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Title: A UV-Based ChIRP Method for the Verifiable Identification of Proteins that Directly Interact with LncRNA: From Cultured Cells to Mouse Testis

Authors and Affiliations:

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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? **NO**

- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **NO**

- 3. Filming location:** Will the filming need to take place in multiple locations? **NO**

- 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: 19

Number of Shots: 58

Interviews

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

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Yiting Ye:** Our research develops cPDIRT, a UV-crosslinking method to map direct lncRNA-protein interactions in both cultured cells and native tissues such as the testis, uncovering Tug1's molecular mechanisms in spermatogenesis.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.2. **Lingxiu Tong:** This protocol enables direct RNA-protein interaction mapping in a wide range of systems, from cultured cells to intact native tissues.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Yiting Ye:** cPDIRT allows researchers to identify and measure proteins that directly interact with specific lncRNAs in both cultured cells and native tissues.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Wanying Jiang:** The most important challenge is achieving efficient UV crosslinking while ensuring high specificity for direct RNA-protein interactions.

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

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Lingxiu Tong:** Our protocol will support future studies to apply this approach to other RNAs and tissues, elucidating direct RNA-protein interactions in development and reproduction.

- 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 Animal Ethics Committee of Nanjing Medical University

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Ultraviolet Crosslinking of Cultured GC2 Cells

Demonstrator: Wanyin Jiang

2.1. To begin, wash the GC2 (*G-C-two*) cells three times with PBS to remove residual culture medium [1]. Distribute 3 milliliters of PBS evenly on the 15-centimeter culture dish [2].

2.1.1. WIDE: Talent washing the cells by adding PBS and tilting the plate.
***Videographer:** Please obtain CU shot as well for this shot.*

2.1.2. Talent distributing the cell suspension evenly onto a 15-centimeter culture dish.

2.2. Irradiate the cells in a 254-nanometer ultraviolet crosslinker at a dose of 4000 millijoules per square centimeter [1]. After crosslinking, collect the cells [2]. Centrifuge the cell suspension at 3000 *g* for 5 minutes at 4 degrees Celsius and discard the supernatant [3]. Weigh the pellet [4] and confirm a minimum pellet mass of 500 milligrams per sample [5].

2.2.1. Talent placing the culture dish into the 254-nanometer ultraviolet crosslinker for irradiation.

2.2.2. Talent collecting the crosslinked cells in PBS.

2.2.3. Talent placing the cell suspension into the centrifuge.

2.2.4. Talent weighing the cell pellet. **Videographer's NOTE: 2.2.4 and 2.2.5 were combined in one clip**

2.2.5. Talent confirming that the pellet mass is at least 500 milligrams per sample.

3. Isolation and Ultraviolet Crosslinking of Testicular Cells

3.1. Wash the decapsulated testes tissue twice with DMEM [1]. Incubate the tissue in DMEM containing 1 milligram per milliliter collagenase IV (4) at 37 degrees Celsius for 5 to 15 minutes until seminiferous tubules are released [2].

3.1.1. Talent washing the decapsulated testes tissue with DMEM.

3.1.2. Talent placing the tissue in DMEM containing collagenase IV for incubation.

- 3.2. Then, centrifuge the suspension at 1000 *g* for 1 minute at 4 degrees Celsius [1] and remove the supernatant [2].
 - 3.2.1. Talent placing the suspension into the centrifuge.
 - 3.2.2. Talent removing the supernatant from the tube.
- 3.3. Wash the pellet with 5 to 10 milliliters of DMEM [1]. Centrifuge the suspension again at 1000 *g* for 1 minute at 4 degrees Celsius [2] and resuspend the pellet in 0.25% trypsin containing 1 milligram per milliliter deoxyribonuclease I (*one*) [3].
 - 3.3.1. Talent washing the pellet with DMEM.
 - 3.3.2. Talent placing the suspension into the centrifuge.
 - 3.3.3. Talent resuspending the pellet in trypsin containing deoxyribonuclease I.
- 3.4. Incubate the suspension at 37 degrees Celsius for 5 to 15 minutes [1]. Then, add an equal volume of DMEM supplemented with 10% FBS to stop the enzymatic digestion [2]. Filter the suspension through a 70-micrometer cell strainer [3].
 - 3.4.1. Talent placing the suspension for incubation.
 - 3.4.2. Talent adding DMEM supplemented with fetal bovine serum to the suspension.
 - 3.4.3. Talent filtering the suspension through a 70-micrometer cell strainer.
- 3.5. Centrifuge the cells at 2000 *g* for 5 minutes at 4 degrees Celsius [1]. Discard the supernatant [2] and wash the pellet with 5 to 10 milliliters of PBS [3].
 - 3.5.1. Talent placing the cells into the centrifuge.
 - 3.5.2. Talent discarding the supernatant. Videographer's NOTE: 3.5.2 and 3.5.3 were combined in one clip
 - 3.5.3. Talent washing the pellet with PBS.
- 3.6. Centrifuge the suspension again at 2000 *g* for 5 minutes at 4 degrees Celsius [1]. Resuspend the single-cell suspension in PBS [2] and distribute it evenly onto a 15-centimeter culture dish [3].
 - 3.6.1. Talent placing the suspension into the centrifuge.
 - 3.6.2. Talent resuspending the single-cell suspension in PBS.
 - 3.6.3. Talent distributing the suspension evenly onto a 15-centimeter culture dish.

- 3.7. Irradiate the cells in a 254-nanometer ultraviolet crosslinker at a dose of 4000 millijoules per square centimeter [1-TXT]. Collect the crosslinked cells [2]. Centrifuge the suspension at 3000 g for 5 minutes at 4 degrees Celsius and discard the supernatant [3].
 - 3.7.1. Talent placing the culture dish into the 254-nanometer ultraviolet crosslinker for irradiation. **TXT: Omit irradiation for control cells**
 - 3.7.2. Talent collecting the crosslinked cells.
 - 3.7.3. Talent placing the suspension into the centrifuge.

4. Lysing the Cells and Preclearing

- 4.1. After weighing the pellet, resuspend it in cell lysis buffer at a ratio of 100 milligrams of pellet per 1 milliliter of buffer [1].
 - 4.1.1. Talent resuspending the cell pellets by adding cell lysis buffer and pipetting up and down. Videographer's NOTE: 4.1.1 and 4.2.1 were combined in one clip
- 4.2. Vortex the sample thoroughly to obtain a homogeneous suspension [1]. Sonicate the lysates using a water-bath sonicator or a focused ultrasonicator until the lysates become visually clear [2-TXT]. Aliquot approximately 10 microliters of the lysate into microcentrifuge tubes for RNA analysis and protein analysis [3].
 - 4.2.1. Talent vortexing the suspension thoroughly.
 - 4.2.2. Talent sonicating the lysates. **TXT: Pulse settings: 15 s ON and 15 s OFF; Total time: 30 min to 2 h**
 - 4.2.3. Talent aliquoting approximately 10 microliters of sample to two labeled tubes for RNA and protein analysis.
- 4.3. For preclearing the lysate, add 30 microliters of pre-washed magnetic beads to the lysate [1]. Incubate the samples at 37 degrees Celsius for 30 minutes with gentle mixing in a hybridization oven [2].
 - 4.3.1. Talent adding pre-washed magnetic beads to each sample.
 - 4.3.2. Talent placing the samples into the hybridization oven.
- 4.4. Place the samples on a magnetic stand for approximately 1 minute until the solution becomes clear [1]. Transfer the supernatant to a fresh tube [2]. Incubate the tube containing the clear supernatant on the magnetic stand again [3] and transfer the

supernatant to a fresh tube to ensure complete removal of residual beads [4].

4.4.1. Talent placing the samples on a magnetic stand.

4.4.2. Talent transferring the supernatant to a fresh tube. Videographer's NOTE: 4.4.2 and 4.4.3 were combined in one clip

4.4.3. Talent placing the samples on a magnetic stand.

4.4.4. Talent transferring the supernatant to a fresh tube.

5. Hybridization and Bead-Based Capture

5.1. Next, add two volumes of hybridization buffer tempered at 37 degrees Celsius to each sample [1]. Add biotinylated DNA probes at a ratio of 1 microliter of 100 micromolar probe stock per 1 milliliter of lysate [2]. Incubate the samples at 37 degrees Celsius with end-over-end rotation for 12 to 16 hours in a hybridization oven [3].

5.1.1. Talent adding hybridization buffer to each sample.

5.1.2. Talent adding biotinylated DNA probes to the lysate.

5.1.3. Talent placing the samples on a rotator placed inside a hybridization oven.

5.2. Next, wash 100 microliters of streptavidin magnetic beads three times with cell lysis buffer just before use [1] and remove the residual buffer after the final wash [2]. Then, briefly centrifuge the hybridization reaction tubes [3].

5.2.1. Talent washing magnetic beads by adding cell lysis buffer.

5.2.2. Talent removing residual buffer from the washed magnetic beads.

5.2.3. Talent placing the hybridization reactions into the centrifuge.

5.3. Resuspend the washed beads in 1 milliliter of hybridized sample [1]. Return the bead suspension to the original tube [2] and incubate at 37 degrees Celsius with mixing for 30 to 40 minutes [3].

5.3.1. Talent resuspending the washed beads in the hybridized sample. Videographer's NOTE: 5.3.1 and 5.3.2 were combined in one clip

5.3.2. Talent returning the bead suspension to the original tube.

5.3.3. Talent placing the bead suspension for incubation.

5.4. After incubation, briefly centrifuge the samples [1]. Place the tubes on a magnetic stand

for 1 to 2 minutes, or until the solution becomes clear [2], then discard the supernatant [3].

5.4.1. Talent placing the samples into the centrifuge.

5.4.2. Talent placing the tubes on a magnetic stand.

5.4.3. Talent discarding the supernatant.

5.5. Then, add 1 milliliter of wash buffer to the beads [1] and place the tubes in a shaking incubator at 37 degrees Celsius for 5 minutes [2]. Transfer 1 to 10% of the washed beads to a fresh tube for ribonucleic acid analysis [3].

5.5.1. Talent adding wash buffer to the beads.

5.5.2. Talent placing the beads for washing in a shaker incubator.

5.5.3. Talent transferring washed beads to a fresh tube for ribonucleic acid analysis.

5.6. Finally, add sodium dodecyl sulfate sample buffer to the remaining beads [1]. Boil the samples at 95 degrees Celsius for 30 minutes to elute proteins and reverse cross-links [2].

5.6.1. Talent adding sodium dodecyl sulfate sample buffer to the beads.

5.6.2. Talent placing the samples for boiling.

Results

6. Results

6.1. ChIRP (*Chirp*)-Mass spectrometry analysis identified more than 190 proteins enriched relative to the negative control in GC2 cells [1-TXT].

6.1.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the left Venn diagram.* **TXT: ChIRP: Comprehensive Identification of RNA-binding Proteins**

6.2. cPDIRT-MS (*C-P-dirt-M-S*) at 4000 millijoules per square centimeters identified a selective set of proteins [1], more than two-thirds of which overlapped with conventional ChIRP-MS [2].

6.2.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the middle Venn diagram.*

6.2.2. LAB MEDIA: Figure 2C. *Video editor: Highlight the right panel.*

6.3. Most proteins identified with at least 2 unique peptides were shared between biological replicates in GC2 cells [1] and mouse testes [2].

6.3.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the overlapping region in the GC2 replicate Venn diagrams.*

6.3.2. LAB MEDIA: Figure 2D. *Video editor: Highlight the overlapping region in the testis cPDIRT-MS Venn diagram.*

6.4. Gene Ontology of reproducibly identified proteins from each dataset revealed biological processes broadly consistent with known functions of Tug1 (*tug-1*) [1].

6.4.1. LAB MEDIA: Figure 2E. *Video editor: Highlight the blue, red, and orange Gene Ontology bars.*

6.5. Comparison of conventional ChIRP-MS (*Chirp-M-S*) in GC2 cells, cPDIRT-MS in GC2 cells, and cPDIRT-MS in mouse testes identified 15 proteins significantly enriched across all datasets [1].

6.5.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the central intersection of the green, dark blue, and light blue circles.*

6.6. Enhanced crosslinking immunoprecipitation sequencing revealed clear and specific binding peaks on the Tug1 transcript for PABPC1 (*P-A-B-P-C-one*) [1] and CCT3 (*C-C-T-three*) [2].

6.6.1. LAB MEDIA: Figure 3C. *Video editor: Highlight the eCLIP-seq peaks labelled 'PABPC1-CLIP1'.*

6.6.2. LAB MEDIA: Figure 3C. *Video editor: Highlight the eCLIP-seq peaks labelled 'CCT3-CLIP1'.*

Pronunciation guides:

1. Crosslinker
Pronunciation link: No confirmed link found
IPA: /'krɒs.lɪŋ.kə/
Phonetic Spelling: kraws·ling·ker
2. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /'sɛn.trəˌfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj
3. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /suː.pəˈneɪ.tənt/
Phonetic Spelling: soo·per·nay·tuhnt
4. Pellet
Pronunciation link: <https://www.merriam-webster.com/dictionary/pellet>
IPA: /'pɛl.ət/
Phonetic Spelling: pel·uht
5. Decapsulated
Pronunciation link: No confirmed link found
IPA: /diːˈkæp.sjəˌleɪ.tɪd/
Phonetic Spelling: dee·kap·syuh·lay·tid
6. Testes
Pronunciation link: <https://www.merriam-webster.com/dictionary/testis>
IPA: /'tɛs.tiːz/
Phonetic Spelling: tes·teez
7. Collagenase
Pronunciation link: <https://www.merriam-webster.com/dictionary/collagenase>
IPA: /kəˈlædʒ.əˌneɪs/
Phonetic Spelling: kuh·laj·uh·nays
8. Seminiferous
Pronunciation link: <https://www.merriam-webster.com/dictionary/seminiferous>
IPA: /ˌsɛm.əˈnɪf.ə.əs/
Phonetic Spelling: sem·uh·nif·er·uhs
9. Tubules
Pronunciation link: <https://www.merriam-webster.com/dictionary/tubule>

IPA: /'tu:.bju:lz/

Phonetic Spelling: too-byoolz

10. Resuspend

Pronunciation link: No confirmed link found

IPA: /,ri:.sə'spend/

Phonetic Spelling: ree-suh-spend

11. Trypsin

Pronunciation link: <https://www.merriam-webster.com/dictionary/trypsin>

IPA: /'trɪp.sɪn/

Phonetic Spelling: trip-sin

12. Deoxyribonuclease

Pronunciation link: <https://www.merriam-webster.com/dictionary/deoxyribonuclease>

IPA: /di.ɑ:k.si.raɪ.boʊ'nu:.kli:.eɪs/

Phonetic Spelling: dee-ok-see-rye-boh-noo-klee-ays

13. Enzymatic

Pronunciation link: <https://www.merriam-webster.com/dictionary/enzymatic>

IPA: /,ɛn.zaɪ'mætɪ.ɪk/

Phonetic Spelling: en-zye-mat-ik

14. Micrometer

Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>

IPA: /maɪ'krɑ:..mə.tə/

Phonetic Spelling: my-krom-uh-ter

15. Strainer

Pronunciation link: <https://www.merriam-webster.com/dictionary/strainer>

IPA: /'streɪ.nə/

Phonetic Spelling: stray-ner

16. Lysis

Pronunciation link: <https://www.merriam-webster.com/dictionary/lysis>

IPA: /'laɪ.sɪs/

Phonetic Spelling: lye-sis

17. Vortex

Pronunciation link: <https://www.merriam-webster.com/dictionary/vortex>

IPA: /'vɔ:r.tɛks/

Phonetic Spelling: vor-tekks

18. Sonicate

Pronunciation link: <https://www.merriam-webster.com/dictionary/sonicate>

IPA: /'sɑ:..nə.keɪt/

Phonetic Spelling: sah-nuh-kate

19. Lysate

Pronunciation link: <https://www.merriam-webster.com/dictionary/lysate>

IPA: /'laɪ.seɪt/

Phonetic Spelling: lye-sayt

20. Ultrasonicator
 Pronunciation link: No confirmed link found
 IPA: /ˌʌl.trəˈsoʊ.nɪ.keɪ.tər/
 Phonetic Spelling: uhl-truh-soh-nuh-kay-ter
21. Aliquot
 Pronunciation link: <https://www.merriam-webster.com/dictionary/aliquot>
 IPA: /ˈæl.ɪ.kwɑːt/
 Phonetic Spelling: al-ih-kwaht
22. Microcentrifuge
 Pronunciation link: No confirmed link found
 IPA: /ˌmaɪ.kroʊˈsen.trəˌfjuːdʒ/
 Phonetic Spelling: my-kroh-sen-truh-fyooj
23. Hybridization
 Pronunciation link: <https://www.merriam-webster.com/dictionary/hybridization>
 IPA: /ˌhaɪ.brə.dəˈzeɪ.ʃən/
 Phonetic Spelling: hye-bruh-duh-zay-shuhn
24. Biotinylated
 Pronunciation link: No confirmed link found
 IPA: /baɪˈɑː.tɪ.nəˌleɪ.tɪd/
 Phonetic Spelling: bye-ah-tih-nuh-lay-tid
25. Streptavidin
 Pronunciation link: <https://www.merriam-webster.com/dictionary/streptavidin>
 IPA: /ˌstreɪ.təˈvæd.ɪn/
 Phonetic Spelling: strep-tuh-vad-in
26. Ribonucleic
 Pronunciation link: <https://www.merriam-webster.com/dictionary/ribonucleic%20acid>
 IPA: /ˌraɪ.boʊ.nuːˈkliː.ɪk/
 Phonetic Spelling: rye-boh-noo-kee-ik
27. Sodium dodecyl sulfate
 Pronunciation link: <https://www.merriam-webster.com/dictionary/sodium>
 IPA: /ˈsoʊ.di.əm/, /doʊˈdes.əl/, /ˈsɒl.fet/
 Phonetic Spelling: soh-dee-uhm · doh-des-uhl · sul-fayt
28. Elute
 Pronunciation link: <https://www.merriam-webster.com/dictionary/elute>
 IPA: /iˈluːt/
 Phonetic Spelling: ee-loot
29. Spectrometry
 Pronunciation link: <https://www.merriam-webster.com/dictionary/spectrometry>
 IPA: /spekˈtrɑː.mə.tri/
 Phonetic Spelling: spek-trom-uh-tree
30. Peptides
 Pronunciation link: <https://www.merriam-webster.com/dictionary/peptide>

IPA: /'pɛp.taɪdz/

Phonetic Spelling: pep·tydz

31. Ontology

Pronunciation link: <https://www.merriam-webster.com/dictionary/ontology>

IPA: /ɑ:n'tɑ:l.ə.dʒi/

Phonetic Spelling: ahn·taal·uh·jee

32. Immunoprecipitation

Pronunciation link: <https://www.merriam-webster.com/dictionary/immunoprecipitation>

IPA: /ɪ,mju:.noʊ.priˌsɪp.ə'teɪ.ʃən/

Phonetic Spelling: ih·myoo·noh·prih·sip·uh·tay·shuhn

33. Transcript

Pronunciation link: <https://www.merriam-webster.com/dictionary/transcript>

IPA: /'træn.skɹɪpt/

Phonetic Spelling: tran·skript