SUPPLEMENTAL DETAILED PROTOCOL:

Biosynthetic mRNA Capture in *Plasmodium falciparum*

Materials:

Generation and verification of *P. falciparum* capable of pyrimidine salvage

1. FCU donor DNA sequence (source: *Plasmodium* vector pHHT-FCU (Maier et al. 2006))
2. PCR amplify yFCU (FCU) with the following primers and your restriction site of choice added
    a. Forward- 5'-CTTAAGATGGTGACAGGGGGAATGG-3'
    b. Reverse - 5'-CGTACGTTAAACACAGTAGTATCTGTCACC-3'
3. *Plasmodium* vector in which FCU is to be inserted (plasmids used in current study: pLN-ENR-GFP (Nkrumah et al. 2006) and pCBM.BSD.5'16.GFP(Eksi et al. 2008))
4. *P. falciparum* strain to be modified (strains used in current study: 3D7 and F12)
5. Anti-yeast Cytosine Deaminase antibody (Pierce, cat. no. PA1-85365)
6. Anti-GFP antibody [9F9.F9] (Abcam, cat. no. ab1218)
7. *Slowfade* Gold antifade reagent with DAPI (Thermo Fisher Scientific, cat. no. S36938)

Thiol-tagging of *Plasmodium* mRNA

1. Transgenic *P. falciparum* parasites expressing FCU
2. 4-Thiouracil (4-TU) (Sigma Aldrich, cat. no. 440736)
    a. Prepare a 200mM stock solution of 4-thiouracil in DMSO. This should be protected from light and stored at -80°C.

RNA Extraction

1. TRIzol® (Thermo Fisher Scientific, cat. no. 15596-026)
2. Chloroform
3. Isopropyl alcohol
4. Sodium acetate
5. DEPC-treated water
6. Ethyl alcohol (75%)

Thio-RNA Biotinylation and Precipitation

1. *N,N*-Dimethylformamide (Sigma Aldrich, cat. no. D4551)
2. EZ-Link™ Biotin-HPDP (Pierce, cat. no. 21341)

a. Dissolve 4mg EZ-Link Biotin-HPDP in 4mL N,N-Dimethylformamide. Heat to 55°C for 5min and vortex well to ensure all Biotin-HPDP is in solution. Store 80µL aliquots at -20°C for up to six months.

3. 10X TE Buffer

    a. Prepare a 10mL solution of 100mM Tris-HCl (pH 7.4) (Fisher, cat. no. BP154) and 10mM EDTA (pH 8.0) (Sigma Aldrich, cat. no. E5134) in DEPC-treated water.

4. 5M NaCl

Detection of Biotinylated RNA

1. OmniPur® Agarose (VWR, cat. no. EM-2125)
2. 1X TAE Buffer

    a. Prepare a 1L solution of 1mM Tris-Acetate (pH 8.5) and 100µM EDTA in DEPC-treated water

3. Ethidium Bromide (10mg/mL)
4. 10X SSC Buffer

    a. Prepare a 1L solution of 1.5M NaCl and 150mM Sodium Citrate

5. Amersham Hybond-N+ (GE Healthcare, cat. no. RPN203B)
6. Handheld UV-light (245nm)
7. Blocking Buffer

    a. Prepare a 250mL solution of 125mM NaCl, 17mM Na_2HPO_4, 7.3mM NaH_2PO_4, and 1% Sodium Dodecyl Sulfate in DEPC-treated water

8. Wash Buffer A

    a. 1:10 dilution of Blocking Buffer in DEPC-treated water

9. Wash Buffer B (10x stock, pH 9.5)

    a. Prepare a 250mL solution of 100mM Tris, 100mM NaCl, 21mM $MgCl_2$

10. Pierce High Sensitivity Streptavidin-HRP (Thermo Fisher Scientific, cat. no. 21130)
11. Pierce ECL (Thermo Fisher Scientific, cat. no. 32209)
12. Radiographic Film (Amersham Hyperfilm MP, cat. no. 28906845)

Magnetic Separation and Precipitation of Biotinylated, Thio-RNA

1. Dynabeads® MyOne™ Streptavidin C1 (Thermo Fisher Scientific, cat. no. 65001)

    a. Prepare beads for RNA manipulation as per manufacturer's protocol.

2. DynaMag™-2 Magnet (Thermo Fisher Scientific, cat. no. 12321D)
3. Binding and Washing Buffer (B & W)

a. Prepare 50mL solution of 1M NaCl, 5mM Tris-HCL (pH 7.5), 500µM EDTA in DEPC-treated water

4. Yeast tRNA (10mg/mL) (Thermo Fisher Scientific, cat. no. AM7119)
    a. Dilute to 5µg/mL in DEPC-treated water
5. Ambion® Linear Acrylamide (5mg/mL), (Thermo Fisher Scientific, cat. no. AM9520)
6. 5% 2-mercaptoethanol (2-ME) (Sigma, cat. no. M3148)
    a. Prepare in DEPC-treated water prior to each use

Microarray Analysis of RNA

1. Oligo(dT) primer (IDT, ReadyMade™ Primer
2. Random primer (nonamer or hexamer) mix
    a. "(N:25252525)(N)(N) (N)(N)(N) (N)(N)N"
3. SuperScript II Reverse Transcriptase (Thermo Fisher Scientific, cat. no. 18064-014)
4. 100mM DTT (supplied with SuperScript II)
5. 5X RT Buffer (supplied with SuperScript II)
6. 50X aa-dUTP dNTPs
    a. For 50X mix: 30µl dATP (100mM), 15µl dCTP (100mM), 15µl dGTP (100mM) 15µl dTTP (100mM), and 30µl aa-dUTP (50mM) (Thermo Fisher Scientific, cat. no. AM8439)
7. Agilent RNA Spike-In Kit, two color (Agilent Technologies, cat. no. 5188-5279)
8. 0.1N NaOH
9. 0.1N HCl
10. DNA Clean & Concentrator™-5 (Zymo Research, cat. no. D4003)
11. 5M Sodium Bicarbonate
12. CyDye Post-Labeling Reactive Dye Packs (GE Healthcare, cat. no. RPN5661)
13. *P. falciparum* Agilent 8x15K Microarray (AMADID #037237) (Agilent Technologies, contact corresponding author for ordering)
14. 10X Gene Expression Blocking Agent (Agilent, cat. no. 5188-5281)
15. 2X Hi-RPM Hybridization Buffer (Agilent, cat. no. 5190-0403)
16. Backing Slides 8 chamber (Agilent, cat. no. G2534-60015)
17. 20X SSPE: dissolve 175.3g of NaCl, 27.6g Sodium phosphate monobasic, and 7.4g of EDTA in 800ml of deionized water. Adjust the pH to 7.4 with 10N NaOH. Adjust the volume to 1L with additional deionized water. Store at RT.
18. Wash buffer A: 700ml deionized water, 300ml 20X SSPE, 0.25ml 20% N-laurylsarcosine. Store at RT.

19. Wash buffer B: 997ml deionized water, 3ml 20X SSPE, 0.25ml 20% N-laurylsarcosine. Store a RT.
20. Acetonitrile (caution: extremely flammable, should be used in a fume hood)
21. Microcentrifuge
22. Hybridization chamber gasket slides: 8 microarrays/slide, 5 slides/box (Agilent, #G2534-60014)
23. Stainless steel hybridization chamber with clamp assembly (Agilent #G2534A)
24. Hybridization oven with rotator for hybridization chambers; temperature set at 65°C and rotation at 10rpm (Agilent, #G2545A and #G2530-60029)
25. Glass slide staining dish (x3) with removable slide rack
26. Magnetic stir plate (x3)
27. Magnetic stir bars, 1" (x3)
28. Forceps for separating and handling glass slide.
29. Powder-free latex gloves
30. Sterile pipette tips
31. Sterile, nuclease free 1.5ml tubes

Procedure:

Generation and verification of *P. falciparum* capable of pyrimidine salvage

Introduction of the FCU fusion transgene will be dependent on the species or strain of *Plasmodium* that is being studied. FCU has been cloned into both pLN-ENR-GFP (Nkrumah et al. 2006) and pCBM.BSD.5'16.GFP (Eksi et al. 2008) which results in both the addition of a 3'-GFP-tag and either the 5'-UTR of *pfs16* or *calmodulin*. The FCU gene (1123bp) was amplified from pHHT-FCU (Maier et al. 2006). Considerations should be made for the stage-specific expression-of-interest and the promoter driving FCU should be adjusted accordingly.

Constructed plasmids were introduced by electroporation using a well-established transfection protocol in *Plasmodium falciparum* (Fidock and Wellems 1997). Expression of the FCU transgene was verified in each line via western blot analysis using anti-GFP (1:2000) or anti-CD (1:500) antibodies (Figure 1). Localization and cell-population expression of FCU was carried out by immunofluorescent microscopy as per well-established protocols (Tonkin et al. 2004) using an Olympus BX61. Cells were incubated with anti-GFP (1:1000) or anti-CD (1:100) followed by the addition of *SlowFade* Gold antifade reagent with DAPI prior to being immobilized on glass slides. Images were taken on an Olympus BX61 fluorescent microscope and processed using Slidebook 5.0 (www.intelligent-imaging.com) (Figure 1).

4-TU Delivery

Stock solutions of 4-TU should be prepared as stated in the Materials section and thawed immediately prior to use. Proper dilutions can be added directly to *in vitro* cultures of *P. falciparum*. The optimal concentration for long-term incubation (up to 48 hours) was determined to be 40µM. However, the amount of 4-TU used to biosynthetically modify parasite transcripts should be reflective of the experimental design and adjusted accordingly. For example, short pulses of 4-TU can be added at a higher concentration to increase the proportion of mRNA that is thiol-modified. This should be determined on a case-by-case basis depending upon the nature of the study (ie: transcript synthesis or decay) and the proportion of the transcripts that are being measured (ie: sub-population analysis). Recently, studies in mice have also shown that intraperitoneal injection of 4-TU at a concentration of 400mg/kg results in sufficient mRNA labeling in as

little as two hours (Gay et al. 2013; Gay et al. 2014). This could be adapted to allow for *in vivo* transcriptional studies of the rodent malarias *P. berghei, P. yoelii, and P. chabaudi*.

Extraction and Preparation of RNA

The following is for *in vitro* cultures of *P. falciparum.* Note: Typically 100mL of transgenic *cam*-FCU-GFP parasite culture (5% hematocrit, 10-15% parasitemia, 10min pulse of 40µM 4-TU) will result in sufficient amounts of labeled RNA for downstream microarray analysis.

1. Pellet culture containing infected and uninfected erythrocytes at 4°C (3000*g* for 5min) and remove supernatant prior to RNA extraction.

2. In order to minimize global genome perturbations due to stress, total RNA isolation is performed immediately following supernatant removal with TRizol® as per manufacturer's instructions.

3. Isolated total RNA should be checked for quality and purity on a NanoDrop© ND-1000 or by your RNA quantitation and quality protocol-of-choice.

    a. A_{260}/A_{280} ratios should be ≥2 and A_{260}/A_{230} ratios should be >2. These values are indicative of good quality RNA that is free of contaminants that would influence downstream processes.

    b. Any samples that do not meet or surpass recommendations should be re-precipitated using common laboratory protocols or column purified using RNeasy MinElute Clean-up Kit (Qiagen, cat. no. 74204).

4. If your RNA is of good quality, it can be stored at -80°C for months, or proceed immediately to the next step. (Note: Set aside 1-2µg for detection on a Northern blot as a control for non-biotinylated RNA.)

Biotinylation and precipitation of thiol-tagged RNA

1. Resuspend thiol-tagged RNA at a concentration of 0.4µg/µl in a final volume of 100µl in a 1.7mL Microfuge tube. Typically, 40µg is enough RNA to yield around 1µg of thiolated RNA for analysis. This is dependent upon the length and concentration of the 4-TU incubation and should be adjusted based upon experimental design.

2. Add 26.6µl of 10x TE buffer.

3. Heat the RNA at 70°C for 2min to denature, then place on ice for 1min.

4. Add 2µl of Biotin-HPDP reagent (1mg/mL) per µg of RNA.

5. The incubation should proceed for 3hrs at 25°C protected from light.

6. Following incubation period, add 1/10 volume of 5M NaCl and 1 volume of 2-propanol for precipitation.

7. Mix well and incubate at 25°C for 5 min. (Note: Alternatively, precipitation can be carried out over night at -20°C)

8. Spin down precipitated RNA for 30min at maximum speed in a 4°C refrigerated microcentrifuge.

9. Remove supernatant by pipetting.

10. Wash pellet with 75% Ethyl alcohol.

11. Centrifuge at 4°C for 10min and remove supernatant.

12. Let air dry for 10min to evaporate residual ethanol. (Note: May have to air dry for a longer period of time depending upon amount of ethanol remaining after pelleting RNA. Any remaining ethanol will interfere with downstream sample processing.)

13. Resuspend in 80µl of DEPC-water or the amount necessary to result in a final concentration of 0.5µg/µl. Mix well to ensure the RNA solubilizes. (Note: Set aside 1-2µg for detection of biotinylation on a Northern blot)

14. Store RNA at -80°C until ready to use.

Northern blot detection of biotinylated RNA

1. Prepare an agarose gel (1% w/vol in 1x TAE with ethidium bromide) in RNase free gel apparatus. (Note: RNase Zap (Thermo Fisher Scientific, cat. no. AM9780) can be used to treat all parts of the gel apparatus)

2. Place gel, buffer, and apparatus in a cold room or refrigerator to equilibrate to 4°C for an hour.

3. Prepare samples by adding 2µg in a final volume of 10µl of DEPC-treated water with RNase-free gel loading buffer. (Note: The amount of RNA run will depend on duration of 4-TU incorporation. In this protocol, it is recommended to run 2µg of biotinylated thiol-RNA. Remember to include the proper controls, such as total RNA from prior to biotinylation. Biotinylated RNA from control cultures that do not express FCU should also be assessed for background 4-TU incorporation.)

4. Prior to loading samples in the wells, centrifuge for 1min at maximum speed to pellet any free Biotin-HPDP that has precipitated out of solution and will increase the background signal.

5. Load samples into the wells and run gel at 200V for 30min at 4°C. (Note: The gel apparatus must be monitored for steam condensation as heat can degrade the RNA. If overheating is occurring, use ice-packs to cover the apparatus.)

6. Disassemble the apparatus, remove gel, and photograph using standard laboratory procedures.

7. Set-up a capillary transfer apparatus according to traditional northern-blotting techniques. (Note: In this protocol, efficient transfer is achieved using 10x SSC transfer buffer and Amersham Hybond-N+ membrane)

8. The transfer should be allowed to proceed overnight at room temperature.

9. The following day, remove membrane with transferred RNA (should be able to observe RNase-free loading buffer on the membrane). Be sure to mark the position of the lanes on the membrane.

10. Crosslink RNA to the membrane at 245nm wavelength with a hand-held UV cross-linker for 1min. (Note: To ensure membrane does not dry out while cross-linking, place on top filter paper that has been saturated with transfer buffer)

11. Incubate the membrane in Blocking Buffer for 30min while shaking. After incubation, discard buffer.

12. Incubate the membrane with streptavidin-HRP (1:10,000 in Blocking Buffer) for 5min with shaking. After incubation, discard buffer.

13. Wash the membrane with “Wash Buffer A” for 20min with shaking. Discard buffer.

14. Repeat step 13.

15. Wash the membrane with “Wash Buffer B” for 5min with shaking. Discard buffer.

16. Repeat step 15.

17. Prepare ECL reagent, add to the membrane and incubate for 1min. Remove the solution.

18. Expose membrane to radiographic film and develop as per standard laboratory protocols.

Biotin-Thiol-RNA Purification

1. Thaw biotinylated RNA on ice. (Note: RNA should be at a concentration of 0.5µg/µl)

2. Wash 2µl of Dynabeads® MyOne™ Streptavidin C1 magnetic beads per µg of RNA. Resuspend in Blocking & Washing (B&W, prepared as per manufacturer's protocol) buffer the same as the initial volume of unwashed beads. (Note: Beads should be washed as per the manufacturers protocol for use with RNA)

3. Beads should be pipetted into a 1.7ml Microfuge tube for use on a DynaMag™-2 Magnet, using one tube per sample with a maximum of 16 samples per purification. (Note: The number of samples processed for purification will vary depending on the capacity of the magnetic apparatus. If your experiment consists of more samples than the stand is able to hold, the remaining samples should be stored at -80°C until the first set of samples are purified.)

4. Add 1µg yeast tRNA per 5µl of MPG slurry to block non-specific RNA interactions with the streptavidin magnetic beads and incubate for 20min at room temp with rotation.

5. Place the tube in a magnetic stand and collect the beads for 1min. Discard supernatant.

6. Wash the beads 3x in B & W buffer.

    a. Resuspend the beads in 1mL of B & W buffer, mix by pipetting and collect beads on magnetic stand for 1min before discarding the supernatant. Repeat 3x.

7. Prepare biotinylated RNA sample for addition to the beads.

    a. Remove free biotin that has precipitated out of solution by centrifuging RNA sample for 1min at maximum speed in a microcentrifuge.

    b. Transfer RNA to a fresh Microfuge tube.

    c. Denature the RNA by heating at 70°C for 2min and place on ice for 1min.

8. Add the biotinylated RNA to washed beads. Use one preparation of beads per RNA sample in individual Microfuge tubes.

9. Incubate at room temp with rotation for 20min. (Note: during this incubation, warm 2mL of B & W buffer per sample to 65°C for step 11d-f below)

10. Place the tube in the magnetic stand and collect for 1min. Carefully remove the supernatant with a pipette and place into a clean Microfuge. Save this sample for precipitation on ice. (Note: This contains the non-biotinylated/non-thiol-tagged RNA)

11. To ensure the removal of non-specific, unlabeled RNA interactions with streptavidin beads, RNA-bound beads should be treated with a series of wash steps as follows.

    a. Resuspend in 1mL B & W buffer and incubate at room temp for 5min with rotation.

    b. Collect beads on the magnetic stand for 1 min. Discard buffer.

    c. Repeat wash step a.

d. Resuspend in 1mL B & W buffer warmed to 65°C. Mix by pipetting and let sit at room temp for 1min. (Note: When the warm buffer is mixed with the beads, the RNA becomes relaxed, thereby removing any non-biotin or secondary RNA-RNA interactions with the beads.)

e. Collect beads on the magnetic stand for 1min. Discard buffer.

f. Repeat wash step d.

g. Repeat wash step a. (Note: this is the last wash and should contain no RNA. If you wish to verify the absence of RNA, save the eluate on ice for later analysis.)

12. Elute the bound RNA with 100µl of FRESHLY prepared 5% 2-ME in DEPC-treated water. This step will provide the reducing conditions necessary to disrupt the disulfide bridge of the biotin-streptavidin interaction, releasing bound RNA from the beads.

13. Incubate the beads for 10min at 25°C with rotation.

14. Collect beads for ≥ 1min. Carefully remove the supernatant with a pipette and place in a clean Microfuge tube. Save this sample for precipitation on ice. (Note: This contains thiol-tagged RNA.)

Precipitation of Purified mRNA

1. Precipitate the samples of unbound RNA (non-biotinylated/non-thiol-tagged RNA) and eluted RNA (thiol-tagged RNA) by adding an equal volume of isopropanol, 1/10 volume Ambion® Linear Acrylamide, and 1/10 volume of 5M NaCl. Mix thoroughly by lightly vortexing.

2. Incubate all samples at room temperature for 5min (or O/N at -20 °C), then spin at maximum speed for 30min in a 4°C refrigerated microcentrifuge.

3. Discard supernatant and wash the precipitated RNA with 100µl of 75% ethanol. Spin at maximum speed for 10min in a 4°C refrigerated microcentrifuge.

4. Remove 75% ethanol by pipetting and place tubes in a speed-vac to dry for 2min (Note: Do not over dry but ensure that all ethanol is evaporated from the sample. Residual ethanol will inhibit downstream enzymatic reactions.)

5. Resuspend the pelleted RNA in RNase-free water (10-15µl).

6. Determine RNA yields by NanoDrop© ND-1000. (Note: A_{260}/A_{280} ratios may be low when analyzing the tagged-RNA; if this occurs, spin the sample for 1 min to pellet residual beads as they might interfere with the readings at A_{280}. Repeat the reading with RNA taken from the upper portion of the sample.)

cDNA Preparation and Dye-Coupling

1. For cDNA synthesis, follow the protocol available at http://llinaslab.psu.edu/wp-content/uploads/2013/12/RT_and_amino-allyl_2010.pdf with the following adjustments:

    a. The yields of thiol-RNA are quite low and the protocol should be adjusted accordingly. Standard amounts of input thiol-RNA for cDNA synthesis range between 500-2000ng. Reactions that include non-thiol-tagged RNA should have an input of 2000ng.

    b. Due to unequal input RNA concentrations, downstream microarray hybridization and analysis could be difficult. To assist with normalization, Agilent RNA Spike-in Kit can be utilized to provide positive controls during cDNA generation and microarray processing.

c. Following reverse transcription, 1µl of purified cDNA should be run on a 1% agarose gel to verify synthesis.

2. For dye-coupling, following the protocol available at http://llinaslab.psu.edu/wp-content/uploads/2013/12/cDNA_Labeling_03_16_11_Llinas.pdf

Microarray Hybridization and Washing

1. For Agilent array hybridization and washing, follow the protocol available at http://llinaslab.psu.edu/wp-content/uploads/2013/12/Agilent_Array_Hybridization_Washing_03_16_11_Llinas.pdf

Processing Microarray Data

1. Optimally, microarray slides should be scanned with an Agilent SureScan scanner at a 5µm resolution and dye-intensities calculated by Agilent Feature Extractor. Alternatively, microarrays can be scanned by any colorometric laser scanners for glass-slides and data extracted by your gene expression analysis software of choice.

2. The output text files can then be imported to R and analyzed using Rnits Microarray analysis (R Bioconductor package, Rnits version 1.2.0) as per commands provided (Sangurdekar 2014).

3. The resultant analysis yields normalized Log2 ratios for each probe or gene depending upon your analysis settings. The gene/probe Log2 ratios are ranked based on p-value of significant change versus the control over time.

References:

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