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Scriptwriter Name: Bridget Colvin
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Title: An In Vitro System to Gauge the Thrombolytic Efficacy of Histotripsy and a Lytic Drug

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Author Questionnaire

1. **Microscopy**: Does your protocol demonstrate the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **Y**

   If **Yes**, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps. Please upload all screen captured video files to your project page as soon as possible.

3. **Interview statements:** Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interview Statements are read by JoVE's voiceover talent.

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **37**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **JoVE's Voiceover Talent**: This protocol provides a benchtop method for assessing the efficacy and mechanisms of action of histotripsy-aided thrombolytic therapy, or lysotripsy, an alternative, non-invasive approach for treating critical deep vein thrombosis **[1]**.

1.1.1. Use 3.5.1. Clot being placed into vessel

REQUIRED:

1.2. **JoVE's Voiceover Talent**: This technique uses bubble activity to have a two-way effect through an increased fibrinolysis due to an enhanced lytic delivery and the hemolysis of red blood cells within the clot **[1]**.

1.2.1. Use 2.5.2 Bubble cloud being located

OPTIONAL:

1.3. **JoVE's Voiceover Talent**: This benchtop approach to treating deep vein thrombosis facilitates the modeling of blood clots, treatment with lysotripsy, and simultaneous imaging during treatment **[1]**.

1.3.1. Use 4.4.1. Test pulse being fired

OPTIONAL:

1.4. **JoVE's Voiceover Talent**: Bubble cloud generation, treatment planning, and image guidance can be further used to investigate in vitro histotripsy-based treatments of other diseases, such as kidney and liver tumors **[1]**.

1.4.1. Use LAB MEDIA: Figure 6 or 3.7.2. Talent adding rt-PA to reservoir

Protocol

2. Histotripsy Source and Imaging Array Setup

2.1. To set up the histotripsy source, mount the source onto a motorized positioning system **[1]**.

2.1.1. WIDE: Talent showing/checking source mounted on system

2.2. Cover the imaging array with a probe cover **[1]** and fix the array coaxially in the aperture of the histotripsy source **[2]**.

2.2.1. Talent covering array
2.2.2. Talent fixing array to aperture

2.3. Connect the imaging array to an ultrasound scanning system **[1]** and completely submerge the histotripsy source and imaging array in the tank **[2]**.

2.3.1. Talent connecting array to u/s
2.3.2. Talent submerging source and array

2.4. Use a syringe to gently remove any air bubbles **[1]** and use the imaging array and scanner to acquire degassed B-mode water images at 20 frames per second **[2-TXT]**.

2.4.1. Bubbles being removed
2.4.2. Talent acquiring images/setting image acquisition parameters **TEXT: See text for full imaging parameter setup details**

2.5. While acquiring the images, adjust the position of the imaging array inside the confocal transducer opening **[1]** until the bubble cloud is located approximately at the center of the image window and record the detected focal location in the imaging window **[2]**.

2.5.1. Talent adjusting array position *Videographer: Important step*
2.5.2. SCREEN: screenshot_1: 00:03-00:09

2.6. Then discontinue the insonation **[1]** and set the voltage applied to the histotripsy source to 0 **[2]**.

2.6.1. Talent stopping insonation by setting voltage to 0

NOTE: Shot 2.6.2. and 2.6.1. were combined together in a single shot.

3. Clot Preparation and Flow Channel Priming

3.1. To prepare a clot for analysis, use pliers to cut the sealed end of a pipette containing a clot **[1]** and let the clot and serum slide into a Petri dish **[2]**.

3.1.1. WIDE: Talent cutting pipette
3.1.2. Clot sliding into Petri dish

3.2. Use a scalpel to cut the clot to a 1-centimeter length **[1]** and use a cleaning wipe to gently blot the cut section to remove any excess fluid **[2]**.

3.2.1. Clot being cut
3.2.2. Cut being blotted

3.3. Use tweezers to carefully place the section of clot onto a scale and record the weight **[1]**.

3.3.1. Talent placing clot onto scale/weighing clot

3.4. Next, manually raise the flow channel out of a degassed reverse osmosis water tank **[1-TXT]** and remove the model vessel from the channel **[2]**.

3.4.1. Talent raising channel **TEXT: See text for flow channel and water tank preparation details**
3.4.2. Talent removing vessel

3.5. Use tweezers to carefully place the clot into the model vessel without damaging the clot **[1]** and attach the vessel to the flow channel **[2]**.

3.5.1. Talent placing clot into vessel *Videographer: Important/difficult step*
3.5.2. Talent attaching vessel to channel *Videographer: Important step*

3.6. Lower the flow channel into the tank such that the proximal end of the stage relative to the reservoir is low compared to the distal side **[1]** and use a pipette to add 30 milliliters of 37-degree Celsius-warmed plasma to the reservoir **[2-TXT]**.

3.6.1. Talent lowering/angling channel
3.6.2. Plasma being added to reservoir **TEXT: See text for plasma preparation details**

3.7. Monitor the temperature of the water tank until it reaches at least 36 degrees Celsius **[1]** and use a pipette to add 80.4 micrograms of rt-PA (R-T-P-A) into the plasma reservoir **[2-TXT]**.

3.7.1. Talent monitoring temperature
3.7.2. Talent adding rt-PA to reservoir **TEXT: See text for rt-PA (recombinant tissue-type plasminogen activator) preparation details**

3.8. To prime the flow channel, use a syringe pump to draw plasma from the reservoir into the channel until the model vessel has been filled **[1].** Then manually level the model vessel to ensure that no air bubbles are present within the imaging window **[2]**.

3.8.1. Plasma being drawn into vessel *Videographer: Important step*
3.8.2. Vessel being leveled.
NOTE: Shot 3.8.2. and its corresponding VO was changed.

3.9. Then manually level the model vessel to ensure that no air bubbles are present within the imaging window **[1]**.

3.9.1. Vessel being leveled

4. Pre-Treatment

4.1. To plan a path for the histotripsy source and imaging array for a uniform histotripsy exposure along the clot length, use the motorized positioners to align the imaging array such that the imaging plane is parallel to the cross-section of the clot **[1]**.

4.1.1. WIDE: Talent aligning array with positioners *Videographer: Important step*

4.2. Using the imaging window as a guide, move the histotripsy source to the proximal end of the clot relative to the reservoir **[1][2]**.

4.2.1. Source being moved closed to proximal end of clot *Videographer: Please frame to allow inset from 4.2.2.*
4.2.2. SCREEN: screenshot_2: 00:07-00:11 *Video Editor: please include as inset in 4.2.1.*

4.3. To determine the insonation path along the clot length, set waypoints along the length of the clot in 5-millimeter increments **[1]**.

4.3.1. SCREEN: screenshot_3: 00:18-00:33
NOTE: The video file to be used for 4.3.1. was changed from 'screenshot_2' to 'screenshot_3'.

4.4. Prior to finalizing each waypoint, fire test pulses from the histotripsy source with the same insonation parameters as demonstrated but with the overall exposure reduced to 10 total pulses. At each waypoint, save the motor positions using the commands designated by the manufacturer **[1]**.

4.4.1. SCREEN: screenshot_3: 00:01-00:11

5. Treatment

5.1. To treat the clot along its length according to the path defined in the pre-treatment step, set the syringe pump to 0.65 milliliters/minute **[1]** and wait for the meniscus of the plasma to move **[2]**.

5.1.1. WIDE: Talent setting pump
5.1.2. Shot of plasma meniscus moving

5.2. Interpolate the treatment path with intermediate steps between the established waypoints with a fixed step size **[1-TXT]** and use the positioners to move the histotripsy source at each path location using the originally set insonation parameters **[2]**.

5.2.1. SCREEN: To be provided by Authors: Treatment path being interpolated **TEXT: i.e., set step size <1/2 width of focal region as measured along clot length**
5.2.2. Source being moved along path *Videographer: Important step* OR SCREEN: To be provided by Authors: Parameters being set

NOTE: No corresponding video file was found for shot 5.2.1. If the videographer has not filmed this shot, skip to 5.2.2.

5.3. Use the imaging window to image the bubble activity during the application of the histotripsy pulse at each path location **[1]**.

5.3.1. SCREEN: screenshot_4: 00:30-00:38

NOTE: The video file for shot 5.3.1. was changed from 'screenshot_3' to 'screenshot_4' and the time stamp was changed from '00:30-00:32' to '00:30-00:38'.

5.4. Next, create a script to set the imaging array to acquire a B-mode image of the clot and model vessel a few seconds before the application of histotripsy pulse at each location **[1]**.

5.4.1. SCREEN: screenshot_4: 00:00-00:22

5.5. Then apply the histotripsy pulse at each way point, acquiring the acoustic emissions in the script to form passive cavitation images after the analysis **[1]**.

5.5.1. SCREEN: screenshot_4: 01:33-01:43

6. Post-Experimental Procedure

6.1. After the analysis, manually raise the model vessel out of the water tank to drain the perfusate via gravity **[1]** and use the syringe pump to draw the plasma solution from the flow channel **[2]** to allow collection of the entire perfusate in a small beaker **[3]**.

6.1.1. WIDE: Talent raising vessel *Videographer: Important step*
6.1.2. Plasma being drawn from channel *Videographer: Important step*
6.1.3. Perfusate being collected into beaker *Videographer: Important step*

6.2. Disconnect the model vessel **[1]** and remove the clot **[2]**.

6.2.1. Talent disconnecting vessel
6.2.2. Talent removing clot

6.3. Then wipe the clot with lab tissues **[1]** and weigh the clot to assess the clot mass loss **[2]**.

6.3.1. Clot being wiped
6.3.2. Talent placing clot onto scale

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
2.5., 3.5., 3.8., 4.1., 5.2., 6.1.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
3.5. It is important that clot is handled very carefully while placing it in the model vessel without damaging/breaking the clot.
5.4. The acquisition of images should be in sync with the histotripsy source pulse to acquire necessary data. For this, triggering of imaging array is set such that the array starts acquiring data few microseconds before the histotripsy pulse reaches the clot.

Results

7. Results: Representative Clot Identification and Lysotripsy

7.1. Upon application of sufficient voltage to the histotripsy source **[1]**, a bubble cloud is generated in the focal region of the transducer and visualized via ultrasound imaging **[2]**.

7.1.1. LAB MEDIA: Figure 3
7.1.2. LAB MEDIA: Figure 3 *Video Editor: please emphasize white "cloud" in image*

7.2. The focal position is defined as the center of the bubble cloud **[1]**.

7.2.1. LAB MEDIA: Figure 3 *Video Editor: please emphasize red cross in center of cloud*

7.3. This control perfusate of a clot exposed to plasma alone **[1]** and this perfusate of a lysotripsy treated clot **[2]** were used to assess the hemoglobin and D-dimer content as demonstrated **[3]**.

7.3.1. LAB MEDIA: Figure 4A *Video Editor: please emphasize Control perfusate*
7.3.2. LAB MEDIA: Figure 4A *Video Editor: please emphasize Treated perfusate*

7.4. The variability in hemoglobin concentration **[1]** can be quantified by optical absorbance **[2]**.

7.4.1. LAB MEDIA: Figure 4B *Video Editor: please emphasize colored wells*
7.4.2. LAB MEDIA: Figure 4C *Video Editor: please emphasize data line*

7.5. Here a clot can be visualized within a model vessel via B-mode imaging **[1]** prior to histotripsy exposure to determine the clot position for segmentation of the passive cavitation image **[2]**.

7.5.1. LAB MEDIA: Figure 5A
7.5.2. LAB MEDIA: Figure 5A *Video Editor: please emphasize clot in middle of vessel*

7.6. As this passive cavitation image co-registered with the B-mode image of the clot confirms **[1]**, the acoustic energy is contained primarily within the clot during the histotripsy exposure **[2]**.

7.6.1. LAB MEDIA: Figure 5B

7.6.2. LAB MEDIA: Figure 5B *Video Editor: please emphasize red-outlined yellow signal in center of image*

7.7. In samples exposed to histotripsy **[1]**, disruption is primarily restricted to the clot center, consistent with the observed locations of bubble activity tracked via passive cavitation imaging **[2]**.

7.7.1. LAB MEDIA: Figure 6 *Video Editor: please emphasize Figure 6B*
7.7.2. LAB MEDIA: Figure 6 *Video Editor: please emphasize disrupted clot in center of Figure 6B*

7.8. With the addition of lytic, mass loss also occurs in regions closer to the periphery of the clot **[1]**.

7.8.1. LAB MEDIA: Figure 6 *Video Editor: please emphasize disruptions around periphery of clot*

Conclusion

8. Conclusion Interview Statements

8.1. **JoVE's Voiceover Talent**: Alternatively, echogenic liposomes containing rt-PA can be used in place of systemic rt-PA delivery to allow the assessment of targeted drug delivery to the clot **[1]**.

8.1.1. Use LAB MEDIA: Figure 5 or 3.7.2. Talent adding rt-PA to reservoir

8.2. **JoVE's Voiceover Talent**: Lysotripsy provides an alternative non-invasive approach to treating many diseases. This in vitro protocol enables assessment of the efficacy of the treatment and its potential success in in vivo application **[1]**.

8.2.1. Use LAB MEDIA: Figure 6 or 5.5.1. acoustic emissions being acquired