GLUCOSE CLAMPING THE CONSCIOUS MOUSE:
A LABORATORY COURSE

Vanderbilt-NIDDK
Mouse Metabolic Phenotyping Center
Nashville, Tennessee

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Preface

The development of methodology to manipulate the mouse genome has led to a dramatic increase in mouse models of disease. This, in turn, has spawned a need to develop technology to study the mouse by overcoming barriers related to their limited size. The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) initiated the Mouse Metabolic Phenotyping Center (MMPC) Program in 2001 to address this need. Vanderbilt University School of Medicine has been an MMPC site since the inception of the program. The objective of the MMPC Program is to develop and apply techniques for studying mouse models of diabetes and related disorders.

*Glucose Clamping the Conscious Mouse: A Laboratory Course* was developed and first conducted in 2005 by the Vanderbilt MMPC in response to requests from the scientific community. There are numerous mouse models with initial phenotypes that indicate a glucose clamp would be informative in understanding the effect of a genetic manipulation or treatment with an exogenous compound. Despite the heavy demand for this technique, convention amongst researchers using this technique has been lacking. This has resulted in an inability to interpret clamp data and compare clamp data between different laboratories. This course is intended to (1) aid those laboratories needing to perform glucose clamps on a regular basis in implementing this challenging technique at their institution, (2) increase the transparency of the technology so that the factors involved in glucose clamping the mouse are better understood by scientists wishing to make sense of the growing literature, and (3) serve as a forum for scientists interested in study of the mouse *in vivo* to compare and exchange methodology.

Since we first compiled the laboratory manual for this course, we have made several adaptations to the original techniques. These modifications were made to decrease stress to the mouse, increase accuracy of the clamp method, or increase surgical and experimental success rates. The method described in this manual and during the weeklong course is the result of a lengthy developmental process that has spanned nearly 20 years. Only the most recent version will completely reflect the state-of-the-art of the Vanderbilt clamp technique. Additions to the manual include, but are not restricted to, a technique for red blood cell replacement, a method for increasing accuracy of glucose flux measurements by mixing [3-\(^3\)H]glucose with “cold” glucose, and specification of new materials and vendors.
As we hope to impart our experiences in glucose clamping the mouse to the participants of this course, we also hope to learn from the participants as they acquire experience. We fully expect for people undertaking this technique to add to and improve upon procedures over time. Our hope is that participants will share their experiences after they return home, so that we may all work together to expedite advancements in understanding, preventing, and treating diabetes and other metabolic diseases.

The pages that follow describe technical aspects of the surgical, experimental, and analytical procedures necessary to perform the glucose clamp in the conscious mouse. Dr. Masakazu Shiota, of our faculty, developed the key surgical and experimental technology. Tasneem Ansari, Staci Bordash, Bingle Bracy, Freyja James, Merrygay James, Alicia Kellarakos, Carlo Malabanan, and Teri Walker contributed to technical descriptions provided in this manual. Please make us aware of sections in the laboratory manual that should be clarified or errors that may exist. Updated versions of this manual and additional Vanderbilt MMPC information are available online (www.vmmpc.org).

We could not accomplish our goals without the innovation, support, and enthusiasm of our sponsors – Instech Laboratories, Inc. and Kent Scientific Corp. We are also grateful to our MMPC Program Coordinator, Eann Malabanan, for handling the logistics of the course. This can be as challenging as glucose clamping a mouse.

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1. Introduction

A glucose clamp is where arterial glucose is maintained at or near a pre-defined set point by a variable infusion of glucose. The rate of the variable glucose infusion is determined by feedback obtained by frequent measurement of blood glucose. There are several terms that are used, sometimes interchangeably, when referring to the glucose clamp. Since many terms are used, even within the same institution, some expressions should be defined at the onset.

The most common application of the glucose clamp technique is where insulin is infused to create hyperinsulinemia, while arterial glucose is clamped at fasting levels. This is the hyperinsulinemic, euglycemic clamp. Since this is a cumbersome expression, it is often referred to simply as an insulin clamp. The term insulin clamp is used in this manual and will be used during the Course. Arterial glucose can also be clamped at a low concentration during a high insulin infusion to test the glucose counterregulatory system or the ability of the body to respond to hypoglycemia. This is a hypoglycemic clamp. Arterial glucose can also be clamped at elevated concentrations without the infusion of insulin. This is called a hyperglycemic clamp and is used to test the secretory capacity of pancreatic β-cells.

It is valuable to be aware of the various applications of the glucose clamp technique. The primary focus here will be on the insulin clamp. Once the tools necessary to conduct an insulin clamp are in hand, other clamp protocols can be easily adapted.

Glucose clamping of the conscious mouse requires four fundamental steps: (1) surgical implantation of catheters, (2) postoperative care of the mouse and catheter maintenance, (3) clamp experiment, and (4) data interpretation. It is important to recognize that there is more than one way to clamp a mouse. While the insulin clamp as performed at the Vanderbilt-NIH MMPC is taught here, there are likely to be other approaches that may also accommodate one’s needs. As such, there are many factors that must be considered in designing and interpreting insulin clamp experiments.
The hope is that participants will leave here with not only the technical skills necessary to glucose clamp the conscious mouse, but also a better understanding as to how to interpret the results that glucose clamps yield.

2. Surgical Catheterization of the Carotid Artery and Jugular Vein

Study of the unstressed mouse requires prior implantation of catheters in the carotid artery and jugular vein. The arterial catheter is used to obtain samples during the clamp and the venous catheter is used as an infusion port. Dr. Masakazu Shiota of Vanderbilt developed the procedure used by the MMPC in the mid-1990’s. The surgical procedure is performed under sterile conditions and takes an experienced surgeon <1 hour. It is critical not only that the catheters work the day of the experiment, but also that mice are stress-free and infection-free.

A. Catheter and MASA™ Construction

i. Supplies

<table>
<thead>
<tr>
<th>Supplies</th>
<th>ID Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silastic tubing 0.012” ID</td>
<td></td>
</tr>
<tr>
<td>Silastic tubing 0.020” ID</td>
<td></td>
</tr>
<tr>
<td>PE-10 0.011” ID</td>
<td></td>
</tr>
<tr>
<td>Stainless steel wire, 0.02” OD</td>
<td></td>
</tr>
<tr>
<td>File</td>
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</tr>
<tr>
<td>Graefe forceps</td>
<td></td>
</tr>
<tr>
<td>Dumont 7b curved shank forceps</td>
<td></td>
</tr>
<tr>
<td>*Silicone medical adhesive</td>
<td></td>
</tr>
<tr>
<td>*Abrasive stone medium grain</td>
<td></td>
</tr>
<tr>
<td>*PE-25 (0.46x0.91mm)</td>
<td></td>
</tr>
<tr>
<td>*Stainless steel tubing 25 ga 0.0123” ID</td>
<td></td>
</tr>
<tr>
<td>*Silastic tubing 0.040” ID, cut 0.7 cm</td>
<td></td>
</tr>
</tbody>
</table>

*for MASA™ construction
**ii. Arterial Catheter Preparation**

1. Cut 10 cm of PE-10 and carefully stretch the tubing to ~13 cm. If the tubing is stretched much beyond this point, it will snap.

2. Cut stretched PE-10 into 1.3 cm pieces.

3. Cut silastic tubing (0.012”ID) into 6 cm pieces.

4. Insert Dumont forceps into the end of a 6 cm piece of silastic. Gently open forceps so the tubing is opened wide.

5. Gently grasp the end of a stretched and pre-cut piece of PE-10 using a pair of Graefe forceps, and insert the tip of the PE-10 into the silastic tubing approximately 3 mm.

6. Slip the Dumont forceps from the silastic tubing, leaving the PE-10 in place. The catheter tip is beveled at the time of surgery.

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*Figure 1: Carotid Artery Catheter. Scheme is not to scale. Note that exact dimensions may vary depending on size of mouse*
iii. Venous Catheter Preparation

1. Cut a 6 cm piece of silastic tubing (0.012” ID).

2. Cut a 1 mm piece of silastic tubing (0.020” ID) for use as restraining bead.

3. Insert the tips of eye dressing forceps into the lumen of the restraining bead and gently hold the tips of the forceps apart to stretch the opening wider.

4. Using another pair of eye dressing forceps, slide the silastic tubing into the lumen of the restraining bead.

5. Pull the silastic tubing through the bead to about 11 mm.

   The bead must lie flat around the catheter.

6. Adjust the bead and then bevel catheter tip at the time of surgery making any necessary adjustments for differences in mouse size.

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**Figure 2: Jugular Vein Catheter. Scheme is not to scale. Note that exact dimensions may vary depending on the size of the mouse.**
iv. MASA™ Preparation

1. Cut two 13 mm stainless steel (ss) tubes using file, smoothing edges with abrasive stone.

2. Cut two 2.5 cm PE-25 tubing, attach to ss tubes from step 1.

3. Slide silastic tubing (0.040” ID) over where both ss tubes and PE-25 attach.

4. Place completed rig in silicone medical adhesive for 24 hours.

5. Catheter plugs for MASA™ are cut from steel wire for use at time of surgery.

Figure 3: MASA™. Scheme is not to scale.
B. Surgical Procedures

i. Supplies

Tubing
Stainless steel, 25ga. Ziggy’s Tubes and Wires # 25R304-36
Silastic tubing 0.012”i.d. Fisher Scientific # 11-189-14
Silastic tubing 0.020”i.d. Fisher Scientific # 11-189-15A
Silastic tubing 0.040”i.d. Fisher Scientific # 11-189-15D
Polyethylene tubing (PE-10). Fisher Scientific # 14-170-12P
Polyethylene tubing (PE-25). Instech # BTPE-T25

Surgical Tools/Suture
Forceps, Dumont 7b curved shank. Fine Science Tools # 11270-20
Hemostats. Fine Science Tools #13020-12
Micro-serrefines. Fine Science Tools # 18055-03 (straight); 18055-05 (curved)
Needle holder. Fine Science Tools # 12060-01
Spring scissors. Fine Science Tools # 15003-08
Scissors, surgical. Fine Science Tools #14058-09
Forceps. Roboz # RS-5136, RS-5132, RS-5110, RS-5111
7-0 nylon suture. Any preferred vendor.
6-0 silk suture. Any preferred vendor.
14-gauge needle, dullled.

Additional Materials and Solutions
Wire, stainless steel. Ziggy’s Tubes and Wires # W020304V-1
Clippers. Oster MiniMax trimmer. Patterson Veterinary Supply #07-842-4245
Nolvasan Surgical Scrub. Fort Dodge Animal Health NDC 0856-1041-03
70% Alcohol. Any vendor.
Isoflurane, USP Liquid for Inhalation. Piramal Heathcare NDC 66794-017-25
Ketofen. Fort Dodge Animal Health NDC 0856-4396-01.
Saline, Bacteriostatic 0.9% Injection USP, 30 mL btl. Hospira, Inc. NDC 0409-1966-07
Heparin, Sodium Injection, USP. 1000 U/mL. Hospira, Inc. NDC 0409-2720-02
Silicone glue. Factor II, Inc. Medical Silicone Adhesive A-100
Preparation of Flush Solution: 200 U Heparinized Saline
Withdraw 6 mL from 30 mL btl of saline.
Add 6 mL of Heparin.

*All surgical equipment must be sterilized.

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**ii. Preparation of the mouse**

1. Anesthetize mouse (40 - 85 mg/kg pentobarbital ip or with isoflurane).

2. Bevel catheters to correct lengths, fill with flush solution and plug.

3. Prepare the animal by removing hair from the surgical site. Perform this procedure in an area separate from where the surgery is to be conducted.

4. Prepare the surgical sites with an appropriate skin disinfectant (betadine followed by alcohol scrub).

5. Administer analgesia per your institution’s policy.

6. Follow aseptic technique per your institution’s policy.
iii. Surgery

1. Make small vertical midline incision 5 mm cephalad to the xiphoid process.

2. Blunt dissect using forceps to expose the left sternomastoid muscle. Reflect this muscle to expose left carotid artery.

3. Gently tease off connective tissue from the carotid artery. It is important at this point to isolate the vagus nerve from the artery without damaging either the artery or the nerve. Isolate artery then ligate cephalic end with silk suture. Another piece of suture is loosely knotted on the caudal end of the exposed artery.

4. Clamp vessel with micro-serrefine and cut just below the ligated end with spring scissors. Carefully insert catheter as far as possible. Grab catheter with forceps, then carefully release micro-serrefine clamp and continue inserting catheter to the silastic-polyethylene junction. At this point the tip of the catheter should be in the aortic arch.

5. Tie both ligatures securely and confirm that the catheter samples. Flush with heparinized saline.

6. Make another incision 5 mm to the right of midline and about 2 mm caudal to the first incision.

7. Blunt dissect to expose right jugular vein.

8. Isolate carefully and ligate cephalic end with silk suture. Loosely tie another piece of suture at the caudal end of the exposed vein.

9. Cut just below cephalic ligature with spring scissors. Insert catheter to the bead, tie, and confirm that it samples. Flush with heparinized saline.

10. Turn mouse over. Make a small incision between the shoulder blades. Tunnel 14-gauge needle under skin through the incision on the back. Thread catheters through the needle to exteriorize them to the back of the mouse.

11. Close ventral incisions with nylon suture.
12. Clamp venous catheter with micro-serrefine at the incision site between shoulder blades. Cut catheter 1 cm above clamp and connect to MASA™. Take care to ensure that there are no holes or kinks in the catheter. Secure venous catheter to MASA™ with silk suture. Repeat for the arterial catheter.

13. Close dorsal incision with nylon and confirm patency of both catheters again. Flush with heparinized saline. Place mouse in warmed, clean cage.

3. Postoperative Care and Catheter Maintenance

Proper attention to the well-being of the mouse and the security of the catheter during the interval between surgery and experiment determines, to a large extent, the quality of the results obtained using an insulin clamp. Described in this section are the tools and reagents needed and the procedures used to maintain the chronically catheterized mouse model during the critical postoperative period. The text below describes some observational tools to assess pain, distress, or infection. Consult your institutional veterinarian if adverse symptoms appear or concerns over the condition of the mouse arise.

A. Supplies

Clean cage (fresh bedding, food, fresh water bottle)
Heating pad
2 x 1-mL syringes (without needle)
Blunt needle with luer hub (25 G x 1/2”)
Microrenathane tubing, ~10 cm
25-ga ss tubing connector, ~1.5 cm long
Flush Solution (200 U/mL heparin in saline)
Hemostats
Hemostats with silicone tubing over tips (also called clamps, used to clamp the lines)
Alteplase Cathflo® Activase® (# NDC 50242-041-64)
B. Postoperative Care of the Mouse

**i. Preparation (as mouse goes into surgery)**

1. Put clean cage on heating pad.

2. Ensure that the water bottle does not leak and contains fresh water. Appropriate food should be placed in the cage.

**ii. Immediate Postoperative Care (as mouse comes out of surgery)**

1. Check the water bottle for leaks, then place animal belly down on warm cage bottom. Watch for excessive bleeding from incisions, the mouse catching its catheter lines in the cage lid, or the animal scratching its sutures open.

2. After animal is fully ambulatory and aware, remove cage from heating pad to the housing area.

**iii. Daily Postoperative Checklist**

1. *Observe animal for signs of infection.* Infection is indicated by suppurative from incision sites, general lethargy, and/or pain.

2. *Watch mouse for signs of pain.* Most healthy animals are ambulatory and eating within 2 hours after the completion of this procedure. Pain may be indicated by a hunched posture and ruffled fur. The mouse may also consume bedding instead of food if in pain. Follow recommendations of veterinarian as to how to alleviate pain and infection.

3. *Check animal for signs of stroke.* The animal will twist abnormally when lifted by the tail, and it may also show limb weakness and difficulty walking. It may have difficulty righting itself in response to a gentle push. Although an animal may recover occasionally, most do not. Euthanasia is suggested.

4. *Weigh mouse.* The mouse may lose weight immediately following surgery, but by the third day the weight loss should stop. By the fifth day, the animal should be within 10% of its original weight. Excessive weight loss may indicate infection, discomfort, and/or stroke.
C. Checking the Catheter Lines prior to Clamping

This can be done on the morning of the clamp to make sure the catheters are working (30 to 60 min before hooking the mouse to the swivel).

1. Fill 2 syringes with heparinized saline and cap with luer hub. Luer hub is attached to microrenathane tubing with connector at the other end (see Figure 4).

2. Remove air bubbles by placing the end connector higher than the rest of the flush syringe and pushing the bubbles out of the line.

3. Allow the heparinized saline solution in the syringe to warm up before putting it into the animal.

4. Place the mouse on the cage top.

5. Clamp off the arterial line (on the mouse’s left side) with silicone tipped hemostats just below the plug.

6. Remove the steel catheter plug using the second pair of hemostats.

7. Slip the connector end of the flush line into the arterial line.

8. Release the silicone tipped hemostats.

Figure 4: Flushing Syringe. Blunted tip of Luer hub is attached to the microrenathane tubing.
9. If the catheter tip is positioned correctly in the aortic arch, then blood can be aspirated easily. If the catheter does not draw easily, it may be necessary to very gently push in a small amount of solution through the catheter to dislodge the tip of the catheter in case it is wedged against the vessel wall*. The catheter has been completely cleared when a plume of blood reaches the syringe. Do not withdraw any more blood than is absolutely necessary to clear the catheters, as blood volume is limited on the day of the experiment.

10. Clamp the microrenathane tube close to the syringe and dispose of syringe.

11. Replace with a new syringe filled with fresh heparinized saline.

12. Unclamp the microrenathane tubing and holding the new syringe upright, flick the new syringe with a fingernail to dislodge any possible new air bubbles upwards and away from the line. GREAT CARE MUST BE TAKEN TO PREVENT INJECTION OF AIR BUBBLES, AS STROKE CAN RESULT.

13. Inject heparinized saline solution until the catheter line is clear and blood-free.

14. Clamp the arterial catheter line with the silicone tipped hemostats below the connector of the flush syringe. Slip the syringe connector from the catheter line and replace it with the steel plugs. Remove the silicone tipped hemostats from the arterial line.

15. The venous line (the one on the mouse’s right side) is cleared in a manner similar to the arterial line. However, because of the low venous pressure, sampling is sometimes not possible. If solution can be infused with minimal resistance, it is probable that the catheter is well positioned in the vein.

* In the case of severe clotting, the catheter can be filled with Alteplase to dissolve the clot as described in the Alteplase package insert. Be aware that any clot will cause a problem if it is pushed into the circulation. Clots must be aspirated and discarded.

Confirm the position of both catheters at the time of necropsy.
4. The Insulin Clamp

The hyperinsulinemic, euglycemic clamp or “insulin clamp” has been used in a variety of species to assess insulin action. In an insulin clamp, the rate of glucose infused to maintain euglycemia is an index of whole body insulin sensitivity. Isotopes can be used during an insulin clamp to distinguish between insulin’s effects on endogenous glucose production and glucose utilization or to examine insulin’s effects on specific tissues and metabolic pathways. The insulin infusion rate used in a study depends on a number of factors such as whether the experiment requires a sensitive analysis of endogenous glucose production, or whether the mouse is known to be unusually insulin resistant (e.g., ob/ob). Finally the insulin clamp technique, especially when isotopes are involved, is most quantitative under steady state conditions. Therefore, clamps should be of a duration that is sufficient for the actions of insulin to obtain steady state (~2 h). The section that follows describes (a) technical procedures used on the day of a clamp experiment, (b) preparation of insulin and tracer infusates, and (c) analyses of plasma and tissue radioactivity. Familiarity with these procedures combined with laboratory demonstration provide the investigator with the necessary background to clamp the conscious mouse.

A. Equipment and Supplies

| Infusion Pumps | Microrenathane tubing (0.033” OD) |
| Stand          | 1.0 mL syringes with blunted needles |
| Dual channel swivel | Microtubes, 0.5 mL and 1.5 mL |
| 3- and 4-way ss connectors | EDTA tubes (20 μL of 0.1M EDTA in 0.5 mL tubes, evaporated) |
| Mouse enclosure | Ice |
| Hematocrit tubes and clay | Timer |
| Saline         | Glucose meter and strips |
| Heparinized saline (10 U/mL) | |
B. Getting Started

1. Weigh mouse and start fast (suggested starting time between 7:00 and 8:00 AM) by placing mouse in a plastic container with fresh bedding.

2. Set up microrenathane tubing to swivel and connectors as needed (see Figure 5). This can be done the day before.

3. Prepare Protocol sheets using mouse weight, insulin dose, sample times and volumes (see Forms on following pages).

4. Collect donor blood (see Donor Blood Preparation).

5. Prepare insulin (see Insulin Preparation).

6. Prepare tracers, if needed (see Tracer Preparation).

7. Fill the arterial line with heparinized saline. Leave the sampling syringe connected to the top of the swivel.

8. If using [3-3H]glucose, prepare the prime syringe: draw tracer infusate into a 1 mL syringe and place it securely into the pump slot.

9. Using the pump, fill the venous line with [3-3H]glucose tracer keep using the 1 mL syringe prepared in step 8. Leave the syringe connected to the tubing and in the pump. Filling these lines prevents infusion of air into the mouse.

10. Hook up the mouse to the microrenathane tubing ~100 min prior to clamp start as shown in Figure 5. The mouse can remain in the plastic container.
11. Begin baseline primed-continuous tracer infusion at least 90 min prior to clamp start. We typically use a 0.8 μCi prime followed by a 0.04 μCi/min continuous infusion. 

12. Once donor blood and insulin are prepared, draw glucose infusate (20% or 50% dextrose mixed with or without [3-3H]glucose), insulin infusate, and donor blood into separate 1 mL syringes, and place them securely into the pump slots. 

13. Connect 10 cm microrenathane tubing to each infusate syringe and connect tubing to a 4-way connector (see Figure 5). Fill each line with glucose, insulin, and donor blood infusates up to the 4-way connector. 

14. Once the last basal sample is taken (see Arterial Sampling), start the pumps for the glucose, insulin, and donor blood, letting infusates drip out of the 4-way connector. Clamp the 3 cm tubing leading into the swivel (A in Figure 5) with a hemostat. Quickly disconnect the primed tracer tubing and connect the four-way connector in its place. Remove hemostat. You should see the blood move in the line towards the swivel. 

15. Stop the pump for the primed tracer. Do not discard the remaining tracer. Place it in 1.5 mL tube on ice to be used during analysis for standards. 

16. Once donor blood reaches the mouse, start the clock.
Figure 5: Scheme illustrating the connection of catheters, dual channel swivel, and infusates in relation to the mouse during the baseline priming period (left panel) and Clamp period (right panel) when clamping with [3-3H]glucose. To clamp without [3-3H]glucose, the top syringe in right panel will contain only glucose and no priming baseline period is required. Diagram is not to scale. The vein port is generally used for 2-deoxyglucose (2DG) tracer injection but can be used for other infusates as well. A indicates the 3 cm microrenathane tubing that needs to be clamped to switch from the priming syringe to the 4-way connector (see step 14 above).
C. Notes on Proper Clamping Technique

1. If a priming dose of insulin is required, calculate the dose needed to reach the target steady state insulin concentration, which is empirically determined. For example, with a 4 mU·kg\(^{-1}\)·min\(^{-1}\) insulin infusion, steady state insulin concentration will be \(\sim 80 \text{ μU/mL}\). Assuming the insulin volume of distribution is 20\% of the body weight, then for a 20 g mouse, the priming dose needed to achieve the target insulin concentration is 16 mU/kg. However, it has been our experience that a priming dose is not required when using an insulin infusion of 4 mU·kg\(^{-1}\)·min\(^{-1}\) and performing the clamp for 2 h.

2. \([3-\text{H}]\text{glucose}\) is added to the glucose infusate to minimize deviations in blood glucose specific activity during the clamp. This technique is highly accurate and prevents large negative measurements of endogenous glucose production.

3. The goal of the clamp is, as one approaches the steady state period (t = 80 to 120 min), to maintain blood glucose within the predetermined range while avoiding large changes in the glucose infusion rate. If this has not been achieved prior to the steady state period (or prior to the 2-deoxyglucose bolus, see below), stop the clock. While the clock is stopped, measure blood glucose every 5 min and make changes to the glucose infusion rate as needed. Once the target glucose level has been attained with minimal changes to the glucose infusion rate, restart the clock and continue with the clamp. In the event that it is necessary to stop the experimental clock, be certain to record the duration that the clock has been paused. *Note: As a clamer becomes more experienced, the need to stop the clock becomes unnecessary.

4. For 2-deoxyglucose, use a 1 mL blunted insulin syringe to administer the 48 μL bolus (see Tracer Preparation), as this syringe has a small dead space. Give the bolus just prior to the steady state period (we administer the bolus at t = 120 min), and chase it with \(\sim 20 \text{ μL}\) of saline to clear the line. Momentarily clamp off other infusion lines while the bolus is being given as it may back up into these lines. Once the bolus of 2-deoxyglucose has been given, the clock cannot be stopped.
D. Blood Sampling

i. Arterial Sampling

1. The sampling syringe attached to the top of the swivel should be filled with ~ 500 µL heparinized saline.

2. Pull back slowly on the plunger to draw blood into the syringe. Draw up ~ 20 – 40 µL of blood into the syringe.

3. Clamp the 3 cm microrenathane tubing connected to the sampling syringe either by pinching with a hand or with clamps with silicon tubing over tips. Clamps can also be used on the 15 cm tubing connected to the bottom of the swivel.

4. Once the arterial line is clamped, remove the sampling syringe and set aside, leaving it vertical such that the ~ 20 – 40 µL of blood does not mix with the heparinized saline.

5. To take glucose samples, release the clamp on the arterial line and allow blood to flow out of the free end of the tubing. A drop of blood will form that can be used on testing strips.

6. To take blood samples for analysis of plasma metabolites:
   • Insert a blunt syringe into the free end of the arterial line prior to releasing the clamp.
   • Release the clamp and draw the desired volume of blood into the syringe.
   • Clamp the arterial line and remove the syringe.
   • Dispense the blood from the syringe into a 0.5 mL EDTA tube and mix by “flicking” the tube.
   • Insert the sampling syringe back into the arterial line. Pull back slightly on the plunger to draw any air bubbles into the sampling syringe. Very slowly, give back the ~ 20 – 40 µL of blood originally taken such that the arterial line becomes lightly red to clear.
   • Centrifuge the blood for 1 min at 16,000 g. Transfer the plasma to an appropriately labeled 1.5 mL tube. Plasma can be temporarily stored on ice but should be permanently stored at –20°C or –80°C.
**ii. Cut Tail Sampling**

Bleeding from the cut tail is an alternative to arterial sampling if carotid artery catheterization proves too challenging due either to the surgeon’s skill level or an intolerance of a given mouse to surgery. The advantage of using the cut tail is that surgery is less involved (for both surgeon and mouse). The disadvantage is that obtaining more than small blood volumes is stressful for the mouse and it is difficult to obtain blood samples at pre-determined intervals. The latter depends on how effectively blood drips from the tail wound.

We recommend you consult with your IACUC regarding this process. Some IACUC protocols will not allow cut tail sampling given the number of samples and volume required to properly clamp a mouse.
E. Preparation of Infusates

i. Donor Blood Preparation

1. Collect ~1 mL of blood from donor mouse in 0.5 mL EDTA tubes.

2. Centrifuge blood (1 min at 16,000 g) and save plasma for preparation of insulin (see below).

3. Resuspend red blood cells (RBC) with heparinized saline (10 U/mL).

4. Centrifuge (1 min at 16,000 g), discard supernatant, and resuspend RBC with an equal volume of heparinized saline. Transfer resuspended RBC (donor blood) to a 1.5 mL tube.
**ii. Insulin Preparation**

1. Using the plasma obtained from donor mice, make a 3% plasma solution in saline by placing 6 mL of saline in a test tube, removing 180 µL and adding 180 µL plasma.

2. Remove 180 µL of this 3% saline-plasma solution, place in a tube and add 20 µL U-100 insulin (making a tenfold dilution to create U-10 solution).

3. Transfer 5 mL of 3% saline-plasma solution to a new test tube.

4. Calculate and add U-10 insulin to achieve desired insulin infusate concentration based on mouse weight, pump infusion rate, and desired insulin infusion rate (see example of an insulin preparation sheet in F. Examples of useful forms).

**iii. [3-^3H]Glucose Infusate Preparation**

1. For baseline infusion, dry down 16 µCi HPLC-purified [3-^3H]glucose per mouse. Reconstitute in 400 µL of saline. Save 20 µL or what remains after the clamp for standards.

2. For clamp period infusion, dry down 40 µCi HPLC-purified [3-^3H]glucose per mouse. Reconstitute in 800 µL of D50 dextrose. Note: If using D20 for the clamp, dry down 16 µCi HPLC-purified [3-^3H]glucose per mouse, and reconstitute in 800 µL of D20 dextrose. Save 20 µL or what remains after the clamp for standards.

**iv. 2-Deoxyglucose (¹⁴C or ^3H) Bolus Preparation**

1. Dry down 13 µCi of 2-deoxyglucose per mouse.

2. Reconstitute in 52 µL of saline. Save 4 µL for standards. The remaining 48 µL is given as the 2-deoxyglucose bolus.
### F. Examples of useful forms

**MOUSE METABOLIC PHENOTYPING CENTER**  
Hyperinsulinemic-Euglycemic Clamp with [3-³H]Glucose

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<td>Body Mass:</td>
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<td>Hematocrit:</td>
<td>Fasting Blood Glucose:</td>
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**Procedure:**

[³H]glucose infusate preparation:

- **Prime:**
- **Pump rate:**

| Insulin infusion rate: | mU·kg⁻¹·min⁻¹ | Dextrose Concentration: | g/100 mL |

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<th>Insulin pump rate:</th>
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<td>Donor Blood pump rate:</td>
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Plasma Volumes Collected for Tracer and Hormones:

Tissues Collected:

Insulin Preparation:

Notes:
Hyperinsulinemic-euglycemic Clamps in Awake Mice

- Food removal at 8 AM on the experiment day for 5-hour fast
- A primed-continuous infusion of 3-[^3]H-D-glucose (0.8 μCi/min for 2 min (10 μL/min) then 0.04 μCi/min (1 μL/min) – 16 μCi 3-[^3]H-D-glucose in 400 μL saline)
- Continuous insulin (Humulin, Eli Lilly) infusion (4 mU/kg/min) for 2.5 hours
- Variable infusion of 50% glucose + 3-[^3]H-D-glucose (40 μCi 3-[^3]H-D-glucose in 800 μL D50) to maintain plasma glucose at euglycemia (target range ___________ mg/dL)

MOUSE #: ___________________  BODY WT (g): ___________________  DATE: ________________

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<th>GLUCOSE (mg/dL)</th>
<th>Time of infusion change</th>
<th>Glucose infusion rate (μL/min)</th>
<th>mg/kg/min</th>
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</table>

G: sample for plasma glucose concentration (5 μL blood for AccuChek)
T: sample for plasma tracer concentration (10 μL plasma)
I: sample for plasma insulin concentration (25 μL plasma)
Example of an insulin infusate preparation sheet

- The insulin infusate is prepared based on the body weight of the mouse. An excel template can be built to facilitate the preparation.
- The insulin is first diluted 10-fold, then further diluted in saline containing 3% plasma (the plasma is collected during donor blood preparation).

**INSULIN INFUSATE PREPARATION**

Updated: September 2016

1. Prepare for each mouse 6 mL of Saline + 3% Plasma
2. Dilute U100 to U10 in Saline + 3% Plasma (10-fold dilution)
3. For each mouse, dilute U10 insulin in 5 mL Saline + 3% Plasma according to calculation

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<thead>
<tr>
<th>Saline + 3% Plasma=</th>
<th>6 mL Saline</th>
<th>180 µL Plasma</th>
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<tr>
<td><strong>Dilute U-100 insulin 1:10 to make U-10:</strong></td>
<td>40 µL U-100 insulin in 360 µL saline +3% plasma</td>
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<table>
<thead>
<tr>
<th>date:</th>
<th>[date]</th>
<th>Mouse ID:</th>
<th>Mouse#:</th>
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<tr>
<td>body weight</td>
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<td></td>
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</tr>
<tr>
<td>insulin</td>
<td>100 U/ml or mU/µl</td>
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<td></td>
</tr>
<tr>
<td>insulin infusion rate</td>
<td>2.5 mU/kg/min or µU/g/min</td>
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<td></td>
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<tr>
<td>insulin pump rate</td>
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<tr>
<td>final volume</td>
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<table>
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<th>*</th>
<th>31 grams</th>
<th>*</th>
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<th></th>
<th>38.8 µL of U-10 insulin</th>
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</thead>
<tbody>
<tr>
<td>1 µl/min</td>
<td>*</td>
<td>10 mU/µL</td>
<td>*</td>
<td>1000 µU/µL</td>
<td>in</td>
<td>5000 µL of Saline + 3% Plasma</td>
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</table>

**Insulin Preparation:**

Add to clean tube | 5000 µL saline + 3% plasma (see above for preparation)
Remove | - 38.8 µL saline/plasma
Add | + 38.8 µL of U-10 diluted insulin
Example of a glucose pump rate sheet

- This sheet converts the Glucose Infusion Rate (in mg/kg/min) into a Pump Rate (in ul/min). This conversion is required so as to input the correct pump rate into the glucose pump each time a change to the Glucose Infusion Rate is required.

- This sheet is unique to each mouse because the conversion takes into account body weight. Print each mouse’s unique sheet and tape near the correct glucose pump so it is visible when changes to the glucose infusion rate are needed.

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<th>pump setting</th>
<th>GIR</th>
<th>pump setting</th>
<th>GIR</th>
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Note: for euglycemic protocol use 50% dextrose, for hypoglycemic protocol use 20% dextrose

Nembutal 35mg/kg (iv) = 217 uL of Nembutal 5mg/mL
dose uL = BW**7

Nembutal 70mg/kg (ip) = 434 uL of Nembutal 5mg/mL
dose uL = BW**14

Diluted Stock 5mg/mL
Updated: September 2016
5. Analysis of Radioactivity (when applicable)

A. Processing [3-3H]glucose in standards and plasma samples\(^1\)

\textit{i. Reagents and supplies}

Saline
Saturated benzoic acid (at least 3.4g benzoic crystals in 1L H2O), stable at RT
0.075N Ba(OH)\(_2\) solution: from 0.3N Ba(OH)\(_2\) diluted 1:3 in H2O (made day of, you need 1300\(\mu\)L/mouse)
0.075N ZnSO\(_4\) solution: from 0.3N ZnSO\(_4\) diluted 1:3 in H2O (made day of, you need 1300\(\mu\)L/mouse)  
- Important: discard stocks of Ba(OH)\(_2\) and ZnSO\(_4\) if precipitate is visible in the bottle, change stocks every 3-4 months.

1.5mL microtubes:
Standards:
- 1 tube per tracer infusate (for 1:200 dilution in benzoic acid) -> 2 tubes/mouse (“prime” + “D50”) 
- 3 CRS “prime” + 3 CRS “D50” tubes per mouse -> 6 CRS microtubes /mouse
Samples: 1 microtube per sample -> 7 tubes /mouse

20mL Borosilicate Scintillation vials:
Standards: 3 CS + 3 CSE + 3 CRS per standard (“prime” and “D50”) -> 18 vials /mouse
Samples: 2 vials per sample (dry and wet\(^2\)), 7 samples per mouse -> 14 vials /mouse

\textit{ii. First day: Sample Deproteinization}

Processing standards
1. Dilute each tracer standard 1:200 in saturated benzoic acid (5\(\mu\)L tracer in 995\(\mu\)L sat. benzoic acid)
2. Add 10\(\mu\)L of this diluted standard to three 1.5mL microtubes labeled CRS.
3. Add 10\(\mu\)L saline + 100\(\mu\)L diluted Ba(OH)\(_2\) first + 100 \(\mu\)L diluted ZnSO\(_4\) to each tube. A white precipitate will appear.
4. Mix well by inverting and leave at +4°C overnight (deproteinization step).
5. Keep the 1:200 tracer dilution at +4°C overnight (will be used for CS and CSE).

Processing plasma samples
Keep pure plasma samples on ice

---


\(^2\) To determine \(^3\)H\(_2\)O accumulation, a measure of glycolytic rate, 40\(\mu\)L of the supernatant are pipetted into a second set of vials and not dried. \(^3\)H\(_2\)O will be the difference in radioactivity between these non-dried samples and the dried samples (correcting for the difference in the volume of supernatant used).
1. Add 10µL of the plasma to a 1.5mL microtube.
2. Add 10µL saline + 100µL diluted Ba(OH)2 first + 100µL diluted ZnSO4.
3. Mix well by inverting and leave at +4°C overnight (deproteinization step). This step can be shortened to a few hours at +4°C. Consistency is recommended. The overnight step works well as the second day can be long.
4. Store the leftover clamp plasma back in -20°C or -80°C.

**iii. Second day**

Work at room temperature

- Spin down (13,000g, 5 min) the deproteinized standards and plasma samples. Do not vortex or disturb the pellet before centrifugating.

**Processing standards**

- From the deproteinized standard tracer:
  o Transfer 100µL of the deproteinized standard supernatant to each of three scintillation vials labeled **CRS** and set aside to dry later (see below).
- From the 1:200 standard dilution:
  o Add 10µL of the 1:200 standard tracer dilution to three scintillation vials labeled **CS** and three vials labeled **CSE**.
  o Add 990 H2O to the **CS** vials and cap.
  o Set aside the **CSE** vials for drying (see below).

**Processing plasma samples**

- Transfer 100µL of the deproteinized plasma supernatant to a scintillation vial labeled **Dry** and set aside to dry later (see below).
- Transfer 40µL of the deproteinized supernatant to a vial labeled **Wet**. Add 960µL H2O and cap.
- **Keep the leftover deproteinized plasma supernatant in the fridge for the glucose assay.**

**Drying standards and samples (CRS, CS and one set of plasma samples)**

- The vials set aside for drying can be dried in a vacuum oven with a H2O trap for approx. 1 hour (do not go over 1h). To set up oven:
  o Fill the inner container with dry ice.
  o Place the vials (no caps!) in the oven, close door. Turn on heat.
  o Turn on the pump, and adjust vacuum between 23 and 25 in.Hg.
  o To turn off, release the vacuum before turning off the pump.
- Once samples are dried and cooled down, add 1mL H2O to the vials.
Counting

- Dispense 10mL of scintillation fluid to all vials, cap, vortex well (5-10 sec per vial), and count with a dual labeling protocol (very important).

Suggested vial order: CS – CSE - CRS prime – CS – CSE - CRS D50 – Dry plasma samples – Wet plasma samples

Enzymatic Glucose Assay

Once vials are counting, run an enzymatic glucose assay on the remaining deproteinized supernatant (a suggested protocol follows).

**iv. Enzymatic Glucose Assay**

The glucose assay is run on the supernatant of the deproteinized plasma samples prepared earlier that day. If needed, samples can be stored overnight at +4°C and the glucose plate run the next day. It is not recommended to wait longer as glucose can be degraded quickly in the deproteinized sample. Glucose in these supernatants degrades even in the -80°C.

**Reaction Buffer: prepare on day of study.**

You need:
- 200mM Tris-HCl pH 7.4 (+4°C, stable)
- 500mM MgCl2 (RT, stable)
- ATP (fridge, Sigma #10519987001)
- NADP (fridge, Sigma #10128058001)
- combined Hexokinase + G6PDH (fridge, Sigma #10737275001)

Volumes indicated in table include standard curve + samples + 2mL extra volume.

<table>
<thead>
<tr>
<th># of mice on plate</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5 (1 plate)</th>
<th>2 plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>200mM Tris-HCL</td>
<td>9.5 mL</td>
<td>13 mL</td>
<td>16.5 mL</td>
<td>20 mL</td>
<td>24 mL</td>
<td>48 mL</td>
</tr>
<tr>
<td>500mM MgCl2</td>
<td>198 µL</td>
<td>271 µL</td>
<td>344 µL</td>
<td>417 µL</td>
<td>500 µL</td>
<td>1 mL</td>
</tr>
<tr>
<td>ATP</td>
<td>28 mg</td>
<td>38 mg</td>
<td>48 mg</td>
<td>58 mg</td>
<td>70 mg</td>
<td>140 mg</td>
</tr>
<tr>
<td>NADP</td>
<td>20 mg</td>
<td>27 mg</td>
<td>34 mg</td>
<td>42 mg</td>
<td>50 mg</td>
<td>100 mg</td>
</tr>
<tr>
<td>HK + G6PDH</td>
<td>49.4 µL</td>
<td>67.6 µL</td>
<td>85.8 µL</td>
<td>104 µL</td>
<td>125 µL</td>
<td>250 µL</td>
</tr>
</tbody>
</table>

**Glucose Standard Curve:**

BaZn diluent:
- 1 mL Ba(OH)2 + 3 mL H2O (solution A)
- 1 mL ZnSO4 + 3 mL H2O (solution B)
- Add equal volumes of A and B in microtubes and spin 5min at 13,000 g.
- Collect the supernatants for use as standard curve diluent (~ 2.3 mL will be required).

**Glucose standards:**

Sigma stock (G6918; 100 mg/dL) kept in the fridge, stable for 1 year.
Standards can be stored at -20°C.
If samples are concentrated, drop the 0.5 standard and add the 30 mg/dL standard (this is rare).

<table>
<thead>
<tr>
<th>Glucose Standard (mg/dL)</th>
<th>BaZn Diluent (µL)</th>
<th>Glucose Stock (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>250</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>398</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>297</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>245</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>285</td>
<td>15</td>
</tr>
<tr>
<td>10</td>
<td>225</td>
<td>25</td>
</tr>
<tr>
<td>15</td>
<td>255</td>
<td>45</td>
</tr>
<tr>
<td>20</td>
<td>200</td>
<td>50</td>
</tr>
<tr>
<td>30 (optional)</td>
<td>175</td>
<td>75</td>
</tr>
</tbody>
</table>

**Enzymatic Assay:**
- Glucose Standard Curve: 20 µL in duplicates.
- Samples: use the deproteinized plasma supernatant from the Somogyi procedure, centrifuge again if needed to tightly pellet the precipitate. 20 µL in duplicates.
- Add Reaction Buffer: 250 µL per well

Mix at room temperature for 15 min.
Read at 340nm (within 30 min of the end of mix).

**Example of plate setup:**

<table>
<thead>
<tr>
<th>Stds duplicates</th>
<th>Mouse 1 duplicates</th>
<th>Mouse 2 duplicates</th>
<th>Mouse 3 duplicates</th>
<th>Mouse 4 duplicates</th>
<th>Mouse 5 duplicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>-15</td>
<td>-15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>-5</td>
<td>-5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>80</td>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>90</td>
<td>90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>110</td>
<td>110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>15</td>
<td>120</td>
<td>120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
B. 2-Deoxyglucose Radioactivity (standard and plasma samples)

i. Standards
1. Directly add 196 µL water to the saved 4 µL of 2-DG Standard, vortex
2. Transfer 50 µL of this dilution to new tube, add 950 µL water, vortex
3. Transfer 100 µL of this dilution into a scintillation vial
4. Add 900 µL of water
5. Add 10 mL scintillation fluid and count

ii. Plasma Samples
1. Add 10 µL of plasma + 10 µL saline + 100 µL diluted Ba(OH)\textsubscript{2} first + 100 µL diluted ZnSO\textsubscript{4}
2. Mix well by inverting
3. Spin down 5 min at 13,000 g
4. Transfer 100 µL of supernatant to a scintillation vial
5. Add 900 µL of water
6. Add 10 mL scintillation fluid and count in liquid scintillation counter using a dual labeling protocol.

C. Tissue 2-Deoxyglucose Phosphate Radioactivity

i. Reagents and supplies
0.3N Ba(OH)\textsubscript{2} (discard Ba(OH)\textsubscript{2} stock if any precipitate is present in the bottle.)
0.3N ZnSO\textsubscript{4} (discard ZnSO\textsubscript{4} stock if any precipitate is present in the bottle.)
0.5% perchloric acid diluted in ddH\textsubscript{2}O
Liquid nitrogen
Ice
20 mL borosilicate scintillation vials
5 mL and 13 mL large polypropylene tubes w/ caps
1.5 mL tubes
5N KOH and 10N HCl
Forceps
Homogenizer
pH meter
**ii. Protocol**

1. Keep tissues and 5 mL tubes in liquid nitrogen and make sure that the end of the forceps that will come in contact with tissue is cooled in liquid nitrogen.

2. Weigh each tissue sample (~60 mg), transfer to 5 mL tube in liquid nitrogen and record weight. *Note: you will not obtain 60 mg in all tissues (e.g., soleus muscle). In those cases, simply weigh out the entire amount of tissue obtained.*

3. For each sample, place on ice the 5 mL tube containing the tissue and homogenize in 1.5 mL 0.5% perchloric acid. Keep the homogenized sample on ice while the remaining tissues are homogenized. *Note: the homogenizer can be a polytron or a bullet blender. In the latter case use 1.5 mL tubes (with 750 µL of 0.5% PCA) instead of 5 mL tubes.*

4. Centrifuge tubes for 20 min at 2,000 g at 4°C (or 15 min at 13,000 g for 1.5 mL tubes).

5. Place tubes on ice and transfer 1.25 mL of the supernatant to 13 mL tubes. If you are unable to extract 1.25 mL of supernatant, note the volume of supernatant you are able to extract.

6. Neutralize each sample (pH ~7.0) using KOH and HCl. Record the total volume of KOH and HCl used to neutralize each sample.

7. Centrifuge tubes for 10 min at 2,000 g at 4°C.

8. Label two scintillation vials (A and B) for each tissue
   - A vials (measurement of 2-deoxyglucose phosphate + 2-deoxyglucose)
     - Add 125 µL of neutralized supernatant and 875 µL ddH₂O
   - B vials (measurement of 2-deoxyglucose only)
     - Pipette 250 µL of neutralized supernatant into 1.5 mL tubes
     - Add 125 µL of stock 0.3N Ba(OH)₂
     - Add 125 µL of stock 0.3N ZnSO₄
     - Centrifuge at 16,000 g for 5min
     - Add 250 µL of supernatant and 750 µL ddH₂O to vials

9. Add 10 mL of scintillation fluid to each vial, shake and measure radioactivity in liquid scintillation counter.

10. 2-deoxyglucose phosphate is the difference between radioactivity in A and B.

---

3 For tissues that have high rates of glucose uptake per mass of tissue, 30mg of tissue is enough (muscles, heart, brown adipose, brain).
6. Considerations in Designing Insulin Clamp Experiments in the Conscious Mouse

There are numerous factors to consider when designing an insulin clamp to study the conscious mouse. Most of these factors apply to clamping any organism while others are specific to the mouse. Below is a list of general considerations.

**Considerations**

- Strain
- Genotype
- Observable phenotype
- Gender
- Breeding and other husbandry issues
- Diet
- Weight and body composition
- Age
- Insulin dose
- Define desired target glucose concentration during clamp
- Tracer application
- Sampling site
- Blood sampling volume
- Volume infused
- Main tissues and metabolic pathways of interests

Please see the following publications for experiments by the Vanderbilt MMPC addressing some of the issues listed above:

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Ayala JE, Bracy DP, Malabanan C, James FD, Ansari T, Fueger PT, McGuinness OP, Wasserman DH.  
*Hyperinsulinemic-euglycemic clamps in conscious, unrestrained mice.*  
J Vis Exp. 2011 Nov 16;(57).

Hughey CC, Wasserman DH, Lee-Young RS, Lantier L.  
*Approach to assessing determinants of glucose homeostasis in the conscious mouse.*  

Ayala JE, Bracy DP, McGuinness OP, Wasserman DH.  
*Considerations in the design of hyperinsulinemic-euglycemic clamps in the conscious mouse.*  

Berglund ED, Li CY, Poffenberger G, Ayala JE, Fueger PT, Willis SE, Jewell MM, Powers AC, Wasserman DH.  
*Glucose metabolism in vivo in four commonly used inbred mouse strains.*  
Diabetes. 57(7):1790-9, 2008.

McGuinness OP, Ayala JE, Laughlin MR, Wasserman DH.  
*NIDH Experiment in Centralized Mouse Phenotyping: The Vanderbilt Experience and Recommendations for Evaluating Glucose Homeostasis in the Mouse.*  

Wasserman DH, Ayala JE, McGuinness OP.  
*Lost in translation.*  

*Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice.*  
7. Use of Tracers to Assess Insulin Action

Isotopic tracers can be used in conjunction with the insulin clamp technique to assess sites of insulin action and insulin’s effects on specific metabolic pathways. In this section, special reference is made to two isotopes commonly used to assess glucose metabolism during an insulin clamp. These are \([3^-\text{H}]\text{glucose}\) which is used to measure whole body glucose turnover and 2-\([\text{\textsuperscript{3}}\text{H}]\text{deoxyglucose}\) (or 2-\([\text{\textsuperscript{1}}\text{4C}]\text{-deoxyglucose}\)) which is used to measure an index of tissue-specific glucose uptake. The principles introduced in this section, however, are of general applicability to other isotopes.

Living systems are in a constant state of change. Although blood glucose is maintained within narrow limits, glucose molecules that comprise the blood glucose pool are continually being exchanged with body tissues. Arterial glucose is in a dynamic steady state. The rate of tissue-blood glucose exchange varies with physiological conditions such as exercise and insulin stimulation. The rate of glucose exchange is also affected by pathological conditions, most notably diabetes.

If isotopic glucose (radioactive or stable) is injected or infused into the body, it disappears in a finite time period. In a steady state, the rate that isotopic glucose disappears is directly related to the rate at which glucose is removed. The use of isotopic glucose to measure glucose turnover requires that the isotope is:

1. Nontoxic and does not affect glucose metabolism
2. Spread evenly throughout the body glucose volume of distribution (well mixed)
3. Not recycled back to glucose once it is removed (no recirculation, irreversible loss)
4. Handled equivalently to that of unlabeled glucose (no “isotope effect”)

If the ratio of isotopic glucose to non-isotopic glucose is measured and the assumptions hold, then the mass glucose turnover rate can be quantified. The goal of this section is to explain how tracers can be used to assess the kinetics of substrates \textit{in vivo}.
A. Glossary of Terms

**Turnover**
Turnover is the renewal rate of a “substance” in a defined system and is abbreviated $R_t$. This renewal can take place in two possible ways:
1. The substance can be synthesized and metabolized in the same tissue.
2. The substance can be synthesized at a site distant from its site of metabolism.

Note: the term turnover has an unequivocal meaning only when the substance is in a steady state. Units: mg/min, µmoles/min, mg/kg/min

**Dynamic steady state (DSS)**
DSS exists when the mass of a substance in a defined system remains constant over time. In a steady state the rate of production or appearance ($R_a$) of a substance equals the rate of removal ($R_d$) of the substance.

$$R_a = R_d$$

Note: it is assumed that the volume of the pool and the dynamics of mixing in the pool in which that substance is dissolved remain constant.

**Pool**
Pool is the space in which the substance of interest is distributed. This space is assumed to be well-mixed.

**Tracee**
Tracee is the substance whose turnover is measured.

**Tracer**
Tracer is the isotopically labeled (radioactive or stable isotope) form of the tracee. Note that while principles of radioactive and stable isotopes are identical specific references in the Laboratory Manual are generally to use of radioactive isotopes.

**Specific activity (SA)**
SA is the ratio of the amount of tracer to tracee in the pool.
The units are usually expressed as dpm/mmol, dpm/g or cpm/g
Where dpm=disintegrations per min; cpm=counts per min
If stable isotopes are used, “enrichment” is defined as the ratio of tracer to tracee.
Fractional turnover rate (k)
k is the fraction of the tracee that is replaced per unit time assuming a single pool.

Half-life (t½)
Half-life is the time it takes to replace one half of the original components of the pool. k and t½ are related by the following equation:

\[ F = e^{-kt} \]

Where F= fraction of the original tracee that remains at any time (t). F=½ at t½. If we solve for t½ in terms of k:

\[ \frac{1}{2} = e^{-kt_{1/2}} \]

\[ \ln(\frac{1}{2}) = -kt_{1/2} \]

\[ -\ln(2) = -kt_{1/2} \]

\[ \frac{-\ln(2)}{k} = t_{1/2} \]

Example: if k=0.1/min, then t½=6.9 min

Volume of distribution (Vd)
Vd is an empirically derived volume (i.e., pool) that the tracee rapidly mixes in. Vd is usually very close to the true pool size if the model which best describes the kinetics of the pool is one compartment (e.g., the extracellular space). Since the body usually cannot be considered a single compartment, the Vd is only an approximation of the pool size. The units are usually mL/kg.

Clearance
Clearance is formally defined as the volume of biological fluid completely cleared of tracee per unit time. It is calculated as the ratio of R and the tracee concentration (units: mL/kg/min or mL/min). It is an index of the tracee uptake normalized by tracee concentration. Note that the ratio of clearance and Vd is equal to k if you assume a single compartment.

\[ k = \frac{Clearance}{V_d} \]
B. Methodological Approaches to Measuring Turnover with Glucose as Tracee

i. Bolus Method

To assess the $R_t$ of glucose one takes advantage of the assumption that the removal mechanism cannot distinguish between tracer and tracee. By adding an isotopic glucose tracer at time=0 and following its removal rate, the rate of removal of glucose can be assessed. Suppose an amount of isotopic glucose defined as $M^*$ is injected.

If $M^*$ mixes instantly in the pool, the cells cannot distinguish it from the tracee and the tracer is irreversibly disposed, glucose utilization rate can be calculated. The plasma concentration of the tracer will decrease in an exponential fashion.

In the figure above, it is assumed that the half-life of glucose is one minute.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>12.5</td>
</tr>
<tr>
<td>6</td>
<td>1.6</td>
</tr>
<tr>
<td>10</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Note: the curve is exponential and follows the equation $F=e^{-kt}$. Since $t_{1/2}$ = 1 min then $k=ln2=0.693$ min$^{-1}$.

To determine the amount of tracer remaining at any time $[M^*]_t$ multiply both sides of the equation by $M^*$ (the quantity of tracer injected).

$$M^*\cdot F=M^*e^{-kt}=M^*_t$$

Note: $M^*_t=M^*$ when $t=0$
Since we are in a DSS, V and total tracee mass (M) remain constant, we can divide both sides of the equation by M.

\[
\frac{M^*}{M} = M e^{-kt}
\]

M*/M is equal to the SA:

\[SA_t = SA_0 e^{-kt}\]

where \(SA_0\) is the specific activity at time = 0 which is obtained by extrapolating back to zero time. Since \(SA_0=M^*/M\) and \(M^*\) is known (the amount of tracer we injected) we can calculate \(M\). \(k\) is calculated by determining the slope of the line plotting \(\ln(SA_t)\) vs. time.

\(R_t\) is equal to the product of \(k\) and \(M\).

\[R_t = k \cdot M\]

**Assumptions:**
1. DSS
2. Tracer acts like tracee (note: beware of tracer contaminants)
3. Mass of tracer is negligible compared to the mass of the tracee in the pool
4. Instantaneous mixing of tracer and tracee
5. One compartmental model (homogeneity of the pool)
6. No recirculation of isotope
7. Loss of tracer reflects irreversible loss of tracee

**Summary:** A series of plasma samples (dpm/mL plasma) are collected over time after the injection of \(M^*\). If the data are plotted as the natural log of dpm over time, the slope is equal to \(k\). The total amount of radioactivity injected (\(M^*\)) is divided by the y-intercept to calculate \(V_d\). \([V_d (mL) = (dpm\ injected)/(dpm/mL\ at\ time\ zero)]\). \(k\) and \(V_d\) are then used to calculate substrate turnover and clearance. While the bolus technique seems easy, it has its limitations. If the bolus technique is used to estimate \(R_t\), a number of samples over a short time must be taken to obtain an accurate curve fit. Only one estimate of \(R_t\) is obtained.
ii. Constant Infusion Method

a. Steady State Conditions

In = Out

Tracer entry is equal to the rate of tracer exit in a steady state. The ratio of the rates of tracer and tracee exiting the system is proportional to the ratio of the tracer to the tracee (i.e., SA) since the body treats them equivalently.

\[ R_a^* = \frac{A^*}{A} \cdot R_a \]

\[ R_a^* = I = R_d^* \]

\[ SA_a = \frac{A^*}{A} \]

Where

- \( I \) = tracer infusion rate
- \( A^* \) = tracer concentration
- \( A \) = tracee concentration
- \( R_a \) = rate of tracee appearance
- \( R_d \) = rate of tracee disappearance
- \( R_a^* \) = rate of tracer appearance = tracer infusion rate = \( I \)
- \( R_d^* \) = rate of tracer disappearance
- \( SA_a \) = specific activity of tracee

Therefore

\[ \frac{SA}{R_a^*} = I = \frac{A^*}{A} \cdot R_a \]

simplify

\[ R_a^* = \frac{I}{SA} \]

Note: in a steady state \( R_t \) is independent of the size or complexity of the pool.

Assumptions:
1. Steady state
2. Tracer acts like tracee
3. No recycling of isotope
4. Loss of tracer reflects irreversible loss of tracee
5. Tracer is infused in trace amounts relative to the amount of tracee
6. The SA of the pool where blood samples are obtained is equal to the specific activity at the site of utilization
A strength of this approach is that one can get multiple estimates of $R_t$ by taking serial samples of specific activity in the plasma pool.

**b. Priming the Pool**

The goal of a primer is to expedite the time required for SA to obtain a steady state associated with a constant tracer infusion. If a bolus of tracer is given, the peak SA occurs right after the tracer is injected ($SA_0$). The SA would fall in an exponential manner following the equation:

$$SA_t = SA_0 e^{-kt}.$$  

If only a constant tracer infusion is given the SA would gradually rise until a steady state is reached. In the adjacent figure, steady state SA is 500 dpm/mg. The rate of rise in SA will follow the equation :

$$SA_t = \left( \frac{I}{M \cdot k} \right) \left( 1 - e^{-kt} \right)$$

[in the adjacent figure this is the “infusion” line]. Note that in a steady state the equation simplifies to the steady state equation. $SA = I/(M \cdot k)$. This is a rearrangement and substitution of the equation $R_t = I/SA$ where $M \cdot k$ was substituted for $R_t$; $M \cdot k = R_t$.

The primer is calculated with the objective that $SA_0 =$ steady state SA. To do this you would give the prime and at the same time start the constant tracer infusion. If you give the correct amount of prime for the given infusion rate the specific activity would be constant. [in the adjacent figure this is the “infusion +prime” line] For $SA_t = SA_0$ to be true the sum of the equations for the prime and constant infusion must equal the steady state equation at any point in time:

$$SA_0 e^{-kt} + \left( \frac{I}{M \cdot k} \right) \left( 1 - e^{-kt} \right) = \frac{I}{M} k$$

The equation can be simplified to:

$$SA_0 \left( \frac{M}{I} \right) = \frac{1}{k}$$
A sound estimate of the prime (P) required to fill the system can be obtained from the preceding relationship. Since $SA_0 \cdot M$ is equal to the amount of prime this equation simplifies to:

$$\frac{P}{I} = \frac{1}{k}$$

$k$ is the fraction of the pool that turns over per unit time. There are generally realistic literature values for $k$ that can be used or $k$ can be estimated from $R_t$ and $V_d$ measured in the laboratory. Thus, the appropriate prime (P) can be calculated once an appropriate constant tracer infusion rate (I) is chosen with an estimate of $k$.

Examples of values in mouse and human for measurement of glucose turnover:

<table>
<thead>
<tr>
<th>Units</th>
<th>Human</th>
<th>mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_d$</td>
<td>mL/kg</td>
<td>200</td>
</tr>
<tr>
<td>$R_t$ (glucose)</td>
<td>mg·kg·min⁻¹</td>
<td>2</td>
</tr>
<tr>
<td>clearance</td>
<td>mL·kg·min⁻¹</td>
<td>100</td>
</tr>
<tr>
<td>$k$</td>
<td>1/min</td>
<td>0.01</td>
</tr>
<tr>
<td>$t_\frac{1}{2}$</td>
<td>min</td>
<td>69.31</td>
</tr>
<tr>
<td>$k^{-1}$</td>
<td>min</td>
<td>100</td>
</tr>
<tr>
<td>$I$</td>
<td>dpm·kg·min⁻¹</td>
<td>1000</td>
</tr>
<tr>
<td>$SA$</td>
<td>dpm/mg</td>
<td>500</td>
</tr>
</tbody>
</table>

If the subject is diabetic and the glucose concentration is 200 mg/dL with comparable $R_t$ (2 mg·kg⁻¹·min⁻¹) a larger bolus (200 min; 200,000 dpm/kg) will be required. Since the exact $R_t$ in any given study will not be known beforehand, historical data and experience can be used to gain a reasonable approximation.

*Note: $V_d$ is unique for each tracee. In the example above we assumed the $V_d$ of glucose was 200 mL/kg. Investigators in the past have used a range from 130 to 200 mL/kg. The $V_d$ for fatty acids and amino acids will be different than that of glucose.*
**iii. Choosing the Appropriate Tracer**

The most commonly used radioactive tracer for glucose is\(^{[3-\text{H}]}\)glucose. When \(^{[3-\text{H}]}\)glucose is metabolized it exchanges \(^3\text{H}\) with \(\text{H}_2\text{O}\) when it passes through the triose phosphate step in glycolysis. The advantage of this tracer is that it is not recycled into glucose (via gluconeogenesis) and the \(^3\text{H}_2\text{O}\) can be removed from samples by evaporation. In contrast, \(^{[14\text{C}]}\)glucose tracers recycle and their use will lead to an underestimation of glucose turnover. \(^{[2-\text{H}]}\)glucose has been used, but this tracer is problematic due to futile cycling between glucose and glucose 6-phosphate in the liver. This leads to a rapid loss of \(^3\text{H}\). This will result in an overestimate of glucose flux to the extent that futile cycling is occurring. Some investigators will, in fact, infuse both \(^{[2-\text{H}]}\)glucose and \(^{[3-\text{H}]}\)glucose and measure the difference in the two estimates of \(R_t\) as a measure of hepatic futile cycling.

**iv. Non-Steady State Analysis of Tracee Flux**

If a non-steady state exists, then either or both the amount of tracer (\(M^*\)) or tracee (\(M\)) in the pool is changing. This can be expressed mathematically by taking the derivative of both sides of the equation \(M^* = SA \cdot M\).

1) \[
\frac{\partial M^*}{\partial t} = SA \frac{\partial M}{\partial t} + M \frac{\partial SA}{\partial t}
\]

2) \[
R_a - R_d = \frac{\partial M}{\partial t} = V_a \cdot \frac{\partial A}{\partial t}
\]

Note the implication of Equation 2 is that when \(R_a\) is not equal to \(R_d\) (IN \(\neq\) OUT) then the mass of the tracee (e.g. glucose) will change (i.e. \(dM/dt \neq 0\)).

3) \[
\frac{\partial M^*}{\partial t} = I - R_d \cdot SA
\]

The implication of Equation 3 is that when the tracer infusion rate (\(I\)) is not equal to the rate the tracer is leaving the body (IN \(\neq\) OUT), then the radioactivity of the tracer (e.g. \(^{3}\text{H}\)glucose) will change (\(\partial M^*/\partial t \neq 0\)).
Substitute Equations 2 and 3 in Equation 1:

\[ I - R_d \cdot SA = SA(R_a - R_d) + M \frac{\partial SA}{\partial t} \]

Solve for I:

\[ I = R_d \cdot SA + SA(R_a - R_d) + M \frac{\partial SA}{\partial t} \]

Simplify:

\[ I = R_a \cdot SA + M \frac{\partial SA}{\partial t} \]

Solve for \( R_a \):

\[ R_a = \frac{I - M \frac{\partial SA}{\partial t}}{SA} \]

Since \( M = V_d \cdot A \), where \( V_d \cdot A \) are the volume of distribution and concentration of the tracee, then

\[ R_a = \frac{I - V_d \cdot A \frac{\partial SA}{\partial t}}{SA} \]

Since \( R_a - R_d = V_d \cdot \frac{\partial A}{\partial t} \), the following relationship can be calculated:

\[ R_d = R_a - V_d \cdot \frac{\partial A}{\partial t} \]

Note if in a steady state \( \frac{\partial SA}{\partial t} = 0 \) then \( R_a = I/SA \).

\[ M = V_d \cdot A \]

Non-steady state equations calculate \( R_a \) and \( R_d \) over a time interval and not at discrete points. In the equations above, the mean \( SA \) and mean \( A \) for a given time interval are used for the defined time interval. Non-steady state equations assume a single compartment of known size \( (V_d) \).
Example:

<table>
<thead>
<tr>
<th>time (min)</th>
<th>[glucose] mg/dL</th>
<th>[(^{15})H-glucose] dpm/mL</th>
<th>I (dpm·kg(^{-1})·min(^{-1}))</th>
<th>SA (dpm/mg)</th>
<th>Rt (mg·kg(^{-1})·min(^{-1}))</th>
<th>Vd (mL/kg)</th>
<th>time (interval)</th>
<th>dA/dt (mg·mg(^{-1})·min(^{-1}))</th>
<th>dSA/dt (dpm·mg(^{-1})·min(^{-1}))</th>
<th>Ra (mg·kg(^{-1})·min(^{-1}))</th>
<th>Rd (mg·kg(^{-1})·min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>-10</td>
<td>100</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>10</td>
<td>200</td>
<td>(-10 - 0)</td>
<td>0.00</td>
<td>0.00</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>10</td>
<td>200</td>
<td>(0 - 20)</td>
<td>0.01</td>
<td>-16.7</td>
<td>16.4</td>
<td>14.4</td>
</tr>
<tr>
<td>20</td>
<td>120</td>
<td>800</td>
<td>1000</td>
<td>667</td>
<td>15</td>
<td>200</td>
<td>(20 - 40)</td>
<td>0.00</td>
<td>0.00</td>
<td>15.0</td>
<td>15.0</td>
</tr>
<tr>
<td>40</td>
<td>120</td>
<td>800</td>
<td>1000</td>
<td>667</td>
<td>15</td>
<td>200</td>
<td>(40 - 60)</td>
<td>-0.01</td>
<td>0.00</td>
<td>15.0</td>
<td>17.0</td>
</tr>
<tr>
<td>60</td>
<td>100</td>
<td>667</td>
<td>10000</td>
<td>667</td>
<td>15</td>
<td>200</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A steady state exists between -10 and 0. Thus, R\(_t\) at -10 and 0 min is equal to the R\(_a\) and R\(_d\) for that interval. For interval 0 to 20 min, glucose levels rose but [\(^{15}\)H]glucose concentration and SA fell. One can see that steady state equations give an overestimate. By calculating R\(_a\) and R\(_d\) using non-steady state equations, it can be seen that glucose levels rose because R\(_a\) rose more than R\(_d\). After an initial non-steady state interval glucose concentration and SA stabilize such that R\(_a\)=R\(_d\)=R\(_t\). Between 40 and 60 min the steady state equation predicts that R\(_t\) did not change even though glucose falls (of course this cannot be true). Non-steady state equations, reveal that the fall in glucose is due to a large increase in R\(_d\), while R\(_a\) does not increase. It is evident that both the SA and the tracee concentration must be constant for the estimate of R\(_t\) to be valid.

Assumptions:
1. Well mixed *single* pool
2. Irreversible disposal
3. Mass of tracer is negligible
4. Tracer behaves like tracee
5. The SA of the pool that is sampled is equal to the SA at the site of utilization.
v. Implications of Non-Steady State

During an insulin clamp total glucose flux increases and SA decreases (remember $\uparrow R_t = \frac{f}{\downarrow SA}$). Endogenous glucose production ($\text{EndoR}_a$) is calculated as the difference between $R_a$ and the exogenous glucose infusion rate. It takes more than 80 min to reach a new steady state during an insulin clamp in humans. If steady state calculations are used prior to 80 min to estimate total glucose turnover the total $R_a$ would be underestimated. The underestimation of $R_a$ would lead to an overestimate of the suppression of $\text{EndoR}_a$ by insulin. In fact, negative estimates of $\text{EndoR}_a$, which are theoretically impossible, may be calculated. If non-steady state equations are used, more accurate estimates of $R_a$ as well as $R_d$, are obtained. However, non-steady state equations can yield inaccurate results if the assumption of a single well-mixed pool is not valid and changes in the rate of glucose infusion are required. The best way to perform the clamp is to: 1) minimize changes in SA and 2) take multiple SA measurements so that non-steady state equations can be optimally applied.

Changes in SA can be minimized by using one of two approaches. One can infuse extra tracer when the clamp is started to minimize the fall in SA. Alternatively, tracer can be mixed with exogenous glucose to obtain an infusion syringe SA that reflects basal SA. This method causes the tracer infusion rate to be varied with glucose infusion rate. This is the method currently employed by the VMMPC.
vi. Estimating Indices of Tissue Specific Glucose Uptake

Isotopic 2-deoxyglucose has been validated as a tracer and been used to derive indices of rates of glucose uptake in a variety of tissues (e.g., skeletal muscle, heart, brain). A bolus of trace quantities of radioactive 2-deoxyglucose (2-DG) is given and a series of blood samples are taken over the course of 20 to 40 min. Tissues are then collected to determine how much radioactive 2-deoxyglucose-6-phosphate (2-DGP) has accumulated in the tissues of interest. Tissue-specific glucose clearance is quantitated by dividing tissue 2-DGP radioactivity by the integrated area of the plasma radioactivity - time curve of 2-DG. This is then normalized to a rate of glucose uptake by multiplying by the average glucose concentration during the decay period.

The tissue-specific clearance of [2-3H]DG, or \( K_g \), and the metabolic index, or \( R_g \) are calculated as described below.

\[
K_g = \frac{[2-3^H]DGP_{\text{muscle}}}{\text{AUC}[2-3^H]DG_{\text{plasma}}}
\]

\[
R_g = K_g \cdot \text{Glu} \cos e_{\text{plasma}}
\]

In the example above where [2-3H]DG is used as isotope. [2-3H]DGP\text{muscle} radioactivity in the muscle is in dpm/g, AUC[2-3H]DG\text{plasma} is the area under the plasma [2-3H]DG decay curve calculated using the trapezoid method (dpm/mL-min), and Glucose\text{plasma} is the average plasma glucose (mM or mg/dL) during the decay period. Typical units for \( K_g \) and \( R_g \) are mL·100g\(^{-1}\)-min\(^{-1}\) and \( \mu \text{mol}·100\text{g}^{-1}\cdot\text{min}^{-1} \) or \( \mu \text{g}·100\text{g}^{-1}·\text{min}^{-1} \), respectively.
You can use this method along with [3-3H]glucose during a clamp to measure both whole body glucose turnover and tissue glucose uptake. However, you will have to use 2[1-14C]deoxyglucose ([14C]DG) instead of [2-3H]deoxyglucose. The processing of the samples and the calculations will be the same whether you use [2-3H]DG or [14C]DG. However, if both [3-3H]glucose and [2-3H]deoxyglucose are used in the same study, the plasma and tissue samples will have multiple 3H containing species which the scintillation counter cannot discriminate between, making the data uninterpretable.

The assumption is that 2-DG can be transported and phosphorylated, but not further metabolized. Thus any 2-DGP that is made by the tissue is trapped. This method is invalid for tissues that contain glucose-6 phosphatase (liver, renal cortex, and possibly intestine) since 2-DGP is not effectively “trapped.” 2-DG is not an ideal tracer if glucose transporters or hexokinases differentiate between 2-DG and glucose. If this is the case, the clearance rate of 2-DG (Kg) will not equal glucose clearance rate. A factor has been used in some studies to correct for differences in the transport and phosphorylation of 2-DG and glucose. This factor is called the lumped constant (LC). LC in muscle and brain are very close to 1 and do not seem to be affected by insulin.

Suggested Reading:

Kraegen EW, James DE, Jenkins AB, Chisholm DJ:  
*Dose-response curves for in vivo insulin sensitivity in individual tissues in rats.*  

De Boisblanc BP, Dobrescu C, Skrepnik N, Nelson S, Spitzer JJ, Bagby GJ:  
*Compartmentalization of glucose utilization after intravenous vs. intratracheal challenge with LPS. Am J Physiol* 270:L452-8,1996.

Wolfe RR, Chinkes DL.:  
*Isotope Tracers in Metabolic Research: Principles and Practice of Kinetic Analysis,*  
**Example for Calculating Glucose Flux in Mice**

The following information is needed to calculate glucose turnover:

A. **Body weight of mouse (grams)**

B. **Glucose infusion rate** must be recorded throughout the study (mg/kg/min)

C. **Tracer infusion rate**:
   1. A precise pump rate (µL/min)
      
      Save some of the infusate and dilute 1:200. Add 10 µL to scintillation vial (in triplicate). Dry down and reconstitute in water and scintillation fluid. This vial is labeled CSE (Chemical Standard Evaporated), you can also pipette some and not evaporate it to see if there is some ³H₂O in the tracer (Chemical Standard). A portion of the diluted infusate (10 µL) is processed in an identical manner as the plasma samples (see below). This is the Chemical Recovery Standard (CRS).

If you are giving exogenous glucose during a clamp study to which [3-³H]Glucose is added, a separate set of standards (CSE and CRS) must be prepared using the glucose containing infusate as well.

D. **Specific activity of plasma glucose at each time point**
   1. **Plasma glucose concentration (mg/dL plasma)**: Note that the blood and plasma concentration may not be the same and that plasma values are needed to calculate glucose fluxes. The Beckman glucose analyzer, which uses plasma, is not practical to use, as it requires taking a large blood sample (~20 µL). The Hemocue (which uses blood) gives a reading very similar to the Beckman which measures “plasma” glucose but still requires ~7 µL blood. Note other blood glucose meters are designed for human not rodent blood and are not accurate enough to assess “plasma” glucose. AlphaTRAK® glucose meters that are calibrated specifically for mice, while more accurate than the meters designed for humans, are still not optimal for a tracer study. Vanderbilt MMPC measures the glucose concentration in the Ba(OH)₂ and ZnSO₄ supernatant using an enzymatic assay.

   2. **Plasma [3-³H]glucose concentration: (dpm/µL plasma)** Plasma samples will have to be treated with Ba(OH)₂ and ZnSO₄, evaporated and counted. Multiply plasma radioactivity by the CSE to CRS ratio to correct for dilutions and assay recovery.
Example calculations

1. **Whole Body Glucose Fluxes**

   Body weight: 36.2 g

   **Baseline (-90 to 0 min):**
   - CSE: 5536 dpm (10 µL of diluted 200-fold baseline infusate)
   - CRS: 2520 dpm (10 µL of infusate added to Ba(OH)$_2$ and ZnSO$_4$
   - CSE/CRS = 2.20
   - Pump rate of tracer: 1.5 µL/min -90 to 0 min

   **Clamp (80 to 120 min):**
   - CSE: 4911 dpm (10 µL of diluted 200-fold glucose infusate)
   - CRS: 2219 dpm (10 µL of infusate added to Ba(OH)$_2$ and ZnSO$_4$
   - CSE/CRS = 2.21
   - Pump rate of tracer: 2.34 µL/min 80 to 120 min (average glucose infusion rate from 80 to 120 min). Use the actual rate at each time point in the calculations.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Glucose mg/dL</th>
<th>Plasma [3-3H] glucose dpm</th>
<th>Glucose infusion rate mg/kg/min</th>
<th>Glucose pump infusion rate µL/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>103</td>
<td>1852</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>80</td>
<td>117</td>
<td>981</td>
<td>28</td>
<td>2.2</td>
</tr>
<tr>
<td>90</td>
<td>117</td>
<td>1018</td>
<td>28</td>
<td>2.2</td>
</tr>
<tr>
<td>100</td>
<td>121</td>
<td>1048</td>
<td>31</td>
<td>2.44</td>
</tr>
<tr>
<td>110</td>
<td>121</td>
<td>1053</td>
<td>31</td>
<td>2.44</td>
</tr>
<tr>
<td>120</td>
<td>121</td>
<td>1067</td>
<td>31</td>
<td>2.44</td>
</tr>
</tbody>
</table>

   \[
   \text{Tracer infusion rate (dpm/kg/min)} = \frac{\text{Pump rate} \times \text{CSE} \times 200}{10} \left(\frac{\text{Body weight t}}{1000}\right)
   \]

   \[
   \text{Specific activity (dpm/mg)} = \frac{\text{plasma (dpm)} \times \frac{\text{CSE}}{\text{CRS}} \times 1000}{10 \times \frac{\text{plasma glucose}}{100}}
   \]

   \[
   \text{Glucose turnover (Rt; mg/kg/min)} = \frac{\text{Tracer infusion rate (dpm/kg/min)}}{\text{Plasma glucose specific activity (dpm/mg)}}
   \]
Endogenous glucose production (Endo $R_t$) = glucose turnover rate - exogenous glucose infusion rate

<table>
<thead>
<tr>
<th>Time (Min)</th>
<th>Plasma Glucose (mg/dL)</th>
<th>Plasma $[^3]$H glucose dpm</th>
<th>Glucose infusion rate (mg/kg/min)</th>
<th>Tracer infusion rate (dpm/kg/min)</th>
<th>SA (dpm/mg)</th>
<th>$R_t$ (mg/kg/min)</th>
<th>Endo$R_t$ (mg/kg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>103</td>
<td>1852</td>
<td>0</td>
<td>4,587,845</td>
<td>395,002</td>
<td>11.6</td>
<td>11.6</td>
</tr>
<tr>
<td>80</td>
<td>117</td>
<td>981</td>
<td>28</td>
<td>5,969,171</td>
<td>185,565</td>
<td>32.2</td>
<td>4.2</td>
</tr>
<tr>
<td>90</td>
<td>117</td>
<td>1018</td>
<td>28</td>
<td>5,969,171</td>
<td>192,564</td>
<td>31.0</td>
<td>3.0</td>
</tr>
<tr>
<td>100</td>
<td>121</td>
<td>1048</td>
<td>31</td>
<td>6,620,354</td>
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<td>1067</td>
<td>31</td>
<td>6,620,354</td>
<td>195,160</td>
<td>33.9</td>
<td>2.9</td>
</tr>
</tbody>
</table>

2. **Tissue Specific Glucose Uptake**

At 120 min a bolus of $[^14]$C2DG was given. Plasma samples were collected for the subsequent 25 min. Tissues were collected at 145 min and analyzed for $[^14]$C2DG-P.

<table>
<thead>
<tr>
<th>Time</th>
<th>Glucose (mg/dL)</th>
<th>$[^14]$C2DG dpm</th>
<th>AUC (dpm·min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>122</td>
<td>136</td>
<td>4648</td>
<td>51587.5</td>
</tr>
<tr>
<td>125</td>
<td>142</td>
<td>2257</td>
<td></td>
</tr>
<tr>
<td>130</td>
<td>139</td>
<td>2879</td>
<td></td>
</tr>
<tr>
<td>135</td>
<td>119</td>
<td>1907</td>
<td></td>
</tr>
<tr>
<td>145</td>
<td>130</td>
<td>1378</td>
<td></td>
</tr>
</tbody>
</table>

Integrated plasma $[^14]$C2DG SA (dpm/μg) = \[
\frac{AUC \cdot 220}{100 \cdot 10} \]

\[
\text{Glucose avg (mg/dl) } \cdot 0.01 \cdot \text{time}
\]

Where AUC is the area under $[^14]$C2DG curve period. When multiplied by 220 and divided by 1000 it will have units of (dpm/μL)*min. AUC is determined by trapezoidal rule. Time is the duration the AUC is measured. For example samples taken between 122 and 145 min would be a period of 23 min.

The rate of tissue glucose uptake (Rg)

\[
R_g = \frac{[^14]C2DGP \text{ in tissue}}{\text{Tissue weight} \cdot \text{Plasma}[^14]C2DGSA \cdot \text{time}} = \text{ug glucose·mg tissue}^{-1} \cdot \text{min}^{-1}
\]
Multiply by 555 to convert to μmol glucose·100 g tissue⁻¹·min⁻¹

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Weight mg</th>
<th>Volume of Base μL</th>
<th>Volume of Acid μL</th>
<th>A dpm</th>
<th>B dpm</th>
<th>A-B dpm</th>
<th>Tissue dpm/mg tissue</th>
<th>Plasma [¹⁴C] 2DG SA dpm/ug</th>
<th>Rg μg/min/mg tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soleus</td>
<td>22</td>
<td>16</td>
<td></td>
<td>817</td>
<td>321</td>
<td>496</td>
<td>6028</td>
<td>274</td>
<td>370</td>
</tr>
<tr>
<td>Gastro</td>
<td>61</td>
<td>16</td>
<td></td>
<td>2448</td>
<td>638</td>
<td>1810</td>
<td>21998</td>
<td>361</td>
<td>370</td>
</tr>
<tr>
<td>Vastus L.</td>
<td>63</td>
<td>16</td>
<td>45</td>
<td>3860</td>
<td>842</td>
<td>3018</td>
<td>37983</td>
<td>603</td>
<td>370</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>60</td>
<td>16</td>
<td>30</td>
<td>5911</td>
<td>1620</td>
<td>4291</td>
<td>53387</td>
<td>890</td>
<td>370</td>
</tr>
<tr>
<td>Heart</td>
<td>63</td>
<td>16</td>
<td></td>
<td>12674</td>
<td>2140</td>
<td>10534</td>
<td>128026</td>
<td>2032</td>
<td>370</td>
</tr>
<tr>
<td>Adipose</td>
<td>55</td>
<td>16</td>
<td></td>
<td>330</td>
<td>105</td>
<td>225</td>
<td>2735</td>
<td>50</td>
<td>370</td>
</tr>
<tr>
<td>Brain</td>
<td>65</td>
<td>16</td>
<td>41</td>
<td>3498</td>
<td>559</td>
<td>2939</td>
<td>36876</td>
<td>567</td>
<td>370</td>
</tr>
</tbody>
</table>

Total amount of radioactivity in the homogenized tissue: Tissue (dpm)

\[
Tissue(dpm) = \left(\frac{measured(dpm) \cdot (1250 + Va + Vb)}{125}\right) \times \frac{1500}{1250}
\]

Where Va is volume (µL) of acid needed to neutralize PCA extract
Vb is volume (µL) of base needed to neutralize PCA extract
Appendix A. Suggested Equipment, Tools and Reagents

The list below is a suggestion of items that are not easy to locate or where experience has indicated that a specific vendor is preferred.

**Tracers**
- [3-³H]-Glucose. Perkin Elmer. NET331C
- 2-[¹⁴C]-deoxy-glucose. Perkin Elmer. NEC 495 or 2-[¹⁴C]-deoxyglucose NEC 720A

**Surgical Tools**
- **Forceps, Dumont 7b curved shank.** Fine Science Tools # 11270-20
- **Hemostats.** Fine Science Tools # 13010-12
- **Micro-serrefines.** Fine Science Tools # 18055-03 (straight); 18055-05 (curved)
- **Needle holder.** Fine Science Tools # 12060-01
- **Spring scissors.** Fine Science Tools # 15003-08
- **Scissors, surgical.** Fine Science Tools # 14058-09
- **Forceps.** Roboz # RS-5136. RS-5110. RS-5132. RS-5111.
- **Cautery.** Cardinal Health # 65410-010

**Miscellaneous Equipment**
- **Glue, silicone.** Factor II, Inc. Medical Silicone Adhesive A-100
- **File, general purpose X slim taper file.** Ace Hardware
- **Swivel, dual channel.** Instech # 375/D/22LT
- **Connector, 3-way.** Ziggy’s Tubes and Wires # HSCY-25 or Instech SCY25
- **Connector, 4-way.** Ziggy’s Tubes and Wires # HSC4-25 or Instech SCX25
- **Blunt needle with luer hub.** Ziggy’s Tubes and Wires # LHN-E011041 25ga x 0.5” or Instech LS25
- **Wire, stainless steel, for plugs.** Ziggy’s Tubes and Wires # W020304V
- **Clamp, extension.** Fisher Scientific # 05-769-7Q
- **Connector, hook.** Fisher Scientific # 14-666-18Q
- **Stand, support.** Fisher Scientific # 14-670A
- **Pump, single channel infusion.** Harvard Apparatus # 70-4500 (Instech HA1100)
- **Heating pad.** Harvard Apparatus # 72-0494
**Tubing**

Stainless steel, 25ga, for connectors. Ziggy’s Tubes and Wires # 25R304-36 or Instech SC25/10, precut 10mm

Microrenathane. Braintree Scientific # MRE-033

Silastic tubing 0.012”i.d. Fisher Scientific # 11-189-14 or Instech BTSIL-025

Silastic tubing 0.020”i.d. Fisher Scientific # 11-189-15A or Instech BTSIL-027

Silastic tubing 0.058”i.d. Fisher Scientific # 11-189-15E

Silastic tubing 0.078”i.d. Fisher Scientific # 11-189-15H

Polyethylene tubing (PE-10). Fisher Scientific # 14-170-12P or Instech BTPE-10

Polyethylene tubing (PE-25). Instech # BTPE-25

**Miscellaneous Chemicals**

0.3 N Ba(OH)$_2$. Sigma # B4059

0.3 N ZnSO$_4$. Sigma # Z2876

Benzoic Acid Sigma # 242381

ATP Sigma # 10519987001

NADP Sigma # 10128058001

Hexokinase + Glucose-6-Phosphate Dehydrogenase (combined) Sigma # 10737275001

Glucose Standard Solution Sigma # G6918

Alteplase Cathflo® Activase® # NDC 50242-041-64
Appendix B. Checklist for Preparing a Clamp Study

Day before experiment:
- Dry down tracers
- Make sure Insulin, D50, Saline (including heparinized), EDTA tubes, timers, glucose strips, glucose meters, liquid nitrogen and donor mice are available
- Prepare blunt needles
- Check swivels and connectors
- Cut tubing
- Set up pumps
- Prep and label tubes (EDTA, plasma, tissue, insulin, etc.) and hematocrit tubes
- Connect blunt needles to syringes
- Make sure mouse buckets are ready with clean bedding for fasting
- Prepare tissue collection station with tools, tubes, coolers, etc.

Day of experiment:
- Weigh mouse, start fast, collect donor mouse
- Check the mouse’s catheter lines with Hep-Saline
- Enter mouse weight in GIR-Pump rate and Insulin prep spreadsheet and print them out
- Check swivels and connectors, attach cut tubing to appropriate ports
- Prepare donor blood
- Reconstitute tracers
- Dilute insulin according to mouse weight
- Prepare infusates in syringes (donor blood, tracers, insulin, D50), place in pumps and connect to tubing
- Run tracer, saline or Hep-Saline through the correct lines, make sure there are no bubbles
- Connect the mouse to the swivel
- After 3 h 30 min of fasting, start [3-3H] glucose tracer, start timer from 0
- At 75 and 85 min, take basal samples
- At 90 min, start the donor blood, insulin and D50-3H glucose. When the blood gets to the mouse, reset the timer to 0
- Clamp the mouse
- Make sure you save some of each tracer infusate to determine standards (freeze at -20°C or -80°C with plasma samples)