing the needle along the inferior-superior axis and taking care not to pierce the septum or left atrium.
13. Release the grasp of the heart and maintain needle position in the left ventricle with the dominant hand. Initiate perfusion. Fluid should flow out of the incision previously made in the right atrium; it will first be dark red and should lighten to eventually become clear. The color of peripheral organs should also lighten, which will indicate a successful perfusion (*see* **Note 10**).
14. Collect desired organs. Carefully remove fat and connective tissue to isolate the organ of interest. Rinse tissue thoroughly with diH_2O, ensuring any tissue particulate, blood, and/or fur is removed.
15. Place each sample into individual, pre-labeled, 2 mL microcentrifuge tubes (*see* **Note 11**).
16. Store organs at −80 °C until ready to process.

3.2 Tissue Mincing, Homogenization, and Probe Sonication

1. Prior to tissue processing, 2 mL locking lid tubes should be pre-labeled and pre-weighed. If the locking apparatus of the tube is missing or appears damaged, the tube should be discarded (*see* **Note 12**).

2. Remove organs, including control tissue, from −80 °C and allow to thaw on ice (*see* **Note 13**). For each study, the same organ for all subjects should be processed on the same day. This will allow for a single standard curve for quantification to be generated.
3. Fill a large beaker with ice water and place under the probe tip at a level that allows the sample to remain cold during sonication.
4. Mince tissue using a scalpel and razor blade. Mincing should be done on a hard surface such as a plastic weigh dish (*see* **Note 14**). Tissue must be completely minced with clean cuts, such that no fibrous or connective tissue remains intact. After mincing, the organ should have a viscous or gelatinous consistency.
5. Transfer the organ to the pre-weighed tube. Re-weigh the tube. Determine the amount of diH_2O needed for 10% weight by volume (w/v) of each sample.
6. Add three stainless steel beads to each tube and add the appropriate volume of water (*see* **Notes 15** and **16**). Once the sample, water, and beads are added, the lid may be closed and locked in place.
7. Place tubes in the high-throughput bead homogenizer. Ensure there is a tight fit with no extra space between the tubes and the apparatus. Run the homogenizer at maximum speed for 5–10 min or until tube contents are liquefied.
8. Remove the tubes from the homogenizer and place on ice for at least 20–30 min to allow any froth generated by homogenization to settle.
9. Use a magnet to remove the steel beads from the tubes. If any tissue is removed with the beads, use tweezers to return the tissue to the tubes. It is normal for some tissue to remain after processing certain organs (e.g., liver). These pieces will be disrupted during probe sonication.
10. Transfer the contents of the microfuge tube to a pre-labeled 15 mL conical tube. We suggest only processing tissue volumes under 3 mL. Otherwise, the sonication energy is not effective.
11. Sonicate the sample at an amplitude of 40% for 10 s. Quickly swirl the conical tube in ice water to ensure contents remain cold.
12. Allow the sample to de-froth for 20–30 min. Once de-frothed, sample fluid should be relatively clear and can be transferred to the pre-labeled sample homogenate tube (*see* **Notes 17** and **18**).
13. Repeat **steps 11** and **12** for each organ sample.
14. Store samples at −80 °C until ready to analyze.

3.3 Quantification

1. Remove samples from −80 °C.
2. Vortex each sample for 10 s immediately before plating.
3. Pipette 50 μL of sample or control homogenate into individual wells of a 96-well plate.
4. Add 10 μL of DMSO to each well to reach a final volume of 60 μL.
5. Read fluorescence of all samples on a plate reader using settings appropriate for detecting the signal of interest (*see* **Note 19**).
6. Based on the sample readings, make a serial dilution of the fluorophore in DMSO.
   (a) Adjust the standard curve to as needed to incorporate all the sample points. Ensure all samples lie within the linear portion of the standard curve.
7. If analysis needs to be delayed, plates can be sealed and stored at −80 °C.
8. Arbitrary fluorescent units read from sample homogenates should then be converted to concentration by comparison to the control curve (example data are provided in Fig. 1).

4 Notes

1. Heparinized saline can be made in advanced if stored at 4 °C. Allow the solution to reach room temperature prior to performing perfusion.

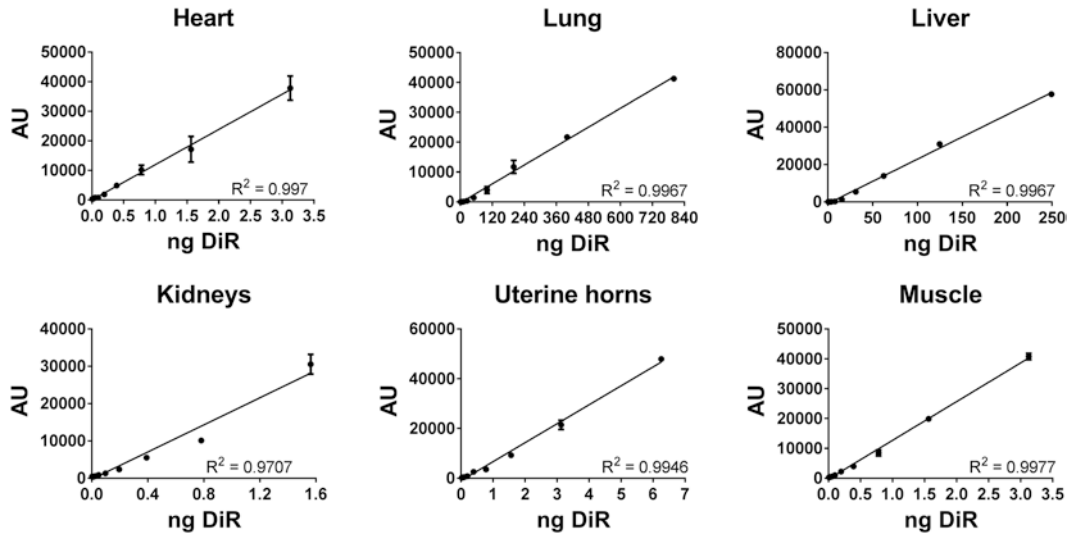

Fig. 1 Example control curves demonstrate a highly reproducible and linear relationship between the quantity of spiked fluorophore in organ homogenate and the fluorescence readout. The fluorophore in this example was DiR, and three replicates are represented per time point

2. Ketamine/xylazine solution should be prepared in a sterile, nonpyrogenic empty vial. The solution can be stored effectively and remain stable up to 2 weeks at 4 °C.
3. We recommend wearing gloves and a laboratory coat for all experiments. A disposable gown, eye protection, and disposable face mask are recommended during perfusion, to protect from splashes.
4. We have observed significant changes to extraction efficiency as a function of temperature. All samples should be held on ice unless otherwise specified. They should also be covered and protected from prolonged exposure to light at all times.
5. Tubing for perfusion can be re-used if it was used with heparin or saline during prior perfusions. Tubing previously used with fixative (e.g., formaldehyde) may still contain traces of the chemical; this tubing should not be re-used, as it may cause the perfusion to fail.
6. The mouse must be deeply anesthetized with the heart still beating for a perfusion to be successful. Too-heavy anesthesia can compromise circulation and should be avoided. Use of inhaled anesthesia may prove difficult, due to the challenge of keeping the mouse's nose in contact with a nose cone while manipulating their body. We have found that injectable anesthesia is both reliable and convenient. Depth of anesthesia should be assessed by performing a toe pinch on both feet prior to beginning perfusion. For the purposes on this procedure, a tail pinch should not be substituted with a touch pinch. The tail is not as sensitive as the feet. Thus a mouse may not respond to a tail pinch but will respond to a toe pinch if it is not yet deeply under anesthesia.
7. Do not freeze blood prior to centrifugation, as the cells will lyse once frozen.
8. A swinging bucket rotor is preferable for centrifugation, because this will place the pellet at the bottom of the tube instead of the sidewall. It is easier to extract plasma this way.
9. After the blood plasma has been collected, the blood pellet can also be processed. The pellet may be frozen in its original tube at −80 °C until the day of processing.
10. Once an efficient perfusion is complete, the liver will have changed from a deep red color to light brown-gray hue. This color change is usually obvious within 10–15 s of perfusion. If the liver does not begin to clear in that time frame, try re-adjusting the needle, or removing and re-inserting the needle into the left ventricle. If the wall dividing the left and right ventricles has been pierced, it may help to clamp between the left and right sides of the heart with tweezers or forceps. We

have found that achieving complete perfusion of the lungs can be difficult, with even very small pockets of inadequately perfused tissue adding high inter-subject variability. To improve perfusion consistency between subjects, we choose to perfuse the lungs separately. After the liver has cleared, re-position the needle toward the pulmonary veins. The lungs will swell with fluid, which will exit via the nostrils. After a count of 3, the needle is removed.

11. Printable tube labels can save a significant amount of time at this stage. We specifically recommend using Cryo-Babies which can withstand storage up to −196 °C (9187–1700, USA Scientific, Ocala, FL, USA).
12. Avoid opening and closing the latch on the lid, which weakens the plastic and can result in breaks. Tubes with broken latches have an increased likelihood of opening during bead homogenization, which may result in sample loss.
13. The number of animals required for a study depends on biological variability in delivery, as well as on the skill and consistency of the perfusion and organ collection. We find that n = 5–6 subjects is often sufficient to detect moderate differences in delivery (e.g., comparing a targeted to a non-targeted formulation in a single organ, with an expectation of 50–100% increase in signal in the targeted group). Control tissue should be collected and pooled to construct control curves, which are spiked with known quantities of nanocarrier after being processed to produce homogenates. Some organs (e.g., brain, liver) are relatively large, and n = 3–4 subjects will suffice. Other organs (e.g., spinal cord and lungs) are small, and up to 12 subjects will be needed to obtain a sufficient quantity. We routinely collect and store control tissue from non-treated subjects to ensure control organs are available and can be processed in parallel with samples. Control and sample tissues should be subjected to the same number of freeze thaws. i.e., do not generate a single batch of control homogenate and then repeatedly dip into the stock on different days (this will produce signal drift).
14. To minimize waste when mincing samples, the plastic weigh dishes can be re-used. Confirm that the plastic has not been damaged with repeated use, rinse in clean water, dry with a laboratory tissue, and wipe with ethanol. Residual ethanol should be allowed to evaporate prior to next use.
15. The use of three beads was determined to be optimal for all organ types. Adding less than three beads will not provide enough force to homogenize the tissue of more fibrous organs. Adding more than three beads will not provide ample room for the beads to move in the tube, which causes the tissue to

clump. The number of beads may need to be re-optimized if a different diameter is used.

16. All tools, including the probe sonicator and beads, should be cleaned in between subjects. To best clean the homogenization beads, it is important to remove every trace of sample. We clean beads by three washes in distilled water followed by three washes in ethanol. The probe sonicator should be rinsed with clean water, dried with a laboratory tissue, and wiped with ethanol. Residual ethanol should be allowed to evaporate prior to next use.
17. It is important to collect as much of the sample as possible. If, after 30 min, the sample had not de-frothed enough to allow for sufficient pipetting, perform a quick centrifugation to collect the remaining sample before moving forward. Additional diH_2O can be added to the empty tube to collect remaining tissue debris, which can then be disrupted by additional sonication.
18. During probe sonication or vortexing, any fat that was not removed will aggregate and form a layer in the sample that may incorporate with any foam that is present. When pipetting the sample, turn the tube to a 45° angle and pipette below the layer of the foam.
19. We find that readings tend to benefit from a brief shake of the 96-well plate on the plate reader for 10 s. Fluorescent gains should be optimized for each organ (i.e., gains do not need to be matched between organs, and each control curve may have a different gain that should be matched to its own organ). If the experiment requires multiple plates to be read for the same organ, ensure the controls are present on all plates to control for plate to plate variability.

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