BriteVu® perfusions can be divided into 5 distinct phases:

1. Subject Preparation
2. Vascular Flush
3. BriteVu® Mix Preparation
4. Subject BriteVu® Perfusion
5. Post-Perfusion Tissue Handling

Protocols are highly variable and based on the subject being studied and research goals. The following guidelines should give researchers a good starting point with terminal contrast perfusions using BriteVu® products. Protocols tailored towards specific species are outlined in the following links:

Bird perfusions
Mouse perfusion
Rat perfusions

Possible Materials Needed

- BriteVu® in a Bottle
- BriteVu® 170 g
- BriteVu® Enhancer
- BriteVu® Special Projects

- Catheters (14-26 gauge IV catheter or 21 to 25 gauge butterfly catheter) and trocars (large subjects only)
- Coloring agents (water soluble food colors, fluorescein dye, etc)
- Cotton tipped applicators
- Dissection kit: mosquito hemostats, forceps, iris scissors, needle holders
- General anesthetics (Isoflourane, Sevoflourane), injectables, etc)
- Glass beaker with plastic coated magnetic stirring bar
- Heparin 1000 U/ml
- Hot plate with magnetic spinner
- IV catheter line (standard size)
- Mixing hot plate
- Needles (18-30 gauge)
- Physiologic solution (9% NaCl, PBS, LRS, etc)
- Radiolucent tape (ie: 3M Transpore)
- Solution
  -Distilled water
  -Phenol
- Spring Clamp Work-holders
- Suture material (3-O' to 5-O' for small subjects and 2-0' or larger for big subjects)
- Syringes (1-60 cc)
- Thermometer (lab grade)
- Tissue or 'Super' glue

1. Subject Preparation

The key to a good contrast perfusion is ensuring a clear pathway for BriteVu® mix to fill the vasculature, airways or other region being studied. As with all perfusions, BriteVu® mix will follow the path of least resistance when perfused into a system. Obstructed pathways may result in suboptimal filling with BriteVu® mix.

Preventing or removing clots and clearing the blood from the circulatory system is essential for vascular perfusions. We have found it best to give live subjects 1000 U of heparin (IV, IP or SQ) per 1 Kg of body weight 30 minutes prior to flushing blood. Subjects should also be kept at their preferred optimal temperature zone or higher (reptiles, amphibians and fish) or normal body temperature (mammals and birds) to ensure a normally functioning circulatory system.

Cadavers, museum specimens and other deceased subjects often require special handling to remove clots from the circulatory system.

2. Vascular Flush

As mentioned above, a good vascular flush is essential to a thorough BriteVu® mix perfusion.

Exsanguination/Flushing Solution

Use warmed (to normal body temperature or slightly higher) 0.9% NaCl, PBS or other physiologic solution. This solution will be used to flush the vasculature and exsanguinate the animal/tissue.

Generally, 30-40% volume/weight of flushing solution is used. For example, a 1 Kg subject will need 300-400 ml of flushing solution. Blood exiting the subject should be pink tinged by the end of a successful flush. The more blood that is cleared from the vascular system, the better the perfusion with BriteVu® mix.

As the flushing solution goes in the subject, it must come out somewhere. Ideally, make the exit site (a cut vessel for example) at a location distant from your entrance site. This will help ensure that that vasculature is flushed throughout the body rather than a small section. The exit site should be at least as large as the catheter or trocar delivering the flush solution. Exits sites that are too large (4-6 + times the entrance site) may drop systemic blood pressure and prevent a thorough flush and subsequent perfusion.

No one flush fits all subjects. See variations listed below and on the species specific white papers.

***Small Rodents (mice, baby rats, hamsters, etc) and cardiac perfusions**: Small rodents may require up to 3 times their body weight in fluids (3 L per Kg of body weight) to remove blood from the vascular system. Also, cardiac perfusions (left ventricular puncture) and drainage (right auricle laceration) often require the same high volume of flush on most any animal.

****Embryonic Animals**: Many do not have clotting factors established. As a result, heparin may not be needed. Estimate the size of the embryo and flush with 30-50% of its estimated body weight.

***Young (developing) animals**: Young animals may have increased blood supply compared to adults and may require more (towards 40% volume/weight) flushing solution.

****Reptiles and amphibians**: Reptiles tend to have lower blood volumes than mammals and birds and may only need 20-30% volume/weight.

*****Cadavers**: Generally, cadavers have clotted blood and may require large volumes of flushing solution. Conversely, some cadavers require relatively little flushing solution to remove the clots (especially if already prepared by other methods). The amount of flushing solution and time required to adequately flush are highly variable.

3. BriteVu Mix Preparation

BriteVu® Contrast Media. As a rule, flush 20-40% BriteVu® mix volume per body weight. As an example, plan to flush 200-400 cc (ml) prepared BriteVu® per Kg of subject body weight. As a variation, up to 3 times the subject's body weight can be perfused (300 cc [ml] for a 100 g animal) to improve capillary perfusion with cardiac puncture (left ventricle) and laceration (right auricle).

Solutions
BriteVu® 170 g and BriteVu® Special Projects come as a powder that is then mixed in a warmed solution to become a homogenous liquid. As the solution cools, the BriteVu® mix solidifies forming a cast.

BriteVu® mix will go through small gauge needles more readily at higher temperatures. As the BriteVu® mix approaches 37ºC, it begins to solidify.

BriteVu® in a Bottle and BriteVu® prepared with BriteVu® Enhancer mixes solidify around 33ºC.

Several protocols have been developed each with their pros and cons:

Protocol # 1: Distilled water and BriteVu® Enhancer

Use 1 part BriteVu® powder in grams to 3-4.5 parts distilled water in cc (ml). Add 1-2% BriteVu® Enhancer based on *BriteVu® weight*.
-Example: 25 grams of BriteVu® is added to 75-112.5 cc (ml) distilled water + 0.25-0.5 cc BriteVu® Enhancer.

1. Heat water to 40-45ºC on a stirring hot plate and then add calculated amount of BriteVu® Enhancer. Let mix for 1 minute and then add calculated amount of powdered BriteVu®.

2. Heat solution to 70-80ºC for 10 minutes. Then cool to 40-65ºC for most perfusions. To preserve histology, perfuse at the lower temperature range.

Pros: Easy, non-toxic, produces best results for most perfusions, goes through a 30 g needle at 37ºC, significantly reduces micro clumps and does not solidify until solution reaches around 33ºC.

Cons: Takes a little longer to solidify (lower temperature of solidification) compared with other mixtures.

Protocol # 2: Distilled water only

Use 1 part BriteVu® powder in grams to 3-4.5 parts distilled water in cc (ml).
-Example: 25 grams of BriteVu® is added to 75-112.5 cc (ml) distilled water.

3. Heat water to 40-45°C on a stirring hot plate and then add calculated amount of powdered BriteVu®.

4. Heat solution to 70-80°C for 10 minutes. Then cool to 45-65°C for perfusion.

Pros: Easy, classic formula, non-toxic, produces great results for most perfusions, goes through a 30 g needle at 40°C.

Cons: May see radiodense particle settling when perfused at high temperatures (> 65°C). When prepared without BriteVu® Enhancer, mix is prone to microclumps that may be visible on micro CT scans < 50µm slice thickness.

Protocol # 3: BriteVu® in a Bottle

BriteVu® in a Bottle is prepared with BriteVu®, BriteVu® Enhancer and distilled water and comes as a solid. BriteVu® is mixed 1 part to 4.5 parts distilled water. Preparation simply requires heating the bottle and contents in a warm water bath.

1. Place BriteVu® In a Bottle into a container of water filled 5" deep (below the neck of the bottle). Do NOT fill such that water covers the neck or top of the bottle.
2. Heat water to 55 deg C. A sous vide precision cooker works perfect to properly heat the water.
3. Keep BriteVu® In A Bottle in the water bath for 35 minutes to ensure proper heating.
4. Using heat resistant gloves, pull the bottle from the water bath and rotate end over end 20 times. Do NOT shake bottle.

5. If needed, the solution can be increased or cooled as per your research protocol.
6. Prior to and after use, clean the Septa Cap surface with alcohol.
7. Use a needle and syringe to penetrate the white Septa Cap and draw out the necessary amount of contrast solution. We do NOT recommend screwing off the Septa Cap or removing the shrink wrap if planning multiple uses.

Pros: Easiest to prepare and use.

Cons: The only downside is that if your protocol requires a more or less radiodense version, BriteVu® in a Bottle contents may need to be modified.

Protocol # 4: Phenol

Use 1 part BriteVu® powder in grams to 3-4.5 parts phenol in cc (ml).
-Example: 25 grams of BriteVu® is added to 75-112.5 cc (ml) phenol.

1. Heat solvent to 40-45^{o}C on a stirring hot plate and then add calculated amount of powdered BriteVu®.

2. Heat solution to 70-80^{o}C for 10 minutes. Then cool to 45-65^{o}C for perfusion.

Pros: Allows for perfusion and limited preservation (not fixation) at the same time.

Cons: Adds an additional toxic chemical to the mix.

Coloring Agents

BriteVu® mix can be colored using most common water soluble coloring agents. Over the counter food coloring agents tend to work best. Fluorescein dye (such as water soluble automotive dye) is also commonly used in BriteVu® solutions. A UV light can be used to highlight vessels perfused with BriteVu® and can be particularly helpful when viewing small peripheral vessels during perfusions.

Simply add the amount of coloring agent needed to achieve the color desired. Water soluble agents should not affect the perfusion results.

***For best capillary perfusion:**

1. Prepare BriteVu® mix using **one of the protocols listed above**.
2. Calculate the amount of BriteVu® solution needed to determine quantity of powder and distilled water. Estimate that final mix will be approximately 110-120% of the distilled water amount. For example, if using 25 g of BriteVu® and 75cc (ml) distilled water, expect to have about 83-90 cc (ml) of useable solution. Note: prolonged heating will result in evaporative solvent loss and less useable solution.
3. When using flushing solution, limit bubbles or air pockets in the flush. The bubbles may result in air traps within the vasculature and prevent complete filling of the BriteVu® solution.
4. As with the flush above, limit bubbles or air pockets in the BriteVu® mix perfusion. The bubbles may result in incomplete vascular filling and visible 'gaps' when viewed on CT and other imaging scans.
5. Immediately after completing the perfusion, soak the subject/tissue in ice water or place in a cooler. This step speeds the rate of solidification and prevents excess leakage.
6. Once the BriteVu® mix has solidified, the subject can be stored cool (not frozen) until scanning. Or, better store the subject and/or tissues in formalin for permanent stabilization.

****For large subject/tissue perfusions**:

Large cold tissues (or whole specimens) may result in premature solidification of BriteVu® mix -especially if the contrast fluid is perfused at low temperatures (< 50ºC) and over too long of a time (greater than 30 minutes). Large animals or tissues should be warmed to between 25-35ºC just prior to perfusion and BriteVu® mix should be perfused at 55-70ºC. Multiple perfusion sites and large bore catheters or trocars can be used to more rapidly deliver BriteVu® mix in large subjects. Large animals/tissues are also prone to greater autolysis if not cooled rapidly (which is more of a challenge than with small tissues).

****For open vascular systems (as with cardiac perfusion and atrial laceration or individual tissues/limbs with open venous or arterial drainage)**:

Open systems are not well pressurized. Subsequently, fluids will travel the path of least resistance and rapidly exit the (open) system without flushing/perfusing the smaller vessels. For these types of perfusions up to 300-400% volume/weight of BriteVu® mix may be needed to adequately perfuse tissues. It is always best to limit the exit site size to better pressurize the system for improved perfusion. This can best be accomplished by using clamps, tourniquets or suture to occlude draining vessels.

*****For best perfusions with follow-up histology**:
Immediately after perfusion, submerge the subject in ice water for 60 minutes. Once the BriteVu® mix has completely solidified, remove excess contrast agent, trim tissues and place in the appropriate amount of fixative. Alternatively, consider cool perfusions (see '**Protocol #1 and # 3**') (<50ºC) and/or phenol based BriteVu® solutions (see '**Protocol #4**'). These protocols will significantly reduce heat damage so that tissues can be studied histologically.

4. Subject BriteVu Perfusion

Step 1:

Completely anesthetize the animal with Isoflurane (or sevoflourane/O_2 and/or other anesthetic protocol) per your approved animal use protocol (IACUC).

Step 2:

Catheterize a peripheral vein using a 14-26 g catheter. Trocars or other devices can be used for even larger vessels. The best location will depend on the species. Place the catheter in the direction of blood flow (towards the heart in veins and both towards and away from the heart in arteries). Some species may require a cut down technique to expose the vein or artery. Catheters may be secured by different means. If using tape, use radiolucent products such as 3M Transpore Tape.

*__If using the jugular vein__: Surgically expose the jugular vein and place a 14-26 gauge IV catheter going in the direction of the heart. Tie off the descending portion of the jugular vein around the indwelling catheter using suture material. Dissect out the proximal portion of the jugular vein and temporarily clamp with hemostats just proximal to the catheter. Excise the jugular vein between the hemostats and catheter and direct the proximal portion of the jugular out and away from the body. Alternatively, place and secure an additional catheter in the proximal portion of the vein and direct blood flow away from the body (without transecting the vein). Apply tissue glue to the catheter hub and descending jugular vein. No fluid should leak out of the jugular vein/jugular catheter interface.

**__If using the ventral midline vein (as in amphibians and lizards/crocodilians)__: Follow the same procedure as with a jugular cut

down described above. Alternatively, place the catheter as above and do NOT transect the ventral midline vein. However, cut a distal limb or tail to allow the flushed blood and (later) BriteVu® to escape.

***__If performing cardiac perfusion (as in mice and some rats)__: Expose the heart via a cranial ventral abdominal approach going through the diaphragm. Place a needle (size appropriate, however most mice can take a 20-23 g needle) into the left ventricle. Butterfly needles can be secured via the 'wings' using spring clamp work-holders. Otherwise, secure the needle to prevent movement. Cut the right auricle. Alternatively, large distal limb or proximal tail vessels can be cut (instead of the auricle) in rats and other larger rodents. It is best to attach a fluid (0.9% NaCl or PBS) filled IV extension line to the needle to limit movement. Fluids can be administered on the other end of the IV extension set with a syringe.

****__If performing cardiac perfusion (as in embryonic animals)__: Visualize the heart through the transparent skin. If necessary, make a small incision through the skin and chest to expose the heart. Place the needle of appropriate size into the left ventricle. If flushing first, then cut the umbilical vessels. Otherwise, BriteVu® can be directly injected in the ventricle and the heart will pump the contrast agent (mixed with blood) throughout the body.

*****__If performing perfusion of an animal cadaver (whole or part)__: Catheterize a main artery supplying the region of interest with a large catheter, trocar or other device (depending on the vessel size). For whole body perfusion, catheterize a main vein (jugular or vena cava), multiple veins or artery(ies). Arteries and veins can be catheterized simultaneously if needed. Flush with copious warm saline until all visible clots are removed.

If flushing a whole animal cadaver, a large distal vein should be cut to provide an exit for flushed fluids. Severed limbs may leak flushed fluids from multiple sites. Once large clots are removed from cadaver limbs,

isolate and clamp draining veins until pressure is increased and smaller vessels become cleared. The clearing process depends on the state and size of tissue(s), coagulation present, any fixative(s) present and other factors. Complete clearing my take up to 24 hours of continuous flushing if capillaries are to be cleared.

Step 3:

Calculate 30-40% of the animal's (or tissue) body weight in grams – this will equal the volume (in cc [ml]) of flushing solution to use. Using the preplaced catheter or needle (as with cardiac perfusion), carefully flush with the flushing solution. The amount of pressure will vary with each subject. It is recommended to first test the pressure with your hand (and syringe). Syringe and other pumps can be used once an acceptable pressure has been determined. Excessive pressure may result in vessel rupture. By the time the total volume of the exsanguination/flushing solution is delivered, the fluid exiting the draining vein(s) should be clear to slightly pink tinged. If there appears to still be significant blood or clots leaving the draining vein, use more of the flushing solution and flush until the exiting fluid is slightly pink tinged.

Step 4:

For best results, perfuse with BriteVu® mix immediately after completing step 3. Calculate 20-40% of the animal's (or tissue) body weight in grams – this will equal the volume (in cc [ml]) of BriteVu® mix to use. Perfuse the vessel (or cardiac chamber) with the pre-calculated volume of BriteVu® mix (e.g. a 1000 gram animal would receive 200-400 cc [ml] solution). As a note, up to 3-4 times the subject's body weight can be perfused (300-400 cc [ml] for a 100 g animal) to improve capillary perfusion. At the same time the catheterized vessel (or heart chamber) is being perfused, direct the draining vessel (or heart chamber) away from the body to reduce tissue contamination with contrast agent.

Contrast contaminated tissues should be carefully cleaned off using warm moistened cotton tip applicators, or other non-destructive cleaning devices prior to solidification.

5. Post-Perfusion Tissue Handling

Once the full amount of BriteVu® mix has been perfused, tie catheter (using suture material) and/or cap off catheters as needed to prevent further leakage. Draining vessels should also be tied off or occluded with a pressure bandage.

Once perfusion is complete, set subject aside in cold water or refrigeration until BriteVu® mix solidifies. Any additional excess gelled BriteVu® mix can be removed. To speed up the solification process and reduce heat induced tissue damage (if applicable for histology), immediately immerse the subject in an ice water bath until BriteVu® mix solidifies.

For best results, perform imaging as soon as possible once BriteVu® mix has solidified (usually 60 minutes after perfusion). If needed, the animal (tissue) can be stored in formalin or phenol and scanned later. Freezing will induce artifacts and is not recommended prior to imaging. Once BriteVu® has solidified; individual tissues may be removed and scanned or stored in fixative and scanned at a later date.