Intravital Microscopy for the Study of Hepatic Glucose Uptake

Megan L. Stefkovich,[1] Sun Woo Sophie Kang,[1] and Natalie Porat-Shliom[1,2]

[1]Thoracic and GI Malignancies Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland
[2]Corresponding author: *poratshliomn@nih.gov*

The liver is central in maintaining glucose homeostasis. Indeed, impaired hepatic glucose uptake has been implicated in the development of hyperglycemia in type II diabetes (T2D) and non-alcoholic fatty liver disease (NAFLD). However, current approaches to evaluate glucose mobilization rely on indirect measurements that do not provide spatial and temporal information. Here, we describe confocal-based intravital microscopy (IVM) of the liver that allows the identification of hepatocyte spatial organization and glucose transport. Specifically, we describe a method to fluorescently label hepatic landmarks to identify different compartments within the liver. In addition, we outline an in vivo fluorescent glucose uptake assay to quantitatively measure glucose mobilization in space and time. These protocols allow direct investigation of hepatic glycemic control and can be further applied to murine models of liver disease. © Published 2021. This article is a U.S. Government work and is in the public domain in the USA.

Basic Protocol 1: Mouse surgical procedure and positioning for liver intravital imaging
Basic Protocol 2: Fluorescent labeling and intravital imaging of mouse hepatic compartments
Basic Protocol 3: Mouse hepatic glucose uptake assay and intravital imaging analysis

Keywords: hepatic glucose uptake • hepatic lobule • hepatocyte • intravital microscopy • mouse liver

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How to cite this article:
Stefkovich, M. L., Kang, S. W. S., & Porat-Shliom, N. (2021).
Intravital microscopy for the study of hepatic glucose uptake.
Current Protocols, *1,* e139. doi: 10.1002/cpz1.139

INTRODUCTION

The liver plays a vital role in regulating glucose homeostasis by coordinating various glucose metabolic pathways, including glycogenesis, glycogenolysis, glycolysis, and gluconeogenesis. Dysregulation of glucose homeostasis is a hallmark of metabolic diseases such as T2D and NAFLD. Despite its importance in disease progression, hepatic glucose uptake cannot be measured directly with spatial and temporal precision using current methods.

CURRENT PROTOCOLS
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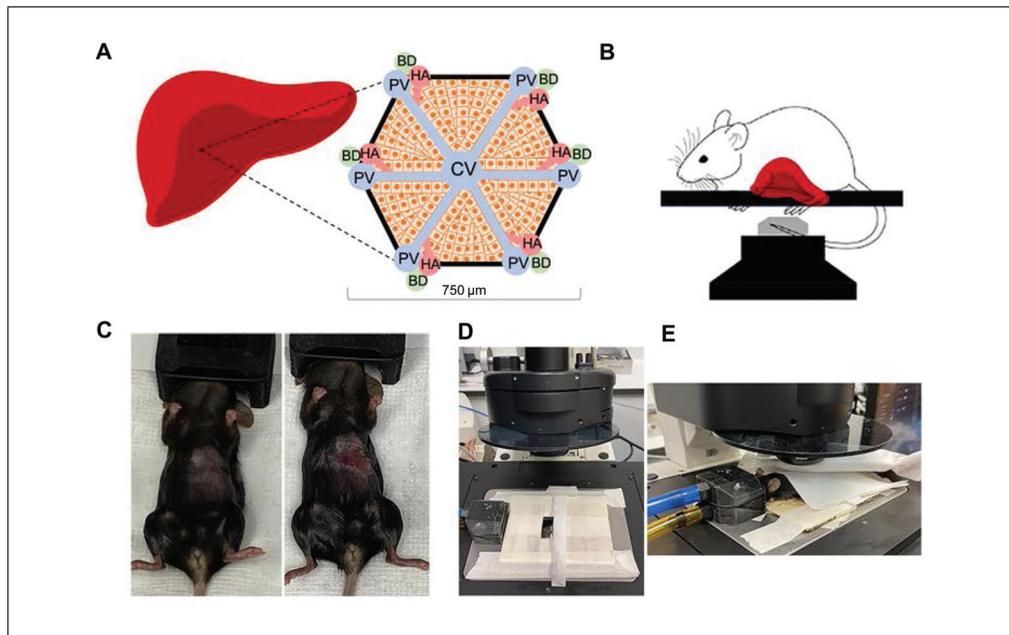
Figure 1 Intravital microscopy setup. (**A**) Schematic representation of the hepatic lobule. HA, hepatic artery; PV, portal vein; BD, bile duct; CV, central vein. (**B**) Anesthetized mouse positioned on the stage of an inverted microscope with the liver surgically exposed. (**C**) Surgical prep under nose-cone-administered anesthesia, demonstrating the shaved area on the abdomen and size of the surgical opening. (**D**) Microscope stage set up for IVM including nose cone for anesthesia, custom-made metal insert with a central 35-mm circle opening coved by a 40-mm coverglass, and cardboard insert to raise the mouse's body away from the exposed liver. A strip of gauze is used to adjust the liver against the coverslip. (**E**) Mouse anesthetized using nose cone and body temperature is controlled using a heat pad.

Hepatocytes are organized into a repetitive hexagonal unit called the hepatic lobule (Fig. 1A). Blood flows directionally from the six corners of the lobule toward a single central vein, generating a gradient of nutrients, oxygen, and hormones across the porto-central axis. Consequently, hepatocytes are exposed to different microenvironments, which affects their gene expression patterns and metabolic activities—a phenomenon known as liver zonation (Ben-Moshe & Itzkovitz, 2019). Specifically, periportal (PV) hepatocytes are known to engage in gluconeogenesis, whereas pericentral (CV) hepatocytes are involved in glycolysis. As such, liver zonation is an important consideration for hepatic glucose homeostasis.

Fluorescent light microscopy has revolutionized the ability to visualize cellular processes in living systems. In the past two decades, the development of fluorescent protein variants, along with advancements in optics and image analysis, have made it possible to track processes in space and time in cultured cells (Lippincott-Schwartz, Snapp, & Kenworthy, 2001). More recently, new technologies to manipulate the genomes of various organisms, as well as new in vivo imaging modalities, have allowed the extension of these light-based approaches to intact tissues (Entenberg et al., 2018; Weigert, Porat-Shliom, & Amornphimoltham, 2013). This collection of light microscopy approaches applied to live, intact tissues is collectively referred to as intravital microscopy (IVM). IVM methods now offer great opportunities to understand liver structure and function (Gola et al., 2021; Meyer et al., 2017; Porat-Shliom et al., 2016; Ritsma et al., 2013; Tavakoli, Tsekouras, Day, Dunn, & Presse, 2019). Furthermore, these approaches will be powerful in resolving the functional heterogeneity of hepatocytes, which is impossible to recreate in vitro.

Table 1 Reagents for Mouse Liver Intravital Microscopy

	Mouse body weight (g)	Board (cm)	Board thickness (mm)	Imaging window (cm)
Small (S)	17-20	12 × 8	1.5	2.5 × 2.3
Medium (M)	22-25	15 × 8	2.5	2.0 × 2.0
Large (L)	>30	15.5 × 8	4.0	3.5 × 1.8

Here, we present a comprehensive protocol for performing intravital microscopy of the murine liver. In Basic Protocol 1, we outline the steps for surgical exposure of the liver and its positioning on the microscope stage. We also discuss considerations taken to avoid tissue damage/ischemia and to monitor mouse body temperature during the procedure. Next, in Basic Protocol 2, we delineate the steps taken to fluorescently label hepatic compartments and to identify PV and CV hepatocytes. Finally, in Basic Protocol 3, we describe a functional assay to evaluate glucose uptake using 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG). Basic Protocol 3 also includes an image analysis strategy to quantify the rate of glucose uptake and its retention within hepatocytes in different parts of the lobule. Together, IVM of hepatocyte spatial organization in combination with dynamic transport of fluorescent glucose provides physiological insight into hepatic glycemic control in real time. These assays can be further applied to different mouse models of liver disease.

MOUSE SURGICAL PROCEDURE AND POSITIONING FOR LIVER INTRAVITAL IMAGING

Here, we describe in detail microscope setup, mouse preparation, mouse anesthesia, and a surgical procedure for liver exposure. Next, we provide guidelines on how to position the mouse on the microscope stage to minimalize motion artifacts due to heartbeat and respiration while maintaining proper perfusion.

IMPORTANT NOTE: All experiments involving animals must be performed under an animal protocol approved by your animal care and ethics committee. Personnel should be properly trained before proceeding.

Materials

    Clidox disinfectant (VWR, MSPP-96118F)
    Mus musculus
    70% (v/v) ethanol
    FORANE (isoflurane, USP) liquid for inhalation, 250 ml (Baxter, 10019-360-60)
    Heparin sodium, 1000 USP U/ml (Hikma, 0641-0391-12)
    Normal saline (0.9% sodium chlorine; Fisher Scientific, cat. no. 7210-16)

    Leica SP8 (or other commercial) confocal microscope
    Custom-made stage cardboard of appropriate dimensions (see Table 1)
    Custom-made aluminum stage insert (3 mm thick) with 35-mm depression
    40-mm coverslips (Bioptech, 40-1313-0319)
    Surgical gauze
    $\frac{1}{2}$-inch. masking tape
    Anesthesia machine: AMD-3 plus (Somni, AMD-17106)
    Reusable chemical heat packs
    Mouse heat pad (ALA Scientific Instruments, HEATINGPAD-1/2)
    Space heat pad (AIMSTM, AIMS™ GGP1)
    Ultra Fine U-100 Insulin Syringes, 31-G (BD, BD328438)
    Hair clipper

Surgical scissors
Cautery System (Gemini, GEM 5917)
Surgical forceps, curved tip
Cotton-tip applicators
Heat pad with rectal probe (ALA scientific instruments, HEATINGPAD-1/2)
Aquasonic 100 ultrasound transmission gel (Parker lab Inc)

Microscope preparation

1. IVM of the liver can be performed on any commercial inverted confocal microscope (Fig. 1B). Standard microscope stages have various inserts to accommodate slides or tissue culture plates. For IVM, a flat surface with a 35-mm circular opening covered with a 40-mm coverglass is required. In some cases, 35-mm-plate inserts can be modified to hold a mouse. Alternatively, a custom-made metal insert, 3 mm in thickness, with a central 35-mm circular opening can be fabricated. The coverglass should be cleaned thoroughly on both sides using 70% ethanol before use.

2. Select the objective based on area size, spatial resolution, and the length of the imaging session. In general, lower magnification is useful for imaging larger areas at lower resolution, whereas higher magnification increases the resolution of smaller structures. One should consider that motion artifact (see below) will be more noticeable when using higher magnifications.

3. Select stage cardboard dimensions based on mouse weight (see Table 1) and tape the stage insert with the imaging window (in the cardboard) directly over the coverslip (Fig. 1D). Minimizing motion artifacts due to respiration and/or heartbeat is central to IVM; the cardboard is used to absorb such motion while also creating a barrier between the body and the microscope stage. Place a small strip of gauze (1×8 cm) across the cardboard imaging window and fix it with masking tape on one side (Fig. 1D).

4. Warm the stage area using chemical heat pads at least 30 min prior to imaging. This will help maintain the mouse body temperature during the imaging experiment.

Mouse anesthesia and surgical procedure

5. Clean anesthesia induction chamber with Clidox disinfectant; this will remove odor of the previous mouse, which reduces stress.

6. Place the mouse in the induction chamber, and turn on the oxygen condenser and 3% isoflurane for 5 min. When the mouse is anesthetized, transfer it onto the heating pad and maintain anesthesia using 1% isoflurane delivered via a nose cone (Fig. 1C).

   This procedure should be done using isoflurane as opposed to other anesthetic agents such as ketamine and xylazine to avoid stimulation of hepatic gluconeogenesis, which may affect the rate of glucose uptake in the liver (Bevan, Rose, & Duggan, 1997).

7. Inject 20 U heparin intraperitoneally (i.p.; dilute 20 µl heparin from 1000 U/ml stock into 30 µl saline). The liver is prone to injury and ischemia; heparin ensures proper perfusion during the imaging study.

8. Shave the mouse abdomen using a hair clipper and remove hair using gauze soaked with 70% ethanol (Fig. 1C, left).

   Removing hair from the surgical site is crucial, as any errant hairs on the liver surface can impede your field of view.

9. Expose the liver by making a 2- to 3-cm incision along the ribcage starting from the xiphoid and ending at the left lateral side of the abdomen. Initially, the outer skin is cut with scissors, and then the muscle layer is cut, using a cauterizer as needed

to avoid bleeding. To avoid burning the liver with the cauterizer, carefully open the abdominal cavity on the lateral side and insert wet gauze (soaked in normal saline) between the liver and muscle layer before starting with the major incision (Fig. 1C, right).

Mice injected with heparin will be susceptible to bleeding. Using a cauterizer to avoid excessive bleeding is crucial for mouse survival.

Positioning of the mouse

10. Position mouse on the microscope stage with the exposed left lateral lobe of the liver laid against the coverslip. Use gauze to separate the liver lobe from the intestines and mouse by placing it between the liver and abdomen (Fig. 1D). Stretching the mouse flat on the microscope stage may help reduce motion (Fig. 1E).

11. Reposition the mouse if breathing causes the liver position to shift on the coverslip. Adjust the liver position very gently using a cotton-tipped applicator soaked in normal saline.

12. Secure the mouse's neck to the stage using tape to prevent motion artifacts upon retro-orbital injection of fluorescent tracers during imaging. Adjust the nose cone so that the mouse remains under the plane of anesthesia while allowing access to the eye. Maintain mouse anesthesia through delivery of 1% isoflurane via a nose cone on the microscope stage.

Higher percentages of isoflurane can make the mouse gasp, causing exaggerated chest movements that destabilize the liver during imaging.

13. Monitor and maintain the mouse body temperature using a heat pad with a rectal probe. Avoid placing the heat pad directly against the skin to avoid overheating. Additionally, avoid tissue dehydration by (1) using a microscope with an enclosed chamber with humidity control or (2) applying Aquasonic 100 ultrasound transmission gel at the edges of the exposed cavity.

FLUORESCENT LABELING AND INTRAVITAL IMAGING OF MOUSE HEPATIC COMPARTMENTS

This protocol describes how to (1) fluorescently label different compartments within the hepatic lobule, (2) identify PV regions, and (3) select the ideal imaging area for time-lapse IVM.

Materials

Appropriate fluorescent dyes (see Table 2)
31-G insulin syringe

Additional reagents and equipment for mouse anesthesia, surgery, and intravital imaging (Basic Protocol 1)

Table 2 Reagents for Fluorescent Labeling of Hepatic Compartments

Fluorescent dyes	Compartment	Concentration	Source (cat. no.)
Alexa Fluor® 647–conjugated anti-mouse/human CD324 (E-cadherin) antibody	PV hepatocytes	0.5 μg/g	Biolegend (147308)
2 M tetramethylrhodamine-dextran	Vasculature	18 μg/g	Thermo Fisher (D7139)
Hoechst 33342	Nuclei	2 μg/g	Thermo Fisher (H357
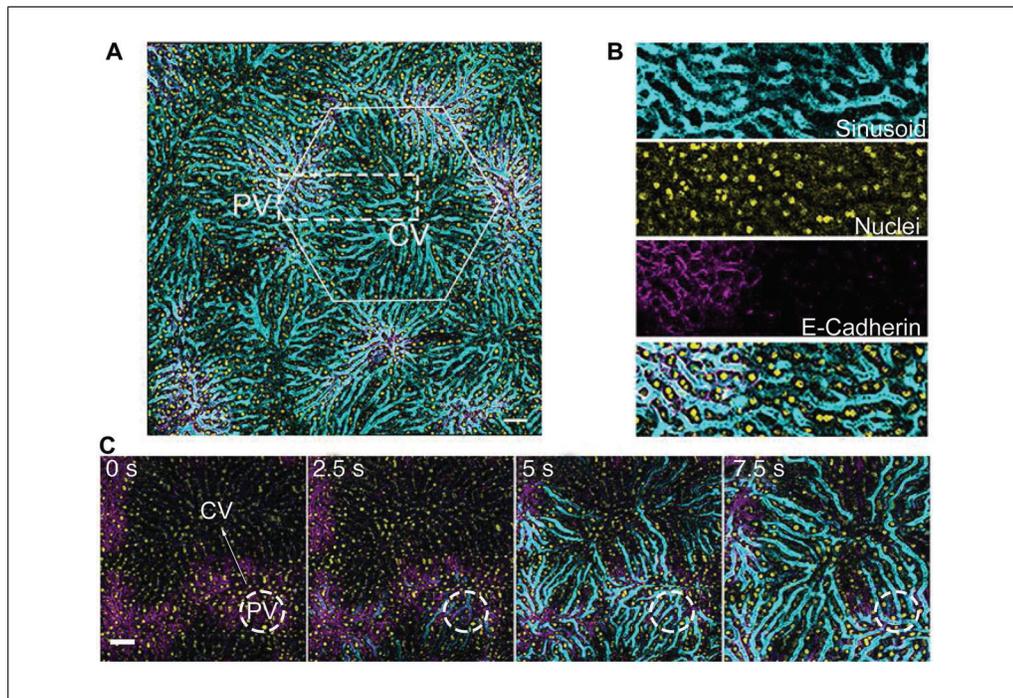
Figure 2 Imaging different compartments in the liver. (**A**) IVM image of the liver parenchyma. Sinusoids are labeled with 2 M dextran (cyan), PV hepatocytes with Alexa-647-conjugated E-cadherin antibody (magenta), and nuclei with Hoechst 33342 (yellow). The white hexagon denotes one lobule, and the dashed rectangle the hepatic acinus. PV, portal vein; CV, central vein. Scale bar, 50 μm. (**B**) Close-up view of the hepatic acinus. (**C**) Selected frames from time-lapse IVM demonstrating how fluorescent blood tracers such as 2 M dextran (cyan) can be used to identify PV and CV regions based on the direction of blood flow. Scale bar, 50 μm.

1. Anesthetize the mouse and maintain anesthesia using nose cone (Basic Protocol 1).

2. Inject 0.5 μg/g Alexa Fluor® 647–conjugated anti-mouse/human CD324 (E-cadherin) antibody diluted in 1× heparin (total volume of 50 μl) via retro-orbital injection using a 31-G insulin syringe.

3. Inject 2 μg/g Hoechst 33342 diluted in 1× heparin (total volume 50 μl), via retro-orbital injection using a 31-G insulin syringe. Use the other eye for Hoechst 33342 injection, as the probe tend to precipitate if combined with other fluorescent probes.

4. Incubate 30-45 min to allow labeling of PV hepatocytes and nuclei, while controlling the body temperature with a heating pad.

5. Prepare microscope and mouse for imaging (Basic Protocol 1).

6. Secure the mouse's neck to the stage with tape.

7. Minimize motion, then retro-orbitally inject 18 μg/g of fluorescent dextran diluted in 10 USP units/ml heparin (total volume 50 μl) using a 31-G insulin syringe.

8. Assess blood flow by observing the fluorescent dextran signal via the eyepiece. Strong labeling of the vasculature should occur within 1 s of injection (Fig. 2).

9. Identify PV from CV areas of the lobule using E-cadherin labeling and the direction of the blood flow. Blood flows from the PV towards the CV regions (Fig. 2C).

10. Select an imaging area that is stable and has a good blood flow. This can be assessed by looking through the eyepiece and acquiring a short IVM time-lapse to evaluate stability over time.
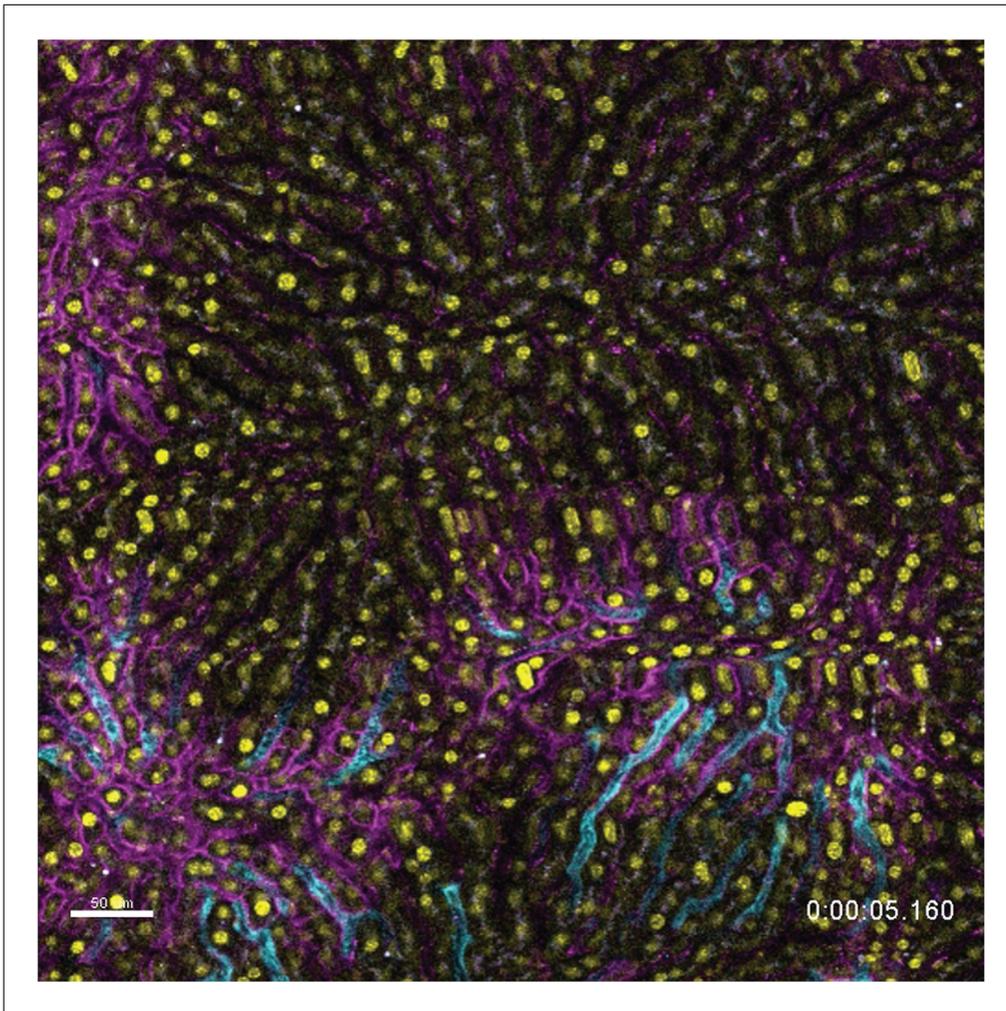
Video 1 The liver was labeled with Hoechst (nuclei; yellow) and Alexa-647-conjugated E-cadherin antibody (magenta) to mark PV regions prior to time-lapse IVM acquisition. Fluorescent dextran (2 M; cyan) was injected while acquiring time-lapse IVM, which can be used to identify PV and CV regions based on the direction of blood flow. Magnification: 20×; 512 × 512; frames were acquired every 2.5 s. Scale bar, 50 μm.

> *It may take 20-30 min for the liver to settle down and form stable contact with the coverslip.*

11. Set up time-lapse IVM to acquire 512 × 512 pixels every 2.5 s for 10 min using the 20× objective (Fig. 2 and Video 1).

MOUSE HEPATIC GLUCOSE UPTAKE ASSAY AND INTRAVITAL IMAGE ANALYSIS

This protocol describes how to use the fluorescent glucose analog 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) to measure glucose mobilization between different compartments in the hepatic lobule of a live, anesthetized mouse. A step-by-step guideline for image analysis follows. This procedure provides an alternative to other methods of measuring hepatic glucose uptake by providing spatiotemporal information. The protocol can be completed in 45-60 min per mouse.

Materials

Dextrose 50% (Hospira, 000622)
2-NBDG (Thermo Fisher, N13195)
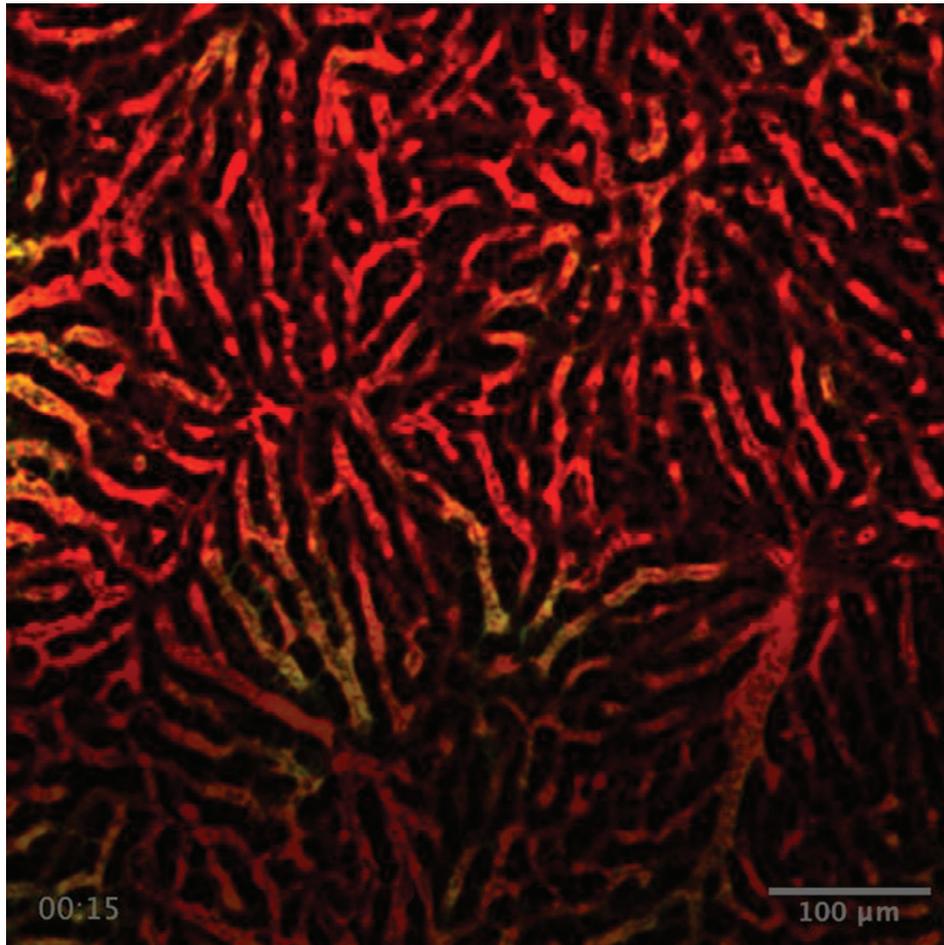
Video 2 Sinusoids were labeled with fluorescent dextran (2 M; red) ahead of imaging. 2-NBDG (green) was injected while acquiring time-lapse IVM. Magnification: 20×; 512 × 512; frames were acquired every 2.5 s. Scale bar, 50 μm.

31-G insulin syringe
ImageJ software or similar
Excel spreadsheet software

Additional reagents and equipment for mouse anesthesia, surgery, and intravital imaging (Basic Protocol 1)

Image acquisition

1. Fast the mice for 4-6 hr prior to imaging.

2. Prepare the microscope and surgically expose the liver as described in Basic Protocol 1.

3. Secure the mouse's neck to the stage with tape.

4. Minimize motion, then retro-orbitally inject 18 μg of dextran per gram of mouse body weight (diluted to a total volume of 50 μl in 10 USP U/ml heparin) using a 31-G insulin syringe.

5. Set up time-lapse IVM to acquire 512 × 512 pixels every 2.5 s for 30 min. Use the 20× objective and select the field of view as described in Basic Protocol 2 (Video 2).
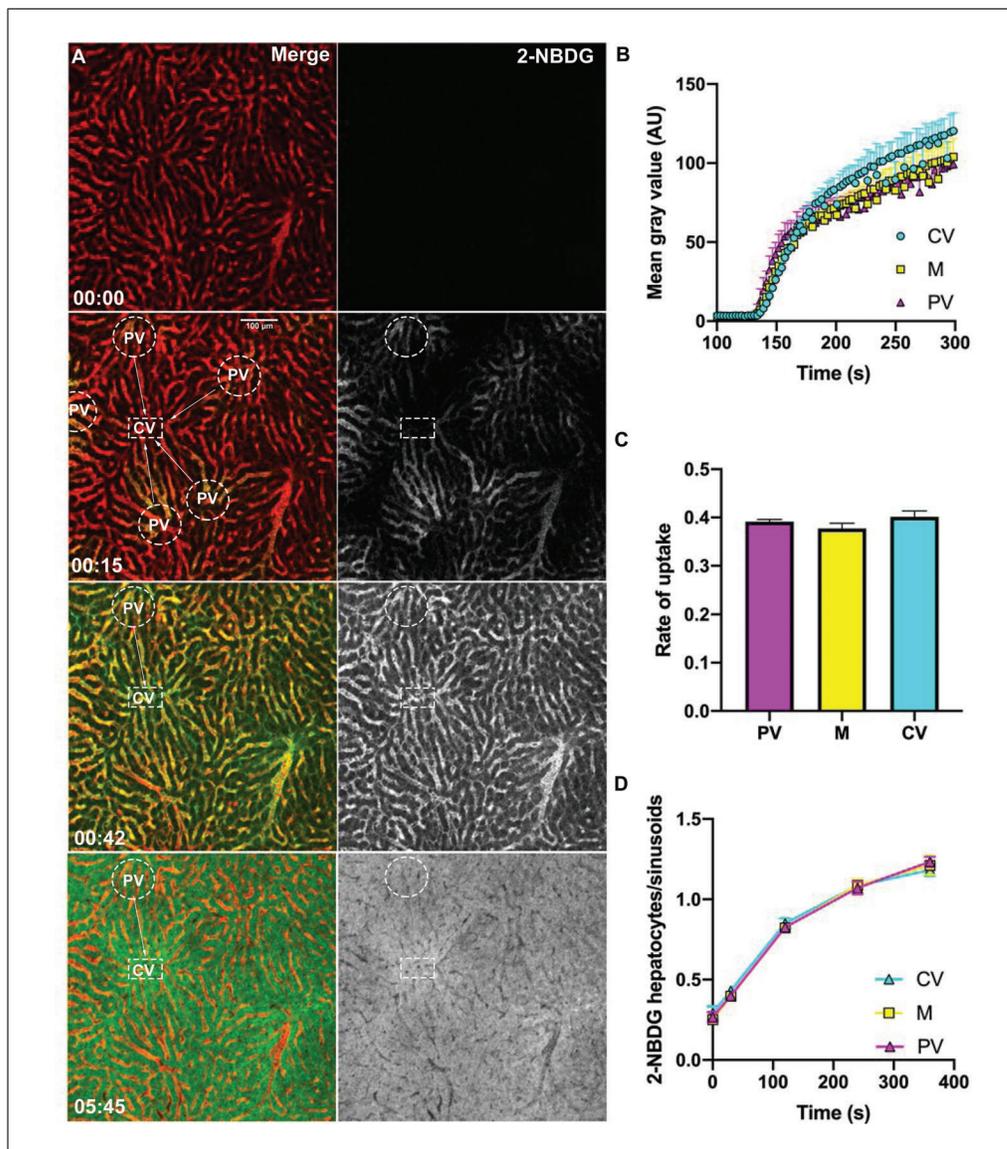
Figure 3 Hepatic glucose uptake and retention quantified using intravital microscopy. Representative frames from time-lapse IVM in which sinusoids were labeled with 2 M dextran (red) and the fluorescent glucose analog 2-NBDG (green in overlay, or gray). Dashed circles around periportal hepatocytes indicate the portal vein (PV) regions; dashed rectangle around pericentral hepatocytes indicate the central vein (CV). White arrows denote the direction of blood flow. Time is shown as minutes:seconds. (**B**) Glucose uptake measured as mean gray value over time in PV, mid-lobular (M), and CV (three ROIs from one mouse). (**C**) Rate of glucose uptake in PV, M, and CV. (**D**) Glucose retention calculated as the ration between the mean gray values in hepatocytes over the mean gray values in the sinusoid. Errors bars, SEM.
Time-lapse IVM.

6. Dissolve 2-NBDG (6.83 μg/g mouse) in 50% dextrose (1 g/kg body weight). Inject 50 μl 2-NBDG retro-orbitally with a 31-G insulin syringe while acquiring time-lapse IVM (Fig. 3A).

   Keep 2-NBDG stock solution on ice, protected from light, for up to 6 hr.

   Expect 2-NBDG signal to appear in sinusoids located in the PV area (Fig. 3A) within 10 s after injection and to be taken up by hepatocytes within 10-15 min.

Image analysis
7. Open the time-lapse LIF (`.lif`) file with ImageJ.
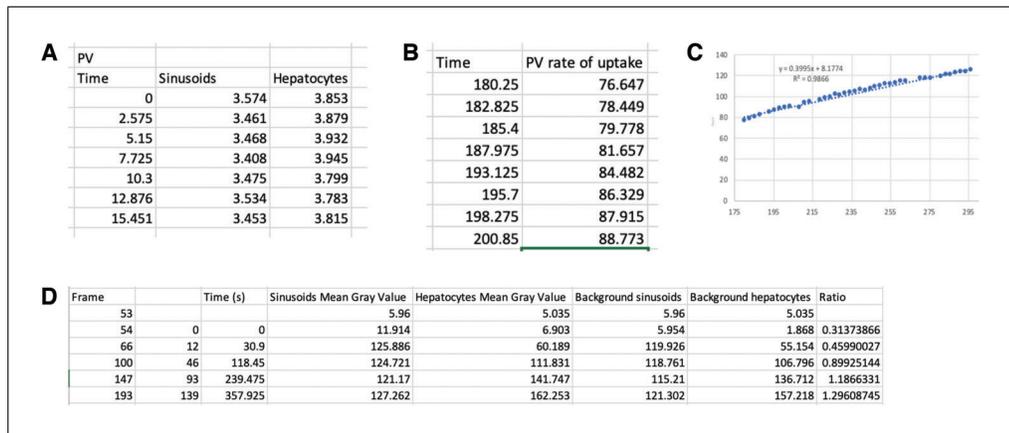
Figure 4 Sample data from intravital hepatic glucose uptake assay. (**A**) 2-NBDG data from the hepatocyte area. (**B**) 2-NBDG uptake data. (**C**) Scatterplot with trend line. The three data points with mean gray values near the top right represent outliers that were deleted. (**D**) Data for sinusoid and hepatocyte mean gray values.

8. Brighten and smooth the dextran signal. Click on Image > Adjust > Brightness/ Contrast and enhance the dextran signal as needed, then click Process > Smooth.

9. Identify PV and CV as described in Basic Protocol 2 using the 2-NBDG channel. Create three ROIs (regions of interest) of the same size on the PV-CV axis: PV, M (mid-lobular), and CV.

   PV regions can be identified by labeling with Alexa-conjugated E-cadherin antibody (Fig. 2C) or by time-lapse IVM, in which 2-NBDG signal appears first at the PV (Fig. 3A).

10. Crop the three ROIs and save them separately.

11. Split the channels

12. Threshold the dextran channel (Image > Adjust > Threshold).

13. Adjust the threshold bar so that only the dextran signal is included (shaded red). Select the thresholder regions (Edit > Selection > Create selection).

14. Save the selections (File > Save as > Selection).

15. Transfer the selection of sinusoids to the 2-NBDG channel by opening the saved selection (File > Open saved selection).

16. Measure 2-NBDG in the sinusoid area (Image > Stacks > Plot z axis profile > Data > Copy all data).

17. Paste the data points into an Excel spreadsheet. The first column (x) is time in seconds and the second column (y) is the mean gray value inside the sinusoid, at each time point.

18. Measure 2-NBDG in the hepatocyte area (2-NBDG channel > Edit > Selection > Make inverse > Image > Stacks > Plot z axis profile > Data > Copy all data).

19. Paste data points into the Excel spreadsheet. The first column (x) is time in seconds and the second column (y) is the mean gray value in the hepatocytes, at each time point (Fig. 4A).

20. Plot the mean gray intensity of the different parts of the hepatic lobule (Fig. 3B).

Calculating the rate of 2-NBDG uptake

21. Identify the time point in the hepatocytes column at which the signal dramatically increases; this is likely the time point where the 2-NBDG was injected. Copy the mean gray values from this column within the 2-min period after that time point. Paste these values into a separate column.

22. Copy the time values for that same 2-min period and paste them into a column (Fig. 4B).

23. Create a scatterplot. If the liver preparation was stable, the scatterplot should be linear.

24. Add a trendline to the scatterplot. Select "Linear" in the Format Trendline box and check "Display equation on chart" and "Display R-squared value on chart." If the points fit a linear equation well, the R^2 value will be close to 1.

   Motion artifacts can result in outlier data points that can be removed. However, the removal of multiple data points will result in an inaccurate linear fit. Therefore, each time-lapse should be evaluated for stability and quality of data. An example is provided in Figure 4C.

25. Compare the slope (in the example above, slope = 0.3995) between zones and mice. A higher value reflects a higher rate of uptake (Fig. 3C).

Calculating 2-NBDG retention in hepatocytes

26. The rate of glucose retention can be calculated as the ratio of the mean gray value of 2-NBDG in hepatocytes to the mean gray value of 2-NBDG in the sinusoids:

   • ratio >1: More 2-NBDG in hepatocytes than in sinusoids;
   • ratio <1: Less 2-NBDG in hepatocytes than in sinusoids;
   • ratio = 1: 2-NBDG signal in hepatocytes = 2-NBDG signal in sinusoids.

27. Select time points from time-lapse IVM from which to calculate ratios. For example: 0 s (defined as the frame before 2-NBDG signal first appears) and 30 s, 2 min, 4 min, and 6 min post 2-NBDG injection.

28. Copy and paste the mean gray values at the chosen time points, and paste them into a new spreadsheet.

29. Subtract the background mean gray value prior to the appearance of the 2-NBDG signal, for both the sinusoid and hepatocyte selections. Next, divide the hepatocyte mean gray value by the sinusoid mean gray value (at each time point) to find the ratio (Figs. 3D and 4D).

COMMENTARY

Background Information

IVM was first described in 1839 (Wagner, 1839), when it was used to observe the movement of leukocytes through blood vessels in the translucent, webbed feet of frogs. The development of novel fluorescent probes since 1994 has made its application to a wide range of intact tissues possible. IVM is particularly suited for studying liver physiology because the liver can easily be slipped out of the body cavity onto a coverslip and is highly amenable to administration of fluorescent probes, eliminating the need to create transgenic animals expressing fluorescently tagged proteins. In these protocols, we have described a novel method for measuring hepatic glucose uptake in real time in live mice using intravital microscopy and the fluorescent glucose analog 2-NBDG. Previous methods have measured hepatic glucose uptake in rats (Tsumura, Tsushima, Tamura, Hasebe, & Kobayashi, 2019) and humans (Mari, Wahren, DeFronzo, & Ferrannini, 1994) by perfusing the livers with stable-isotope-labeled glucose and quantifying the ratio of the carbon isotopes to determine net uptake. Positron emission tomography (PET) has also been used to measure glucose uptake in the liver

(Honka et al., 2018). Although these methods allow quantification of hepatic glucose uptake in vivo, they lack spatial and temporal resolution.

Here we describe protocols for IVM of the liver, labeling of liver compartments, and evaluation of glucose mobilization using the fluorescent tracer 2-NBDG. Collectively, these protocols allow the investigation of zone-specific differences in hepatic glucose transport. Applying these assays to liver disease models will provide invaluable information on hepatocyte regulation of glucose homeostasis at the cellular level. The increased spatial resolution also permits examination into the roles that non-parenchymal liver cells, the microenvironment, or the vasculature may have in the regulation of glucose transport. For example, hepatic glucose uptake might be impaired in the liver due to inadequate vascularization rather than dysfunctional glucose transporters, a distinction that is impossible to test in isolated hepatocytes. This assay is not without limitations, however, as it cannot be used to determine downstream fates of glucose, such as glycogen deposition. Once taken up by hepatocytes, the NBD group is cleaved from the glucose before the glucose is phosphorylated in the first reaction of glycolysis (Yoshioka et al. 1996).

Critical Parameters

Basic Protocol 1: Liver exposure and positioning on the microscope stage are key to obtaining robust results. While surgically exposing the liver, special attention should be paid to avoid excessive bleeding and damage to the liver parenchyma. In addition, positioning of the mouse on the microscope stage is equally critical for two main reasons. First, stable preparation for imaging is a prerequisite for extracting quantitative information from the images. Second, tissue damage may occur during the positioning of the mouse on the microscope stage. Therefore, these steps are particularly sensitive to user manipulation and require precision and practice.

Basic Protocol 2: Labeling of the different hepatic compartments is critically dependent on the fluorescent probe concentrations and the effective delivery of the probes. The concentrations for the fluorescent probes described here were determined empirically in wild-type (C57Bl/6J) mice. Applying this protocol to other lines and/or disease models may require adjusting these concentrations. In addition, retro-orbital injection, like any other procedure, requires precision and practice. It is therefore advisable to master the technique before performing experiments.

Basic Protocol 3: The ability to quantify the rate of glucose uptake is dependent on the stability of the region imaged. Minimizing motion artifacts is extremely important but should be done with caution. Quantification of glucose retention in the liver, however, can still be done even if significant movement persists because individual frames can be analyzed independently. Another key aspect of this assay is ensuring that probes are sufficiently bright once their signal appears in the liver, because a dim signal will impede accurate thresholding.

Troubleshooting

Table 3 lists problems that may arise with these procedure along with their possible causes and solutions.

Statistical Analysis

A Student's *t*-test should be used in Basic Protocol 3.

Understanding Results

Basic Protocol 1

The goal is to surgically expose the liver and position the mouse on the microscope (Fig. 1B and E) to allow visualization of the hepatic lobule (Fig. 1A). To achieve this, the abdomen should be shaved, and the incision should expose the largest liver lobe. Bleeding is to be expected once the muscle layer is cut and can be controlled using a cauterizer (Fig. 1C). The microscope stage should be heated, and a cardboard insert taped to elevate the mouse's body away from the exposed liver. A narrow strip of gauze soaked in saline attached to the insert can keep the liver hydrated and separated from the other internal organs (Fig. 1D). The mouse should be positioned on the stage such that its abdomen is flat against the insert and its nose just inside the nose cone to maintain anesthesia while allowing access to an eye for retro-orbital injection. Gauze placed over the mouse and heating pad connected to a rectal probe help control body temperature. In addition, ultrasound gel around the edges of the incision prevents the liver from drying out (Fig. 1E).

Basic Protocol 2

Retro-orbital injection is a convenient mode to administer fluorescent probes to label hepatic compartments. The sinusoids are labeled with high-molecular-weight dextran (2 M K_d), a probe that is retained for the duration

Table 3 Troubleshooting

Problem	Possible cause	Solution
Mouse not becoming anesthetized	Isoflurane tank needs to be refilled or output is too low.	Fill tank or increase isoflurane output to 2%.
	If administering anesthesia via i.p. injection, an insufficient volume may have been injected.	Inject an additional half dose, especially if the mouse weighs >20 g.
Excessive bleeding during surgical prep	Damage to muscle layer or liver parenchyma occurred.	Cauterize to stop/prevent bleeding. Be mindful of avoiding heat damage to the liver while cauterizing. If direct damage to the liver occurs or bleeding persists, euthanize the mouse.
Inability to find stable field of view	Isoflurane levels are too high, causing the mouse to gasp.	Lower isoflurane levels to 1-1.5%
	Liver is too close to the body cavity.	Use a saline-soaked cotton-tipped applicator to gently separate the liver from the other internal organs. Liver damage can be minimized by gently squeezing the sides of the mouse's abdomen without use of the applicator to coax the liver away from the rest of the body. Soak a strip of gauze in saline and tape it to the insert such that the liver is positioned below it and the rest of the internal organs rest on top.
	Liver-coverslip interface is poor.	Ensure that the liver is moist but not submerged in liquid on the coverslip. Use Kimteck tissue to absorb any excess fluids. Allow 10 min for the tissue to settle and for the formation of stable interface between the tissue and the coverslip.
	Mouse is a heavy breather.	Adjust the mouse's body to minimize breathing motion. This may involve shifting the mouse further up the stage insert so that the ribcage is elevated away from the liver. Choose areas of the liver close to the edges of the lobe, as these are usually less prone to motion artifacts.
Poor signal from the dextran or 2-NBDG probe	Insufficient probe was injected.	Increase the probe concentration to be injected.
	The probe was not correctly injected into the capillary bed behind the mouse's eye.	Ensure that the syringe is inserted at the corner of the eye closest to the nose and behind the eyeball to get to the back of the eye socket. If the first retro-orbital injection fails, try injecting through the other eye.
	Laser power is too low.	Increase laser power while minimizing photobleaching.
	Blood flow in the selected area is poor.	Evaluate the fluorescence signal in other areas of the tissue. If the signal is poor throughout the tissue, this may indicate extensive disruption to blood flow and may require euthanasia of the mouse. It is not uncommon to have local disruptions in blood flow; therefore, if fluorescent signal is good in other regions, they can be imaged.

(*Continued*)

Table 3 Troubleshooting, *continued*

Problem	Possible cause	Solution
Liver damage	Poor blood flow resulted in ischemia.	Identify an area with normal perfusion. Blood flow can be seen indirectly, without any labeling through the eyepiece.
	The liver was subjected to excessive handling.	Handle the liver with cotton-tipped applicators as little as possible. Use gravity to coax the liver out of the body cavity and onto the coverslip. Gently squeeze the sides of the mouse to further extrude the liver. If using an applicator, soak the cotton tip in saline.
Poor labeling of PV hepatocytes with E-cadherin or Hoechst 33342	Retro-orbital injection did not work.	Reinject the probe.
	Probe concentration is too low.	Increase the probe concentration 10×.
	Blood flow is poor due to damage.	Try the procedure again while minimizing tissue damage.

of the imaging experiment (Fig. 2A-C). Similarly, Hoechst 33342 and Alexa Fluor® 647 anti-mouse/human CD324 are used to label the nuclei and E-cadherin, respectively. Expect the nuclei of all hepatocytes to be evenly labeled with Hoechst, whereas the E-cadherin antibody should selectively label PV hepatocytes (Fig. 2B and C and Video 1). The hepatic lobule receives blood from six portal veins that drain into a single central vein (Fig. 1A). When using a 20× objective, multiple lobules can be visible within the field of view (Fig. 2A and Video 1). Portal veins can be identified using the E-cadherin label and/or the site where dextran appears first when acquiring IVM time-lapse (Fig. 2C and Video 1).

Basic Protocol 3

After analyzing the rate of 2-NBDG uptake, the slope of the linear increase in 2-NBDG mean gray value can be compared between mice and shown as a bar graph, as in Figure 3C. 2-NBDG retention is best shown as a line graph, as in Figure 3D, to allow comparison of 2-NBDG retention at different time points and between mice. The retention is calculated in Basic Protocol 3 as the ratio of 2-NBDG mean gray value in the hepatocytes compared to the sinusoids. A ratio <1 indicates that more 2-NBDG is present in the sinusoids than in the hepatocytes, a ratio of 1 indicates that the 2-NBDG levels are about the same in sinusoids and hepatocytes, and a ratio >1 indicates that 2-NBDG levels are higher in hepatocytes than in sinusoids.

Interestingly, although CV hepatocytes are known to rely on glycolysis more than PV hepatocytes (Rui, 2014), we have found that the rate of glucose uptake and retention is comparable between the three zones (Fig. 3C and Video 2). This unexpected result may be due to the strategic distribution of GLUT family transporters along the hepatic lobule. The glucose concentration is higher in PV than CV hepatocytes after a meal (Ricken et al., 2015), and GLUT2, which has a low affinity for glucose, is primarily found in PV hepatocytes (Ogawa et al., 1996), whereas GLUT1, with a high affinity for glucose, is primarily found in CV hepatocytes (Bilir et al., 1993). The low affinity of GLUT2 for glucose around the PV leaves some glucose in the blood for use by the CV hepatocytes, which can rapidly take up glucose even at low concentrations due to the high affinity of the GLUT1 transporter. The organization of glucose transporters in this fashion may be crucial for maintaining glucose homeostasis, as the liver appears to clear 30%-40% of the glucose entering the portal vein (Pagliassotti & Cherrington, 1992). Inadequate glucose uptake due to loss of glucose transporter zonation or expression may contribute to high circulating blood glucose levels and lead to insulin resistance, which could be investigated by using this protocol in disease models.

Time Considerations

Basic Protocol 1 can be completed in 45-60 min.

Basic Protocol 2 can be completed in 30-45 min.

Basic Protocol 3 can be completed in 45-60 min.

Acknowledgments

Author Contributions

Megan Stefkovich: Conceptualization, Data curation, Formal analysis, Methodology, Writing-original draft, Writing-review & editing, Sun Woo Sophie Kang: Data curation, Methodology, Writing-original draft, Natalie Porat-Shliom: Conceptualization, Formal analysis, Methodology, Visualization, Writing-original draft, Writing-review & editing

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

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