BriteVu perfusions can be divided into 5 distinct phases:

1. Subject Preparation
2. Vascular Flush
3. BriteVu 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. Protocols tailored towards specific species are outlined in the following links:

Bird perfusions
Human cadaver perfusions
Lizard perfusions
Mouse perfusion
Rat perfusions
Snake perfusions
Tortoise/Turtle perfusions

Possible Materials Needed

- BriteVu®

- 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
- Emulsifier/Surfactant
  -Dawn Ultra dish soap
  -70% isopropyl alcohol
  -Cascade Complete dishwashing powder/granules
- Syringe Filter (https://www.coleparmer.com/i/advantec-43303020-polypropylene-filter-holder-for-47-mm-membranes/0662322)
- 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)
- Solvent
  -Distilled water
  -Phenol
  -70% isopropyl alcohol
- Spring Clamp Workholders

- 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 to fill the vasculature, airways or other region being studied. As with all perfusions, BriteVu will follow the path of least resistance when perfused into a system. Obstructed pathways may result in suboptimal filling with BriteVu.

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. Studies are

currently ongoing to determine the best means of vascular clearance and are in part discussed in articles within this page.

2. Vascular Flush

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

Exsanguination/Flushing Solution
Use warmed (to normal body temperature or slightly higher) 0.9% NaCl, PBS or other physiologic solution. Add a surfactant if deemed necessary. 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.

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.

Surfactants

Surfactants help lyse blood cells, prevent and break up some clots and aid the removal of flush solution. When adding a surfactant, flush with the same volume as calculated for plain physiologic solutions. Use one *or* the other.

1. 1% Dawn Ultra dish soap. Add 10 cc (ml) of Dawn Ultra to 1 L of physiologic solution and use as a vascular flush. Dawn helps remove minor clots. Be sure to avoid flushing bubbles into the studied system.
2. 0.1% Cascade Complete dishwashing granules. Add 1 g of Cascade Complete to 1 L of physiologic solution and use as a vascular flush. Cascade is more powerful than Dawn at breaking up clots. However, Cascade may prevent BriteVu from solidifying and cause settling after perfusion. After flushing with the determined amount of Cascade solution, follow with a plain physiologic solution flush (25-50% of calculated amount).

***Small Rodents (mice, baby rats, hamsters, etc) and cardiac perfusions**: Small rodents may require up to 4 times their body weight in fluids (4 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**: 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 Preparation

BriteVu Contrast Media. As a rule, flush 20-40% BriteVu 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 4 times the subject's body weight can be perfused (400 cc [ml] for a 100 g animal) to improve capillary perfusion with cardiac puncture (left ventricle) and laceration (right auricle).

Solvents

BriteVu comes as a powder that is then mixed in a warmed solvent to become a homogenous solution. As the solution cools, BriteVu solidifies forming a cast.

As another rule, BriteVu will go through small gauge needles more readily at higher temperatures. As BriteVu approaches 37°C, it begins to solidify. One exception is the Alcohol protocol (# 4) lowers the solidification temperature.

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

Protocol # 1: Distilled water only

Use 1 part BriteVu powder in grams to 2.75-3 parts Distilled water in cc (ml).
-Example: 25 grams of BriteVu is added to 69-75 cc (ml) distilled water.

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

2. 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)

Protocol # 2: Distilled water and 1-2% Dawn Ultra dish soap

Use 1 part BriteVu powder in grams to 2.75-3 parts Distilled water in cc (ml). Add 1-2% Dawn Ultra dish soap.
-Example: 25 grams of BriteVu is added to 69-75 cc (ml) distilled water + 0.69 to 1.5 cc Dawn Ultra dish soap.

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

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

Pros: better emulsifies BriteVu than straight distilled water and allows for higher temperature perfusions (> 65°C for 1% and > 70°C for 2%) with no to minimal radiodense particle settling

Cons: will make the BriteVu solution thicker at lower temperatures (< 50°C) making it more difficult to pass it through a 30 g needle at 40°C

Protocol # 3: Phenol

Use 1 part BriteVu powder in grams to 2.75-3 parts phenol in cc (ml).
-Example: 25 grams of BriteVu is added to 69-75 cc (ml) phenol.

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

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

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

Cons: adds an additional chemical to the mix

Protocol # 4: Distilled water (65 parts) and 70% isopropyl alcohol (35 parts)

Use 1 part BriteVu powder in grams to 3.4-3.7 parts 70% isopropyl alcohol in cc (ml).
-Example: 25 grams of BriteVu is added to 85-93 cc (ml) solvent (55-60 cc [ml] distilled water + 30-33 cc [ml] 70% isopropyl alcohol).

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 20 minutes. Then cool to 40-70^{o}C for perfusion.

Pros: completely emulsifies BriteVu preventing radiodense particle settling at low and high temperatures, lowers the temperature of solidification allowing for cool perfusions (allowing preservation of histologic features) and acts as a limited preservative (not fixative)

Cons: takes longer to prepare, prolongs solidification time

Coloring Agents
BriteVu can be colored using most common water soluble coloring agents. Over the counter food coloring agents tend to work best. Fluorescein dye (such as 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 using one of the protocols listed above.
2. Calculate the amount of BriteVu solution needed to determine quantity of powder and solvent. Estimate that final solution will be approximately 110-120% of the solvent amount. For example, if using 25 g of BriteVu and 75cc (ml) solvent, 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 solution perfusion. The bubbles may result in incomplete vascular filling and visible 'gaps' when viewed on CT 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 BriteVu 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 setting of BriteVu -especially if the contrast fluid is perfused at low temperatures (< 50^{o}C) and over too long of a time (greater than 30 minutes). Large animals or tissues should be warmed to between 25-35^{o}C just prior to perfusion and BriteVu should be perfused at 55-70^{o}C. Multiple perfusion sites and large bore catheters or trocars can be used to more rapidly deliver BriteVu 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). Consider substituting water with phenol or alcohol (see '**Protocol # 3 or # 4**') and scanning and collecting tissues shortly after perfusion.

*****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 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 BriteVu has completely solidified, remove excess contrast agent, trim tissues and place in the appropriate amount of fixative. Alternatively, consider cool perfusions (<50^{o}C) and/or phenol or alcohol solvent based BriteVu solutions (see '**Protocol #3 or #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 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. 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 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 small needle (size appropriate) into the left ventricle. Butterfly needles can be secured via a pin through each wing going either through or around the subject and into a semisolid background (such as Styrofoam). 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 a 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). Alternatively, catheterize a main vein (jugular or vena cava) or multiple veins. Arteries and veins can be catheterized simultaneously if needed. Flush with copious warm saline until all visible clots are removed. The addition of heparin (99 parts saline and 1 part 1000 U/ml Heparin) may or may not be helpful. Also consider adding surfactants such as Dawn Ultra dish soap or Cascage dishwasher granules.

If flushing a whole 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 and arteries until pressure is increased and smaller vessels become cleared. The clearing process depends on the state and size of tissue(s), degree of coagulation, 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 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 solution to use. Perfuse the vessel (or cardiac chamber) with the pre-calculated volume of BriteVu (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 solution 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 at room temperature until BriteVu solidifies. Any additional excess gelled BriteVu can be removed. To speed up the solidification process and reduce heat induced tissue damage (if applicable for histology), immediately immerse the subject in an ice water bath until BriteVu solidifies.

For best results, perform imaging as soon as possible once BriteVu 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.