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Title: Isolation of Subcellular Ribosome Subpopulations Based on Recombinant Peptide Tag-specific Location-restricted Illumination-enhanced Biotinylation

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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? **Yes – the first portion will be filmed in a tissue culture room and the second portion will be filmed in a separate dark room.**

If **Yes**, how far apart are the locations? Same floor, ~100 feet 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. **Yes**

If **Yes**, please provide the **full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author willing to participate.

Kitra Cates, Postdoctoral Fellow [Stanford University School of Medicine]

Current Protocol Length

Number of Steps: 28

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. **Kitra Cates:** Our research investigates how ribosome composition and mRNA translation vary across subcellular domains to regulate local protein synthesis.
 - 1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Kitra Cates:** Current methods lack spatial and temporal precision; ALIBi addresses this through optogenetically controlled proximity labeling of local ribosomes.
 - 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. **Kitra Cates:** ALIBi enables the identification of ribosome-bound proteins and mRNAs in specific subcellular regions, revealing spatial differences in translation regulation.
 - 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. **Kitra Cates:** The key challenge is maintaining specific proximity labeling through careful control of targeting construct expression and illumination conditions.

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

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.5. **Kitra Cates:** Following this protocol, affinity-purified ribosomes and associated mRNAs can be analyzed using quantitative proteomic and transcriptomic approaches.

- 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.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

What value do you expect this video article to have for other researchers working in your field?

- 1.6. **Kitra Cates, Postdoctoral Fellow** (authors will present their testimonial statements live)

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

How does the video format of your article help you to share your work with the broader scientific community?

- 1.7. **Kitra Cates, Postdoctoral Fellow** : (authors will present their testimonial statements live)

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

Ethics Title Card

This research has been approved by the Administrative Panel on Biosafety (APB) at Stanford University

Protocol

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

2. Activation of ALIBi and Cell Lysis

Demonstrator: Kitra Cates

- 2.1. To begin, obtain biotinylated Ribosomal Protein L31 fused to FLAG-TEV-AviTag (*Flag-T-E-V-AviTag*) or L31-FTA (*L-thirty one-F-T-A*) mouse embryonic stem cells culture or mESCs (*M-E-S-C-S*) [1] and transfect them with AviTag-specific Location-restricted Illumination-enhanced Biotinylation or ALIBi (*AL-ee-bye*), GFP (*G-F-P*) control, or full-length biotin ligase or BirA (*bir-a*) plasmids [2].

2.1.1. WIDE: Talent sitting with culture tubes on the workbench. **Videographer:** Please obtain CU shot as well for this shot. NOTE: 2.1.1c: C U shot, 2.1.1a: Wide shot

2.1.2. Shot of transfecting the L31-FTA mouse embryonic stem cells with the plasmids. NOTE: Please use the second shot for this (includes mixing a conical tube by inversion), not the shot of pipetting drop-wise onto a 10-cm plate. 2.1.2b: C U, 2.1.2a: an alternating way to do this

- 2.2. Prechill the centrifuge to 4 degrees Celsius [1].

2.2.1. Talent setting the centrifuge temperature to 4 degrees Celsius.

- 2.3. With the lights off, transfer the transfected plates to a temporary 37 degrees Celsius incubator while preparing the transilluminator in the main incubator to minimize temperature fluctuations [1].

2.3.1. Talent transferring transfected plates into a temporary incubator with the room lights off.

- 2.4. Using 70 percent ethanol, wipe down the blue light transilluminator [1]. After connecting the transilluminator to power, place it in the main incubator [2]. Allow the incubator conditions to re-equilibrate while continuing the setup [3].

2.4.1. Talent wiping the blue light transilluminator with 70 percent ethanol.

2.4.2. Talent placing the transilluminator inside the main incubator.

2.4.3. Shot showing the transilluminator positioned in the incubator while conditions stabilize.

- 2.5. Warm an aliquot of media intended for biotin and cycloheximide or CHX (*C-H-X*) dosing to 37 degrees Celsius [1]. If preparing control samples without supplemental biotin, warm a separate aliquot for cycloheximide only [2].

- 2.5.1. Talent warming the media aliquot.
- 2.5.2. Talent preparing a separate warmed media aliquot for control samples.
- 2.6. Chill an aliquot of DPBS (*D-P-B-S*) intended for CHX dosing [1].
 - 2.6.1. Talent placing the DPBS aliquot on ice.
- 2.7. Prepare the RNase (*R-N-ase*) removal buffer, salt wash, lysis buffer, and wash buffers [1]. Using a pipette, mix buffers containing dense or viscous components to ensure even mixing [2]. Chill the prepared buffers on ice before use [3].
 - 2.7.1. Talent preparing the required buffers.
 - 2.7.2. Talent pipetting the completed buffers to mix thoroughly.
 - 2.7.3. Talent placing the prepared buffers on ice.
- 2.8. Resuspend the stock bottle of streptavidin beads by gentle pipetting or rocking [1]. Transfer 450 microliters of beads into a protein low-binding tube for each 10-centimeter plate of mESCs [2].
 - 2.8.1. Talent resuspending streptavidin beads by gentle pipetting.
 - 2.8.2. Talent transferring beads into a protein low-binding tube.
- 2.9. Place the resuspended beads on the magnetic rack for 1 minute, or until the beads have fully migrated [1]. Aspirate and discard the bead storage buffer without touching the bead pellet [2]. Wash the beads 2 times with 900 microliters of RNase removal buffer [3].
 - 2.9.1. Talent placing the resuspended beads on the magnetic rack
 - 2.9.2. Shot of aspirating and discarding the bead storage buffer.
 - 2.9.3. Talent adding RNase removal buffer to the beads. NOTE: 2.9.3a shows mixing
- 2.10. Wash the beads once with 900 microliters of salt wash [1].
 - 2.10.1. Talent adding salt wash buffer to the beads.
- 2.11. Wash the beads 2 times with 900 microliters of Wash I (*wash-one*) Buffer [1]. Store the beads in the Wash I Buffer at 4 degrees Celsius while performing activation and cell lysis [2].
 - 2.11.1. Talent adding Wash I Buffer to the beads.
 - 2.11.2. Talent placing the tube containing beads in wash buffer I at 4 degrees Celsius for storage.
- 2.12. Add biotin and CHX to the warmed aliquot of media and keep at 37 degrees Celsius until use [1]. Add CHX to the cold DPBS aliquot and keep it chilled until use [2].
 - 2.12.1. Talent adding biotin to the warmed media.

- 2.12.2. Talent adding cycloheximide to the warmed media.
- 2.13. Aspirate the media from the transfected plates [1]. Immediately add 10 milliliters of warm media containing CHX with or without biotin to each plate [2].
- 2.13.1. Talent aspirating media from the plates. NOTE: May be it's vocally slated as 2.13.1 + 2
- 2.13.2. Talent adding the prepared warm media to each plate.
- 2.14. Place negative control plates in a dark incubator [1]. Place the remaining plates directly on top of the transilluminator [2]. Turn on the transilluminator and incubate the plates for 15 minutes [3].
- 2.14.1. Talent placing control plates in a dark incubator.
- 2.14.2. Talent positioning plates on the transilluminator. NOTE: 2.14.2 and 2.14.3 are filmed in a single shot
- 2.14.3. Talent turning on the transilluminator.
- 2.15. Wash each plate 2 times with 10 milliliters of cold DPBS containing CHX [1]. Place the plates on ice between and after washes [2]. After aspirating the second wash, tilt the plate and aspirate any residual DPBS [3].
- 2.15.1. Talent adding cold DPBS to the plate.
- 2.15.2. Talent placing the plates on ice.
- 2.15.3. Talent aspirating residual DPBS from the tilted plate.
- 2.16. Keeping the plate flat on ice, add 2 milliliters of ice-cold lysis buffer and incubate the plate on ice for 5 minutes [1]. Tap the plate 2 to 3 times during incubation to dislodge cells [2].
- 2.16.1. Talent adding ice-cold lysis buffer to the plate while keeping it on ice.
- 2.16.2. Talent tapping the plate during incubation.
- 2.17. Using a micropipette, pipette the lysate up and down across the plate surface to dislodge any remaining cells [1]. Transfer the lysate to a chilled, labeled protein-low-binding tube [2].
- 2.17.1. Talent pipetting the lysate across the plate surface.
- 2.17.2. Talent transferring the lysate into a chilled labeled tube.
- 2.18. In difficult-to-lyse organelles, sonicate all samples for 45 seconds at 4 degrees Celsius, and if required, transfer the lysate into a larger tube before sonication [1]. Transfer the sonicated lysate into a 2-milliliter tube [2].
- 2.18.1. Talent placing samples for sonication.
- 2.18.2. Talent transferring the sonicated lysate into a 2 milliliter tube.

2.19. Incubate the lysate on ice for 15 minutes [1].

2.19.1. Talent placing the lysate on ice.

2.20. Centrifuge the lysate at 17000 *g* for 3 minutes at 4 degrees Celsius [1]. Transfer the supernatant into a chilled protein low-binding tube without disturbing the pellet [2].

2.20.1. Talent placing the lysate tube into the centrifuge.

2.20.2. Talent transferring the supernatant into a chilled protein low-binding tube while leaving the pellet undisturbed.

3. Affinity Purification and Elution

3.1. Using a magnetic rack, pellet the washed streptavidin beads [1]. Aspirate the Wash I Buffer [2].

3.1.1. Talent placing the tube containing washed streptavidin beads on a magnetic rack. NOTE: 2nd try was better

3.1.2. Talent aspirating the Wash I Buffer.

3.2. Transfer 1,200 microliters of clarified lysate to the tube containing the washed beads [1]. Tap the tubes gently to resuspend the beads [2]. Store the remaining clarified lysate at minus 80 degrees Celsius [3].

3.2.1. Talent transferring clarified lysate to the tube containing the washed beads.

3.2.2. Talent gently pipetting to resuspend the beads. NOTE: We completed a "tapping to mix" shot earlier (around 2.9-10) that could also be used here

3.2.3. Talent placing the remaining clarified lysate at minus 80 degrees Celsius for storage.

3.3. Incubate the lysate with the beads at 4 degrees Celsius in the dark on a tube rotator for 1 hour [1].

3.3.1. Talent placing the tube containing lysate and beads on a tube rotator.

3.4. During the incubation, prepare the tobacco etch virus or TEV (*T-E-V*) elution buffer [1].

3.4.1. Talent preparing the TEV elution buffer.

3.5. Pellet the beads on a magnetic rack [1]. Transfer the supernatant to a chilled protein low-binding tube [2]. Store the supernatant at minus 80 degrees Celsius [3].

3.5.1. Talent placing the tube on a magnetic rack to pellet the beads.

3.5.2. Talent transferring the supernatant to a chilled protein low-binding tube.

3.5.3. Talent placing the supernatant at minus 80 degrees Celsius for storage.

- 3.6. Wash the beads 2 times with 900 microliters of Wash I Buffer [1] and once with 900 microliters of Wash II Buffer [2].
 - 3.6.1. Talent adding Wash I Buffer to the beads.
 - 3.6.2. Talent adding Wash II Buffer to the beads
- 3.7. Add 450 microliters of TEV elution buffer to the beads [1].
 - 3.7.1. Talent adding TEV elution buffer to the beads.
- 3.8. Incubate the beads in TEV elution buffer for 1 hour at room temperature on a tube rotator [1].
 - 3.8.1. Talent placing the tube containing beads in TEV elution buffer on a tube rotator.
NOTE: 3.8.1a: running the rotator, 2nd try was better/ slower
- 3.9. Pellet the beads on a magnetic rack [1]. Transfer the eluate to a protein low-binding tube [2]. Store the eluate at minus 80 degrees Celsius if proteomics analysis or western blot is intended [3].
 - 3.9.1. Talent placing the tube on a magnetic rack to pellet the beads.
 - 3.9.2. Talent transferring the eluate to a protein low-binding tube.
 - 3.9.3. Talent placing the eluate at minus 80 degrees Celsius for storage.

Results

4. Results

- 4.1. Cells containing all system components showed a L31-FTA band [1], which was stronger with full-length BirA [2] and faint or absent when components were omitted [3].
 - 4.1.1. LAB MEDIA: Figure 2A. *Video editor: Emphasize the band in the lane 20 at 25 kDa in the streptavidin blot.*
 - 4.1.2. LAB MEDIA: Figure 2A. *Video editor: Emphasize the band in the lanes 1-4 at 25 kDa in the streptavidin blot.*
 - 4.1.3. LAB MEDIA: Figure 2A. *Video editor: Emphasize the faint bands in the lanes 5-19 and 21-24 at 25 kDa in the streptavidin blot.*
- 4.2. Endogenous biotinylated carboxylase proteins were detected in all lysates at 70 to 80 kilodaltons [1] and 120 to 130 kilodaltons [2].
 - 4.2.1. LAB MEDIA: Figure 2A. *Video editor: Emphasize the faint bands around 70–80 kDa that appear across all lanes in the streptavidin blot.*
 - 4.2.2. LAB MEDIA: Figure 2A. *Video editor: Emphasize the faint bands around 120–130 kDa that appear across all lanes in the streptavidin blot.*
- 4.3. Anti-V5 (*Anti-V-five*) probing confirmed expression of the split BirA fragments [1].
 - 4.3.1. LAB MEDIA: Figure 2A. *Video editor: Emphasize the bands in the α -V5 blot.*
- 4.4. Biotinylated L31-FTA decreased in the flow-through relative to the lysate [1].
 - 4.4.1. LAB MEDIA: Figure 2B. *Video editor: Emphasize the bands in the streptavidin blot.*
- 4.5. No biotinylated L31-FTA was present in the eluate following TEV-mediated elution [1].
 - 4.5.1. LAB MEDIA: Figure 2B. *Video editor: Circle and highlight lane 3 of the streptavidin blot and show the absence of a visible band.*
- 4.6. Anti-FLAG (*Anti-flag*) probing showed a cleaved L31-FTA band at a lower molecular weight [1] than intact L31-FTA [2].
 - 4.6.1. LAB MEDIA: Figure 2B. *Video editor: Emphasize the band in lane 3 of the α -FLAG blot, positioned slightly lower on the blot.*
 - 4.6.2. LAB MEDIA: Figure 2B. *Video editor: Emphasize the band in lanes 1 and 2 of the α -FLAG blot.*
- 4.7. Ribosomal protein S12 or Rps12 (*R-P-S-twelve*) probing demonstrated the presence of the small ribosomal subunit in the eluate [1].

- 4.7.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the band in lane 3 of the α -Rps12 blot.*
- 4.8. Anti-V5 probing showed that proteins that do not bind the ribosome were absent from the eluate [1].
 - 4.8.1. LAB MEDIA: Figure 2B. *Video editor: Circle and highlight lane 3 of the α -V5 blot and show the absence of visible bands.*
- 4.9. When 2.5-fold more lysate was used with the same 450 microliters of streptavidin-coated beads [1], a substantial amount of biotinylated L31-FTA remained in the flow-through [2].
 - 4.9.1. LAB MEDIA: Figure 2C. *Video editor: Emphasize the bands in lane 1 of the streptavidin blot.*
 - 4.9.2. LAB MEDIA: Figure 2C. *Video editor: Emphasize the bands in lane 2 of the streptavidin blot.*
- 4.10. Endogenous biotinylated proteins were partially depleted in the flow-through [1].
 - 4.10.1. LAB MEDIA: Figure 2C. *Video editor: Emphasize the bands around 70–80 and 120–130 kDa in lane 2 of the streptavidin blot.*
- 4.11. Endogenous biotinylated proteins were absent from the eluate [1] but were recovered after heating post-elution beads in Laemmli buffer [2].
 - 4.11.1. LAB MEDIA: Figure 2C. *Video editor: Circle and highlight the lane 3 of the streptavidin blot and show the absence of the bands near 70–80 kDa and 120–130 kDa.*
 - 4.11.2. LAB MEDIA: Figure 2C. *Video editor: Emphasize the bands in the lane 4 of the streptavidin blot near 70–80 kDa and 120–130 kDa .*
- 4.12. Anti-FLAG probing detected cleaved L31-FTA at a lower molecular weight [1] than intact L31-FTA [2].
 - 4.12.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the bands in the eluate lane (Lane 3) of the α -FLAG blot (high exposure) and α -FLAG blot (low exposure)*
 - 4.12.2. LAB MEDIA: Figure 2C. *Video editor: Highlight the bands in the lysate lane (Lane 1) of the α -FLAG blot (high exposure) and α -FLAG blot (low exposure)*
- 4.13. Anti-V5 probing showed that the split BirA fragments were absent from the eluate [1].
 - 4.13.1. LAB MEDIA: Figure 2C. *Video editor: Circle and highlight the lane 3 in the α -V5 blot and show the absence of visible bands.*

1. Biotinylated
Pronunciation link: <https://www.merriam-webster.com/dictionary/biotin>
IPA: /baɪˈɑː.tən.əˌleɪ.tɪd/
Phonetic Spelling: bye·ah·tuh·nuh·lay·tid
2. Ribosomal
Pronunciation link: <https://www.merriam-webster.com/dictionary/ribosomal>
IPA: /raɪˈboʊ.sə.məl/
Phonetic Spelling: rye·boh·suh·muhl
3. Embryonic
Pronunciation link: <https://www.merriam-webster.com/dictionary/embryonic>
IPA: /ˌem.briˈɑː.nɪk/
Phonetic Spelling: em·bree·on·ik
4. Transfect
Pronunciation link: <https://www.merriam-webster.com/dictionary/transfection>
IPA: /trænˈsfekt/
Phonetic Spelling: trans·fekt
5. Plasmid
Pronunciation link: <https://www.merriam-webster.com/dictionary/plasmid>
IPA: /ˈplæz.mɪd/
Phonetic Spelling: plaz·mid
6. Ligase
Pronunciation link: <https://www.merriam-webster.com/dictionary/ligase>
IPA: /ˈlaɪ.geɪs/
Phonetic Spelling: lye·gays
7. Cycloheximide
Pronunciation link: <https://www.merriam-webster.com/dictionary/cycloheximide>
IPA: /ˌsaɪ.kloʊˈhek.sɪ.mɑɪd/
Phonetic Spelling: sy·kloh·hek·sih·myde
8. Streptavidin
Pronunciation link: <https://www.merriam-webster.com/medical/streptavidin>
IPA: /strepˈtæv.i.dɪn/
Phonetic Spelling: strep·tav·ih·din
9. RNase
Pronunciation link: <https://www.howtopronounce.com/rnase>
IPA: /ˈɑːr.ɛn.eɪs/
Phonetic Spelling: ar·en·ays
10. Aspirate
Pronunciation link: <https://www.merriam-webster.com/dictionary/aspirate>
IPA: /ˈæs.pə.ɪt/
Phonetic Spelling: ass·puh·rayt
11. Microliters
Pronunciation link: <https://www.merriam-webster.com/dictionary/microliter>
IPA: /ˈmaɪ.kroʊ.liː.tər/
Phonetic Spelling: my·kroh·lee·ter

12. Organelles
Pronunciation link: <https://www.merriam-webster.com/dictionary/organelle>
IPA: /ˌɔr.gəˈnel/
Phonetic Spelling: or·guh·nel
13. Sonicate
Pronunciation link: <https://www.merriam-webster.com/dictionary/sonicate>
IPA: /ˈsɑː.nə.keɪt/
Phonetic Spelling: sah·nuh·kayt
14. Lysate
Pronunciation link: <https://www.merriam-webster.com/dictionary/lysate>
IPA: /ˈlaɪ.seɪt/
Phonetic Spelling: lye·sayt
15. Affinity
Pronunciation link: <https://www.merriam-webster.com/dictionary/affinity>
IPA: /əˈfɪn.ə.ti/
Phonetic Spelling: uh·fin·uh·tee
16. Elution
Pronunciation link: <https://www.merriam-webster.com/dictionary/elution>
IPA: /iˈluː.ʃən/
Phonetic Spelling: ee·loo·shuhn
17. Proteomics
Pronunciation link: <https://www.merriam-webster.com/dictionary/proteomics>
IPA: /ˌproʊ.tiˈɑː.mɪks/
Phonetic Spelling: proh·tee·ah·miks
18. Endogenous
Pronunciation link: <https://www.merriam-webster.com/dictionary/endogenous>
IPA: /enˈdɑː.dʒə.nəs/
Phonetic Spelling: en·dah·juh·nuhs
19. Carboxylase
Pronunciation link: <https://www.merriam-webster.com/dictionary/carboxylase>
IPA: /kɑːrˈbɑːk.səˈleɪs/
Phonetic Spelling: kar·bok·suh·lays
20. Laemmli
Pronunciation link: <https://www.howtopronounce.com/laemmli>
IPA: /ˈlem.li/
Phonetic Spelling: lem·lee