FINAL SCRIPT: APPROVED FOR FILMING

Submission ID #: 68596
Scriptwriter Name: Sulakshana Karkala
Project Page Link: https://review.jove.com/account/file-uploader?src=20921828

Title: A Microscopic 2,3,5-Triphenyltetrazolium Chloride Assay for Accurate and Reliable Analysis of Myocardial Injury

Authors and Affiliations:
Zheheng Ding[1*], Hui Zhang[1,2,3,4,5*], Pascal Bouvain[1], Zhaoping Ding[1], Ulrich Flögel[1]

[1]Institute of Molecular Cardiology, Medical Faculty and University Hospital Düsseldorf, Heinrich Heine University of Düsseldorf
[2]Department of Anesthesiology and Perioperative Medicine, Shanghai Fourth People's Hospital, School of Medicine, Tongji University
[3]Shanghai Key Laboratory of Anesthesiology and Brain Functional Modulation, Shanghai Fourth People's Hospital, School of Medicine, Tongji University,
[4]Clinical Research Center for Anesthesiology and Perioperative Medicine, Shanghai Fourth People's Hospital, School of Medicine, Tongji University
[5]Translational Research Institute of Brain and Brain-Like Intelligence, Shanghai Fourth People's Hospital, School of Medicine, Tongji University

[*]These authors contributed equally to this work

Corresponding Authors:
Zhaoping Ding (ding@uni-duesseldorf.de)
Ulrich Flögel (floegel@uni-duesseldorf.de)
Email Addresses for All Authors:
Zheheng Ding (zheheng.ding@uni-duesseldorf.de)
Hui Zhang (15077169795@163.com)
Pascal Bouvain (bouvain@hhu.de)
Zhaoping Ding (ding@uni-duesseldorf.de)
Ulrich Flögel (floegel@uni-duesseldorf.de)

Author Questionnaire

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

   NOTE: All scope shots were filmed by the videographer

   SCOPE: 3.4.1 – 3, 3.5.1, 3.6.1 – 2, 3.7.1 – 2, 4.1.1 – 2, 4.2.1, 4.4.1 -2, 5.1.3

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

3. **Filming location:** Will the filming need to take place in multiple locations? **Yes, 100 m apart**

4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length
Number of Steps: 30
Number of Shots: 58

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~What is the scope of your research? What questions are you trying to answer?~~

1.1. **Zheheng Ding:** My research focuses on improving TTC staining methods to enhance image quality for accurate detection of myocardial ischemia or reperfusion injury.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:4.3*

~~What are the most recent developments in your field of research?~~

1.2. **Zheheng Ding:** Recent advances include improved TTC staining using cryosectioning and microscopic imaging to enhance myocardial injury visualization.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What significant findings have you established in your field?~~

1.3. **Uli Flögel:** We demonstrated that microscopic TTC assay significantly improves myocardial injury visualization accuracy.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:7.1*

~~How will your findings advance research in your field?~~

1.4. **Uli Flögel:** Our method enhances image clarity and boundary definition, enabling precise, reliable infarct zone identification and advancing myocardial injury assessment accuracy.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:7.1*

~~What new scientific questions have your results paved the way for?~~

1.5. **Uli Flögel:** Our standardized method opens new questions on optimizing staining dynamics, correlating imaging precision with molecular changes, and improving cardiac repair assessment.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the *LANUV* at Institute of Molecular Cardiology in Heinrich-Heine University Düsseldorf. All experiments conducted in accordance with the ARRIVE guidelines and the NIH Guide for the Care and Use of Laboratory Animals

Protocol

2. Langendorff Retro-Perfusion System Setup

Demonstrator: Zhaoping Ding

2.1. To begin, secure a Langendorff retro-perfusion apparatus horizontally on a 150-millimeter Petri dish with adhesive tape to prevent movement **[1]**. Position the cannula tip 1 millimeter above the dish bottom, aligning it with the aortic root when the heart is placed flat **[2]**.

2.1.1. WIDE: Talent taping the retro-perfusion setup horizontally on a 150-millimeter Petri dish.

2.1.2. Talent adjusting the cannula tip to sit 1 millimeter above the Petri dish bottom.

2.2. Prefill the system with ice-cold PBS using a 10-milliliter syringe through the PBS port **[1]**. Use a 3-milliliter syringe to prefill the TTC port with freshly prepared 1 % staining solution **[2-TXT]**.

2.2.1. Talent using a 10-milliliter syringe to fill the PBS port with ice-cold phosphate-buffered saline.

2.2.2. Talent using a 3-milliliter syringe to fill the TTC port with TTC solution.
TXT: TTC: 2,3,5-Triphenyltetrazolium Chloride; Ensure 6 mL per heart

NOTE: Please move 2.2 above 2.1

2.3. Check the perfusion system for any unintentional air bubbles **[1]**. If bubbles are visible, withdraw and refill PBS while gently tapping the three-way stopcock in the upright cannula position **[2]**.

2.3.1. Shot of the perfusion system being checked for bubbles.

2.3.2. Talent identifying air bubbles, gently tapping the stopcock, and flushing PBS to remove bubbles.

2.4. Next, form a loose half-square knot using a 6-0 *(Six-Oh)* silk suture **[1]** and place it on the distal end of the cannula to secure the aortic root later **[2]**.

2.4.1. Talent tying a loose half-square knot with 6-0 silk suture.

2.4.2. Talent placing the half-knot over the cannula tip.

3. Mouse Heart Excision and Cannulation for Retro-Perfusion

3.1. With micro-dissecting forceps and scissors, remove the pelt from the mid-abdomen to the mid-forepaw region of a euthanized and secured mouse **[1-TXT]**. Then make a 2-centimeter transverse incision into the abdominal muscle wall just below the diaphragm to allow for subsequent thoracotomy **[2]**.

3.1.1. Talent using forceps and scissors to remove the pelt. **TXT: Euthanasia: Cervical dislocation under isoflurane anesthesia; Secure limbs with tape**

3.1.2. Talent making a precise transverse incision through the abdominal wall.

3.2. Now, use fine scissors to spread the diaphragm and expose the heart **[1]**, then make bilateral incisions through the skin, muscle, and ribs to open the thoracic cavity **[2]**. Cut the diaphragm back bilaterally and lift the sternum to fully expose the heart **[3]**.

3.2.1. Talent spreading the diaphragm using fine scissors and exposing the heart.

3.2.2. Shot of incisions being made through the skin, muscle and ribs.

3.2.3. Talent cutting diaphragm and lifting sternum to reveal the heart.

3.3. Immediately pour 20 milliliters of ice-cold PBS onto the heart surface to arrest the heartbeat **[1]**. Then excise the heart along with the surrounding tissues **[2].** Transfer the tissue mass into a 50-milliliter beaker containing ice-cold PBS for about 1 minute to allow the heartbeat to subside **[3]**.

3.3.1. Talent pouring PBS over the heart with a steady stream.

3.3.2. Talent excising heart and surrounding tissue.

3.3.3. Shot of the excised tissue being placed in beaker filled with ice-cold PBS.

3.4. Next, transfer the heart onto a 150-millimeter Petri dish **[1].** Trim the aortic root free of connective tissue under a binocular microscope using fine forceps and scissors **[2]**. Then gently remove the thymus and expose the ascending aorta **[3]**.

NOTE: All scope shots were filmed by the videographer

3.4.1. SCOPE: Talent placing the heart in a dish.

3.4.2. SCOPE: Shot of the aortic root being trimmed under microscope.

3.4.3. SCOPE: The thymus is being removed and the aorta is being seen.

3.5. Trim the aortic root by dissecting the connective tissue **[1]**.

3.5.1. SCOPE: The connective tissue around the aortic root is being dissected.

3.6. Pick up the aortic stump with fine tweezers and mount it over the cannula that is already connected to a prefilled perfusion apparatus **[1-TXT]**. Now, secure the aorta using the pre-formed loose knot on the cannula **[2]**.

3.6.1. SCOPE: Talent using tweezers to position the aortic stump over the cannula. **TXT: Use fine tweezers to stabilize the aortic root during insertion**

3.6.2. SCOPE: Talent tightening the knot around the aorta

3.7. Inspect the cannula and confirm heart filling by observing ventricle bulging during PBS perfusion **[1]**. Leave the extra-cardiac tissues including lungs and esophagus in place to support the heart in a natural and appropriate position **[2]**.

3.7.1. SCOPE: Shot of ventricle bulging with PBS perfusion.

3.7.2. SCOPE: Talent arranging surrounding tissues to support the heart in position.

4. Triphenyltetrazolium Chloride (TTC) Staining Procedure

4.1. Cover the heart with moist tissue paper once the cannulation is secured **[1]**. Begin rinsing the heart with ice-cold PBS using a pre-connected 10-milliliter syringe until the cardiac eluate runs clear **[2-TXT]**.

4.1.1. SCOPE: Talent placing a piece of moist tissue paper over the cannulated heart.

4.1.2. SCOPE: Talent gently perfusing the heart with PBS using a 10-milliliter syringe and monitoring for clear outflow. **TXT: Rinse rate: 2 - 3 mL/min, 3 min; Avoid applying excessive pressure during perfusion**

4.2. Inspect the heart under a binocular microscope to confirm there is no leakage at the aortic root and that it inflates properly under perfusion pressure **[1]**.

4.2.1. SCOPE: Shot of the heart being inspected and inflating properly.

4.3. Now, turn the three-way stopcock toward the TTC solution and slowly infuse 1 milliliter of staining solution through the TTC port over 30 seconds **[1-TXT]**.

4.3.1. Talent rotating the stopcock and slowly injecting TTC solution through the TTC port. **TXT: Reinfuse 1 mL TTC every 5 min for 15 min**

4.4. Inspect the heart surface microscopically. At this stage, viable myocardium should appear deep red, while necrotic tissue appears light gray **[1]**. Then remove all non-cardiac tissues using fine scissors **[2]** and transfer the stained heart into a 15-milliliter conical tube containing 3 milliliters of TTC solution **[3]**.

4.4.1. SCOPE: View of the heart surface showing a contrast between deep red viable tissue and light gray necrotic tissue.

4.4.2. SCOPE: Talent trimming away lungs and esophagus.

4.4.3. Talent placing the heart in a conical tube with TTC solution.

4.5. Place the conical tube at 4 degrees Celsius overnight to allow a second round of staining by immersion **[1]**.

4.5.1. Talent placing the conical tube in a 4 degrees Celsius refrigerator.

5. Tissue Embedding, Cryosectioning, and Fixation Protocol

5.1. To create a tissue block, first fill a blunted, curved 20-gauge cannula with optimal cutting temperature compound using a 1-milliliter syringe **[1]**. Excise the tricuspid valve using fine scissors then insert the cannula into the right ventricle **[1]**. Infuse 50 to 80 microliters of OCT *(O-C-T)* compound into the cavity and stop the infusion when backflow is observed **[3-TXT]**.

5.1.1. Talent using a 1-milliliter syringe to load OCT compound into a curved cannula.

5.1.2. Talent excising the tricuspid valve and positioning the cannula into the right ventricle.

5.1.3. SCOPE: Talent slowly infusing OCT compound into the right ventricle and stopping upon backflow. **TXT: Similarly infuse the mitral valve**

5.2. Carefully place the heart into a cylindrical aluminum foil capsule filled with 300 microliters of OCT compound in an vertical, apex-down position **[1]**. Then immerse the capsule in a 2-methylbutane solution pre-chilled to between minus 30 and minus 40 degrees Celsius **[2-TXT]**.

5.2.1. Talent positioning the heart inside the capsule and adjusting it for vertical alignment.

5.2.2. Talent placing the capsule in the chilled 2-methylbutane solution. **TXT: After block solidifies, label and store at - 80 °C**

5.3. Mount the tissue block onto a cryostat chuck using OCT **[1]**. Place the chuck into the cryostat and set the temperature to approximately minus 24 degrees Celsius **[2]**.

5.3.1. Talent applying OCT compound and securing the tissue block onto the cryostat chuck.

5.3.2. Talent inserting the chuck into the cryostat and adjusting the temperature control panel.

5.4. Slice the tissue block until the apex of the heart is visible **[1]**. Then set the section thickness to 50 micrometers and collect every second slice to maintain 100-micrometer intervals between sections **[2]**.

5.4.1. Talent trimming the tissue block inside the cryostat to expose the heart apex.

5.4.2. Talent adjusting slice thickness settings and collecting alternate sections.

5.5. Align the collected slices sequentially on glass microscope slides, placing eight sections per slide for a total of ten slides per heart **[1]**. Dry the slides with a hairdryer on the lowest setting using room-temperature airflow for approximately 10 minutes **[2]**.

5.5.1. Talent arranging and placing tissue sections neatly on microscope slides.

5.5.2. Talent using a handheld hairdryer to dry slides gently without applying heat.

5.6. Submerge the slides in a staining chamber filled with Zamboni's fixative, containing 2 percent paraformaldehyde and 0.4 percent picric acid by weight **[1]**. Fix the heart tissue at 4 degrees Celsius overnight to ensure the tissue surface remains free of air bubbles formed during fixation **[2-TXT]**.

5.6.1. Talent immersing prepared slides into a staining chamber filled with Zamboni's fixative.

5.6.2. Talent placing the staining chamber in a refrigerator at 4 degrees Celsius. **TXT: Post fixation, rinse slides 3x in PBS**

5.7. Mount the slides using mounting medium and cover the sections with coverslips to finalize preparation for image acquisition **[1]**. Store the completed slides in a slide box at room temperature **[2]**.

5.7.1. Talent applying mounting medium and placing coverslips carefully on each slide.

5.7.2. Talent placing the prepared slides into a labeled slide storage box.

6. Imaging Acquisition and 3D Reconstruction Workflow

6.1. To image the sections, set up a light microscope using a 1.25X objective lens and run the CellSens imaging software **[1]**. Position the heart section in the center of the field of view **[2]**.

6.1.1. SCREEN: SCREEN6.2.1---6.2.2.mp4 00:00-00:10

6.1.2. SCREEN: SCREEN6.2.1---6.2.2.mp4 00:11-00:14

6.2. Adjust the camera's exposure time, focus, and white balance as needed **[1]**. Then capture images of each section and save all images from the same heart in a dedicated folder **[2]**.

6.2.1. SCREEN: SCREEN6.2.1---6.2.2.mp4 00:15-00:26

6.2.2. SCREEN: SCREEN6.2.1---6.2.2.mp4 00:27-00:43

6.3. Next, copy the R script to the RStudio platform and run the automated algorithm to assess myocardial infarction. The algorithm calculates infarct sizes for each slice and computes total infarct volume **[1]**. Export the dataset to an Excel file and analyze the data as needed **[2]**.

6.3.1. SCREEN: SCREEN-6.3.1---6.3.2.mp4 00:43-01:25

6.3.2. SCREEN: SCREEN-6.3.1---6.3.2.mp4 03:30-03:39, 03:52-04:00

6.4. Import all images from each heart into the ImageJ platform for 3D reconstruction **[1]**. Stack all images in sequential order using the pop-up menu of ImageJ **[2]**.

6.4.1. SCREEN: SCREEN-6.4.1---6.5.2.mp4 00:09-00:25

6.4.2. SCREEN: SCREEN-6.4.1---6.5.2.mp4 00:33-00:45

6.5. Then create a 3D construction using the surface plot function **[1]**. Lastly, open the Volume Viewer window and adjust parameters for 3D projection **[2]**.

6.5.1. SCREEN: SCREEN-6.4.1---6.5.2.mp4. 00:46-00:56

6.5.2. SCREEN: SCREEN-6.4.1---6.5.2.mp4 01:41-01:55

AUTHOR'S NOTE: Please move steps 6.4 and 6.5 above shot 6.3

Results

7. Results

7.1. In each section, deep red staining of viable cardiomyocytes was clearly distinguishable from yellow necrotic tissue, yielding a sharply defined border **[1]**.

7.1.1. LAB MEDIA: Figure 2A. *Video editor: Zoom in on the labeled regions "viaCM" and "necCM"*

7.2. Compared to conventional staining, the microscopic protocol provided clearer and sharper boundaries and an improved viable-to-infarct signal ratio **[1]**. Filling the ventricular cavities with OCT maintained the heart's physiological shape and preserved the endocardial layer without altering infarct size **[2]**.

7.2.1. LAB MEDIA: Figure 2C. *Video editor: Please highlight the image labeled "microscopic TTC"*

7.2.2. LAB MEDIA: Figure 2D. *Video editor: Please highlight the image labeled "with OCT" and the grey column .*

7.3. Deep red formazan precipitates were found in the cytoplasm of viable cardiomyocytes and overlapped with cardiac troponin I staining **[1]**.

7.3.1. LAB MEDIA: Figure 3. *Video editor: Please highlight the black and white image labeled "cTnL" and the image labeled "TTC"*

7.4. In the mouse myocardial infarction model with 50 minutes of ischemia, the average global infarct size was 32.14%, matching the planimetric size above the mid-ventricular level **[1]**. Infarct size varied across heart levels, peaking at 71.21% in the apical region and gradually declining to zero near the ligation site **[2]**.

7.4.1. LAB MEDIA: Figure 4B (right) *Video editor: Highlight the right stacked bar chart labeled "Sample"*

7.4.2. LAB MEDIA: Figure 4B (left). *Video editor: Trace the line plot from left to right*

7.5. The microscopic TTC assay revealed infarct areas even 3 days post-myocardial infarction, with yellow staining in the necrotic core and pinkish-grey in the periphery **[1]**. At 7 days post-myocardial infarction, fibrotic scar tissue showed distinguishable TTC staining between myocytes and non-myocytes **[2]**.

7.5.1. LAB MEDIA: Figure 5. *Video editor: Highlight the "3D post-MI" image*

7.5.2. LAB MEDIA: Figure 5. *Video editor: Highlight the "7D post-MI" heart section*

7.6. Thirty minutes of cardiac ischemia induced a wavefront pattern of injury starting in the endocardium and progressing toward the epicardium **[1]**. A transmural infarct was consistently induced when ischemia duration exceeded 40 minutes **[2]**.

7.6.1. LAB MEDIA: Figure 6. *Video editor: Highlight the "30-min MI" image*

7.6.2. LAB MEDIA: Figure 6. *Video editor: Sequentially highlight the "40-min MI", "50-min MI" and "60-min MI" heart images*

Pronunciation Guide:

1. Triphenyltetrazolium
   Pronunciation link: No confirmed link found
   IPA: /ˌtrɪˌfɛnilˌtɛtrəˈzoʊliəm/
   Phonetic Spelling: trih-feh-neel-teh-truh-zoh-lee-uhm
2. Myocardial
   Pronunciation link: https://www.merriam-webster.com/dictionary/myocardial
   IPA: /ˌmaɪ.oʊˈkɑːr.di.əl/
   Phonetic Spelling: my-oh-kar-dee-uhl
3. Ischemia
   Pronunciation link: https://www.merriam-webster.com/dictionary/ischemia
   IPA: /ɪˈskiː.mi.ə/
   Phonetic Spelling: ih-skee-mee-uh
4. Reperfusion
   Pronunciation link: https://www.merriam-webster.com/dictionary/reperfusion
   IPA: /ˌriː.pɚˈfjuː.ʒən/
   Phonetic Spelling: ree-per-fyoo-zhun
5. Infarct
   Pronunciation link: https://www.merriam-webster.com/dictionary/infarct
   IPA: /ˈɪn.fɑːrkt/
   Phonetic Spelling: in-farkt
6. Langendorff
   Pronunciation link: No confirmed link found
   IPA: /ˈlæŋənˌdɔːrf/
   Phonetic Spelling: lang-en-dorf
7. Perfusion
   Pronunciation link: https://www.merriam-webster.com/dictionary/perfusion
   IPA: /pɚˈfjuː.ʒən/
   Phonetic Spelling: per-fyoo-zhun
8. Cannula
   Pronunciation link: https://www.merriam-webster.com/dictionary/cannula
   IPA: /ˈkæn.jə.lə/
   Phonetic Spelling: kan-yuh-luh
9. Aorta
   Pronunciation link: https://www.merriam-webster.com/dictionary/aorta
   IPA: /eɪˈɔːr.tə/
   Phonetic Spelling: ay-or-tuh
10. Thoracotomy
    Pronunciation link: https://www.merriam-webster.com/dictionary/thoracotomy
    IPA: /ˌθɔːr.əˈkɑː.tə.mi/
    Phonetic Spelling: thor-uh-kaa-tuh-mee

11. Diaphragm
    Pronunciation link: https://www.merriam-webster.com/dictionary/diaphragm
    IPA: /ˈdaɪ.ə.fræm/
    Phonetic Spelling: dye-uh-fram
12. Tricuspid
    Pronunciation link: https://www.merriam-webster.com/dictionary/tricuspid
    IPA: /traɪˈkʌs.pɪd/
    Phonetic Spelling: try-kus-pid
13. Paraformaldehyde
    Pronunciation link: https://www.merriam-webster.com/dictionary/paraformaldehyde
    IPA: /ˌpær.ə.fɔːrˈmæl.dəˌhaɪd/
    Phonetic Spelling: pah-ruh-for-mal-duh-hyde
14. Picric
    Pronunciation link: https://www.merriam-webster.com/dictionary/picric
    IPA: /ˈpɪk.rɪk/
    Phonetic Spelling: pik-rik
15. Cardiomyocytes
    Pronunciation link: No confirmed link found
    IPA: /ˌkɑːr.di.oʊˈmaɪ.ə.saɪts/
    Phonetic Spelling: kar-dee-oh-my-uh-sytes
16. Formazan
    Pronunciation link: https://www.merriam-webster.com/dictionary/formazan
    IPA: /ˈfɔːr.məˌzæn/
    Phonetic Spelling: for-muh-zan
17. Planimetric
    Pronunciation link: https://www.merriam-webster.com/dictionary/planimetric
    IPA: /ˌplæn.əˈmɛ.trɪk/
    Phonetic Spelling: plan-uh-meh-trik