

Figure S1: The effects of 4-TU incorporation and capture throughout *P. falciparum* 3D7^{cam} development within human erythrocytes. A) 4-TU has a concentration dependent effect on development. *P.f.* 3D7^{cam} growth in the presence of various concentrations of 4-TU (0, 20, 40, 80, 160 μ M) was monitored every 12hrs for 72hrs by Giemsa-stained thin blood films. Parasitemia percentages (infected/uninfected erythrocytes) are represented as an average of two independent experiments \pm s.d. across this timeframe, 40 μ M (red bars) or less had no effect on parasite growth. B) Transcript abundance profiles during the IDC were not altered by the addition of 40 μ M 4-TU. Total RNA isolated from 3D7^{cam} and 3D7^{pfs16} incubated for 12h with 40 μ M 4-TU was compared by DNA microarray. The Pearson correlation (r) of the $\text{Log}_2(\text{Cy}3/\text{Cy}5)$ ratio for each gene across the array at 0, 12, 24, and 36h was calculated. The median correlation of the log_2 ratio of each gene over time in either 3D7^{cam} (top panel) or 3D7^{pfs16} (middle panel) compared to a previously published 3D7 mRNA abundance timecourse (Kafsack et al. 2012) was 0.850 and 0.846, respectively. The median correlation between total RNA abundance of 3D7^{cam} and 3D7^{pfs16} was determined to be 0.815. C) To ensure that 4-TU is efficiently incorporated throughout the IDC, highly synchronized *P.f.* 3D7^{cam} was grown in the presence of 40 μ M 4-TU for various lengths of time (0, 1, 2, 4 hours) during three major developmental stages during the IDC (ring, trophozoite, and schizont). Following incubation, total RNA was extracted, biotinylated and separated on an agarose gel (2 μ g/lane) (top panel). Incorporation of 4-TU throughout the IDC was assessed by Northern blot and probed with streptavidin-HRP (bottom panel). D) Thiolated RNA can be separated from the parasite total RNA using streptavidin magnetic beads. Biotinylated RNA from both wild-type 3D7 and 3D7^{cam} eluted from streptavidin magnetic beads was run on an ethidium bromide stained gel as follows: (1) total RNA, (2) flow-through/non-biotinylated RNA, (3) last wash of 4, and (4) β -mercaptoethanol eluted/thiol-tagged RNA.

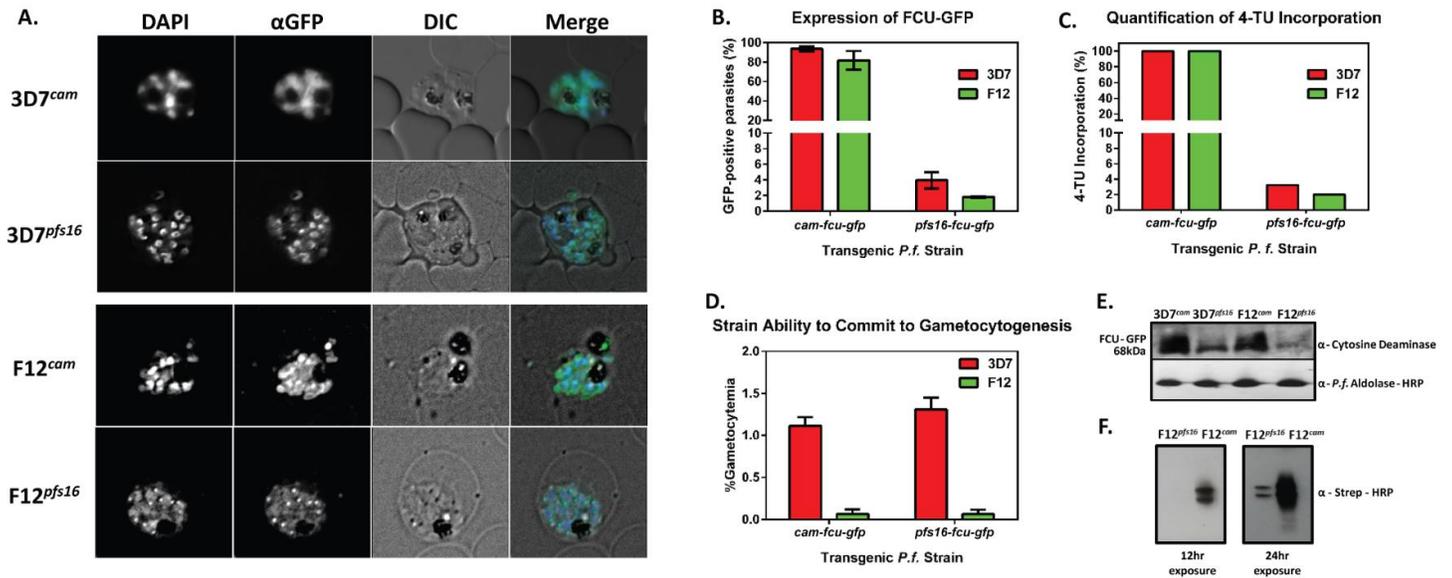


Figure S2: Confirmation of Various Transgenic Strains of *P. f.* Expressing FCU-GFP. A) Fluorescent microscopy images of each parasite line expressing *cam*- and *pfs16-fcu-gfp* (green) and DAPI dsDNA dye (blue) verified the presence of the protein fusion throughout the parasite. B) *P. falciparum* strains 3D7 (red) and F12 (green) were transgenically modified to express FCU-GFP under the control of the *calmodulin* or *pfs16* promoters. The proportion of mixed-stage asexual parasite populations expressing FCU-GFP was determined by calculating the percentage of FCU-GFP positive parasites (GFP positive/non-fluorescent) by fluorescence microscopy. Results represent the average of two independent experiments \pm s.d. C) Thiol-incorporation and biotinylation are decreased in strains expressing *pfs16-fcu-gfp*. Percent incorporation of 4-TU into the total pool of RNA was quantified using ImageJ from Northern blots of 2 μ g of biotinylated RNA from 3D7 and F12 expressing FCU-GFP under the control of *cam* or *pfs16* probed with streptavidin-HRP. Blots utilized for this quantification are as follows 3D7 (Figure 3C) and F12 (Figure S2E). The level of thiolated-RNA in *pfs16-fcu-gfp* expressing parasites was calculated as a percentage of total labeling in *cam-fcu-gfp* expressing lines and normalized based on the length of time the film was exposed. D) Each transgenic strain maintained their parental gametocyte production phenotype. The percentage of stage III gametocytes was determined by Geimsa-stained thin-blood smears. Percentages represent the average of two biological replicates \pm s.d. E) Western blot analysis of parasite protein extracts probed with α -cytosine deaminase confirmed a 68kDa band representing expression of either *cam*- or *pfs16-fcu-gfp* in *P. f.* strains 3D7 and F12. As a loading control, the membrane was probed with α -*P. f.* aldolase-HRP. F) Confirmation of promoter-driven thiol-tagged RNA in F12 *P. f.* transgenic lines (*pfs16-fcu-gfp* and *cam-fcu-gfp*). Total RNA from each parasite line grown in the presence of 40 μ M 4-TU for 12 hours was extracted and biotinylated. The presence of biotinylated, thiol-tagged RNA was assessed by Northern blot and probed with α -streptavidin-HRP (bottom panel). Film exposure times are noted and varied depending upon the time required to visualize biotinylation of RNA from F12^{*pfs16*}.

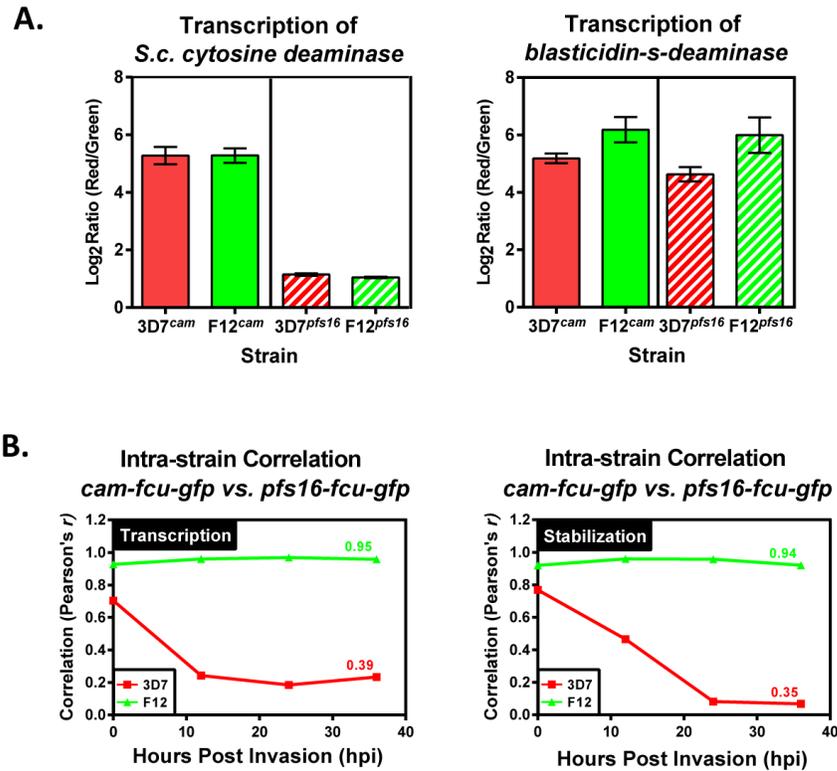
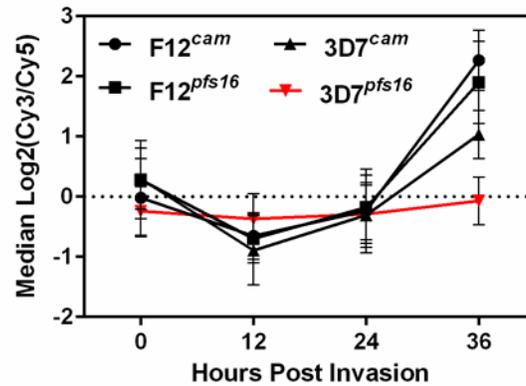


Figure S3 related to Figure 4: The expression level of *fcu-gfp* are promoter dependent. A) Nascent transcription of *cytosine deaminase*, a portion of the *fcu-gfp* fusion gene driven by either 5'*cam* or 5'*pfs16*, is represented as the median normalized Log₂(red/green) ratios in each line over time (error bars represent the s.d. of four time-points). In addition, transcription of *blasticidin-s-deaminase* (*bsd*, driven by 5'*hsp86*) is represented in the same manner. The gene product of *bsd* is the drug selectable marker of the episomal plasmid containing *fcu-gfp* and transcription should not be strain-specific. B) Intra-strain Pearson correlation coefficient (*r*) of all transcribed (left panel) and stabilized (right panel) mRNAs across the DNA microarray timecourse from either *cam-fcu-gfp* or *pfs16-fcu-gfp* expressing 3D7 (red) and F12 (green) parasites. The overall median *r* is noted for each strain.

Median Log₂(Cy3/Cy5) of Genes in Cluster 8



GO-term enrichment of Genes in Cluster 8

GO number	GO term	P-value
0040011	Locomotion	4.94e-10
0051828	Entry into other organism	9.69e-10
0052126	Movement in host	9.69e-10
0044409	Entry into host	9.69e-10

Figure S4 related to Figure 4: Asexual-specific genes transcriptionally silent in 3D7^{pfs16} committed gametocytes. A) The median Log₂(Cy3/Cy5) ratios of 319 genes in Cluster 8 plotted over time from 3D7^{cam}, 3D7^{pfs16}, F12^{cam} and F12^{pfs16} with \pm s.d. B) GO-term enrichment of genes in Cluster 3 and associated p-value score.

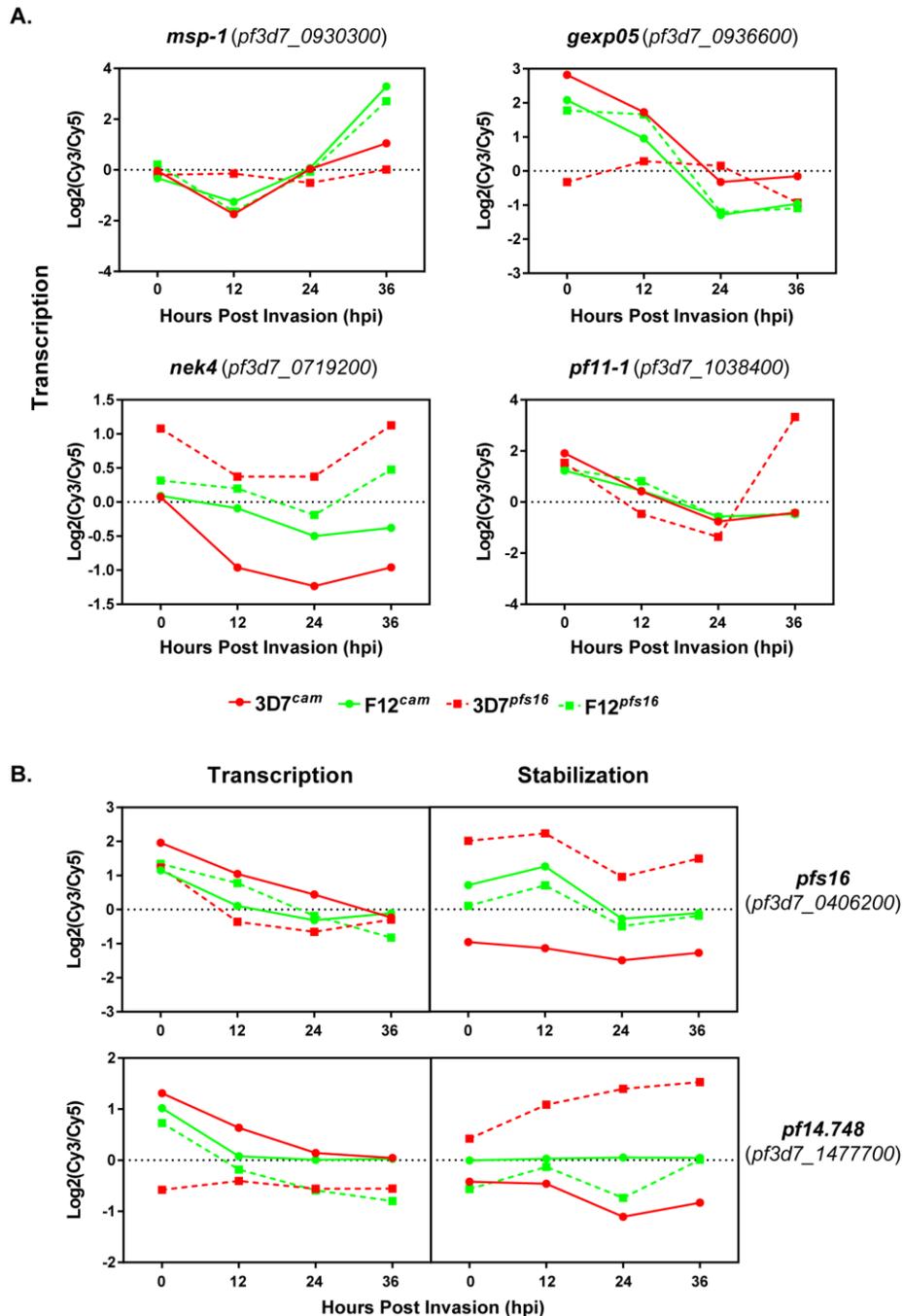


Figure S5: Biosynthetic capture reveals temporal and population-specific mRNA dynamics. A) Nascent transcription genes which are characteristic of either asexual development (*msp1*), gametocyte commitment (*gexp05*), or gametocyte maturation (*puf2*, *pf11-1*) were plotted over time for all strains (3D7^{cam}, solid red circles; 3D7^{pfs16}, dashed red squares; F12^{cam}, solid green circles; and F12^{pfs16}, dashed green squares). B) Transcription and stabilization dynamics for two gene markers of gametocyte commitment, *pfs16* and *pf14.748*, are plotted over time for each strain. Each plot demonstrates that transcription of *pfs16* occurs in all strains; however, in parasites that become gametocytes (3D7^{pfs16}) this transcript is stabilized. Similarly, *pf14.748* is stabilized in 3D7^{pfs16} parasites which are becoming gametocytes but not in F12^{pfs16}.

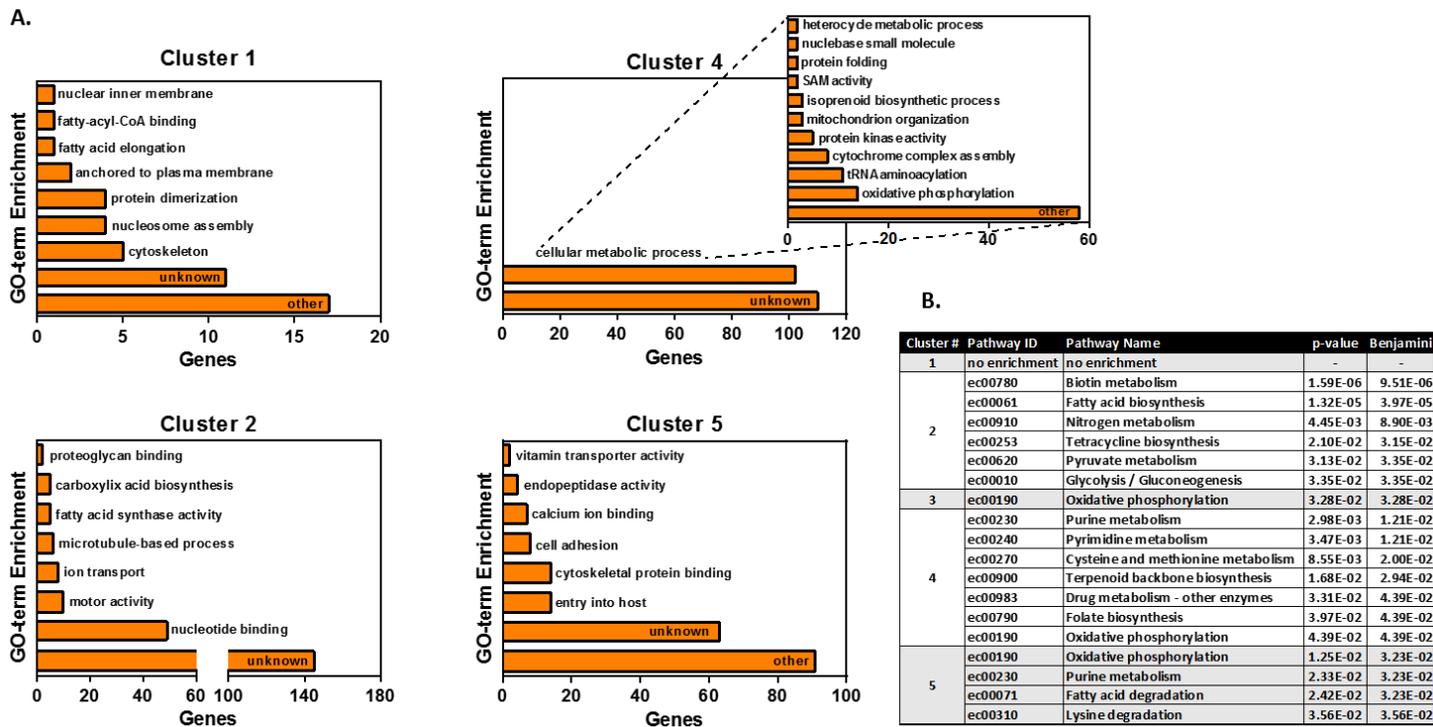


Figure S6 related to Figure 5: Molecular, biological, and metabolic enrichment analysis of gametocyte-specific genes. A) Molecular Process and Biological Function GO-term enrichment ($p\text{-value} \geq 0.005$) of the 808 gametocyte-specific transcribed and stabilized genes identified in 3D7 (\log_2 fold change $pfs16/cam \geq 1$, $p\text{-value} \leq 0.001$) by cluster. B) KEGG pathway enrichment ($p\text{-value} \geq 0.05$) of gametocyte-specific transcribed and stabilized genes (\log_2 fold change $pfs16/cam \geq 1$, $p\text{-value} \leq 0.001$).

<u>5' enriched motif</u>	<u>z-score</u>	<u>p-value</u>	<u>Potential Interacting Protein</u>
	191.0	1.33e-55	N/A
	47.8	6.89e-16	PF3D7_1350900 ^α
	28.7	3.69e-10	PF3D7_1139300 ^α
	26.4	9.57e-10	PF3D7_0420300, PF3D7_0802100, PF3D7_1305200, PF3D7_1456000 ^α
	24.1	5.51e-9	PF3D7_1222600 (AP2-G) ^{α, β}
<u>3' enriched motif</u>	<u>z-score</u>	<u>p-value</u>	<u>Potential Interacting Protein</u>
	18.6	1.25e-6	PF3D7_0417100 (PUF2) ^γ
	13.9	8.38e-6	N/A

Figure S7 related to Figure 5: Gametocyte-specific genes are enriched for potential regulatory motifs. Genes identified as gametocyte-specific identified in 3D7 were analyzed for DNA sequence motif enrichment within their 5' and 3' UTRs (Elemento et al. 2007). Known *trans*-regulatory factors or RNA-binding proteins previously demonstrated to interact with each enriched motif are noted in red. ^α (Campbell et al. 2010); ^β (Kafsack et al. 2014); ^γ (Cui et al. 2002)

Campbell TL, De Silva EK, Olszewski KL, Elemento O, Llinas M. 2010. Identification and genome-wide prediction of DNA binding specificities for the ApiAP2 family of regulators from the malaria parasite. *PLoS Pathog* **6**: e1001165.

Cui L, Fan Q, Li J. 2002. The malaria parasite *Plasmodium falciparum* encodes members of the Puf RNA-binding protein family with conserved RNA binding activity. *Nucleic Acids Research* **30**: 4607-4617.

Elemento O, Slonim N, Tavazoie S. 2007. A universal framework for regulatory element discovery across all genomes and data types. *Mol Cell* **28**: 337-350.

Kafsack BF, Rovira-Graells N, Clark TG, Bancells C, Crowley VM, Campino SG, Williams AE, Drought LG, Kwiatkowski DP, Baker DA et al. 2014. A transcriptional switch underlies commitment to sexual development in malaria parasites. *Nature* **507**: 248-252.

SUPPLEMENTAL MATERIALS AND METHODS:

Verification of transgene expression by immunofluorescence microscopy. FCU-GFP expression in transgenic parasite lines was verified by immunofluorescence imaging analysis. Mixed stage transgenic *P. falciparum* infected erythrocytes were fixed with 4% formaldehyde/0.0075% glutaraldehyde (Polysciences, Inc.) using the method of Tonkin *et al.* (Tonkin *et al.* 2004). Fixed cells were permeabilized with 0.01% Triton-x 100 (Sigma-Aldrich) and blocked with 10% normal goat serum and 3% bovine serum albumin (BSA). To detect FCU-GFP localization, the suspension was incubated with a 1:1000 dilution of a mouse anti-GFP antibody (Thermo Fisher Scientific) followed by secondary antibody Alexafluor-488 rat anti-mouse (Molecular Probes) diluted to 1:500. Cells were resuspended in Slowfade Antifade reagent with DAPI to stain parasite nuclei (Thermo Fisher Scientific) and mounted on slides using Fluoromount-GTM (Southern Biotech). Fluorescent microscopic images were obtained with an Olympus BX61 system and deconvoluted using SlideBook 5.0 software (Intelligent Imaging Innovations).

Gametocyte generation. To determine the ability of each strain to produce mature gametocytes, a standard protocol for gametocyte induction was utilized (Eksi *et al.* 2005). In brief, 1% synchronous ring-stage parasites in 5% hematocrit were induced to generate gametocytes by nutrient deprivation. More specifically, 50% of 24 hour spent medium was removed and replaced with fresh medium to initiate gametocyte development. Medium was refreshed 48 hours later and subsequently maintained under standard conditions. Five days post-induction, Giemsa-stained thin-blood smears were assessed for percentage gametocytemia (numbers of gametocytes/numbers of uninfected red blood cells). Gametocyte percentages are plotted for each strain and error bars represent the standard deviation of two independent experiments.

Assessment of FCU-GFP transgene ability to salvage 4-thioluracil via Northern blot. Incorporation of 40 μ M 4-TU throughout 3D7^{cam} intraerythrocytic development was carried out by incubating highly synchronized ring-, trophozoite- and schizont-stage parasites for one, two and four hours. Incorporation of 4-TU into the total RNA pool was verified by northern blot as described above.

After generation of transgenic FCU-GFP expressing lines F12^{cam} and F12^{pfs16}; ability to incorporate 4-TU was verified by adding 40 μ M 4-TU to mixed-stage parasite culture and incubating for 12 hours. Incorporation of 4-TU into the total RNA was performed as described above. The length of film exposure time was varied based on the visualization of biotinylation.

Effect of 4-TU on parasite growth. Highly synchronized parasite cultures of 3D7^{cam} (2.5% parasitemia, 5% hematocrit) in the presence of increasing concentrations of 4-TU (0, 20, 40, 80 and 160 μ M). Growth was monitored by assessing the change in percent parasitemia every 12 hours for 1.5 life cycles by Geimsa-stained thin blood smears. The optimal concentration of 4-TU was decided by the highest concentration that had the least effect on parasite growth as compared to growth in culture medium containing no 4-TU.

LC-MS detection of pyrimidine salvage driven thiol-incorporation. The ability of transgenic parasites to synthesize or salvage pyrimidine was assessed by whole metabolite LC-MS/MS analysis. Parasite cultures (10% trophozoites, 5% hematocrit) were grown in complete medium supplemented with 40 μ M ¹⁵N-uracil for 10 and 30 minutes. Alternatively, FCU-mediated salvage into the pyrimidine pool was determined by incubating wild-type 3D7 and 3D7^{cam} with 40 μ M ¹⁵N-uracil and 24mM ¹³C-bicarbonate modified complete medium (lacking unlabeled bicarbonate) for 10 min and 30 min in the presence or absence of 10nM atovaquone, an indirect inhibitor or *de novo* pyrimidine synthesis. Whole parasite metabolite extraction was carried out as previously described with minor modifications (Olszewski and Llinas 2013). Briefly, parasites were saponin (0.01%) lysed, collected by centrifugation and washed in ice-cold 1xPBS. Whole metabolites were extracted and stabilized by the addition of 1mL ice-cold 90% methanol to the parasite pellet. The methanol/parasite suspension was centrifuged for 10min at 4°C and supernatant transferred to a clean tube which can be stored at -80°C. Just prior to analysis, metabolite extracts were dried under nitrogen gas flow at room temperature. Following evaporation, samples were reconstituted in 200 μ l HPLC-grade water and analyzed. Metabolites detected by liquid chromatography-mass spectrometry (LC-MS) were quantified using the MAVEN software program (Clasquin et al. 2012) (version 2011.6.17) which aided in the determination of total UMP pool and proportions that were heavy labeled via *de novo* biosynthesis (¹³C-UMP) versus pyrimidine salvage (¹⁵N-UMP). ¹³C-UMP and ¹⁵N-UMP intensity values were corrected for natural abundance and converted to percentages of total UMP. Unlabeled UMP percentages were normalized to 100%, representing the total pyrimidine pool and the labeled UMP intensity values are represented as a percentage thereof. Labeling was performed in triplicate and the error bars represent the standard deviation of two independent biological replicates.

Growth Inhibition Assay. Genetic supplementation of a *de novo* pyrimidine synthesis metabolic bypass was assessed by growth in the presence of various concentrations of atovaquone. All parasite growth inhibition assays were performed in 96-well plates as described by Smilkstein *et al.* (Smilkstein et al. 2004). 3D7 wild-type and 3D7^{cam} *P. falciparum*-infected erythrocytes at 1.0% initial parasitemia and 4% hematocrit were exposed to various concentrations of atovaquone in the

presence and absence of 40 μ M uracil for 48 hours. Following drug treatment, plates were placed at -80° overnight and thawed at room temperature to promote cell lysis. Once lysed, parasite cultures were incubated with SYBR green I dye (Sigma) (0.4 μ L/mL) in 100 μ L of buffer (20mM Tris-HCl, pH 7.5; 5mM EDTA; 0.08% Triton X-100; 0.008% saponin). Fluorescence was quantified using a BioTek Synergy MX plate reader equipped with Gen5 software. Fluorescent quantification using SYBR Green detection of nucleic acids served as a measure of cell proliferation and was plotted as a percentage growth compared to an untreated control using GraphPad Prism 6.

Reverse Transcription and Microarray Analysis. Total RNA, unbound, and bound fractions of mRNA are precipitated by traditional methods (0.1vol NaCl, 0.1vol linear acrylamide and 3vol 100% EtOH) followed by resuspension in 20 μ l DEPC-treated water. The concentration of each sample is determined by NanoDrop ND-1000. Starting with 1.0-2.5 μ g of RNA, single-strand aminoallyl-containing cDNA synthesis and Amersham CyDye-coupling (GE Healthcare) was carried out as previously described (Bozdech et al. 2003) with the addition of control RNAs to aid in the normalization between samples and arrays (two color RNA spike-in kit, Agilent Technologies). To prevent Cy5 degradation by ozone (Branham et al. 2007), all steps starting with dye resuspension were carried out in an ozone-free environment. Final cDNA concentration and dye-incorporation was assessed on a NanoDrop ND-1000 spectrophotometer. To reduce sample and time-point bias, cDNA from each time-point was labeled with Cy5 and combined with an equal amount of Cy3-labelled cDNA reference pool generated from equal amounts of ring, trophozoite, and schizont stage mRNA and hybridized to *P. falciparum* custom arrays (Agilent Technologies 60mer SurePrint platform, AMADID #037237 (Kafsack et al. 2012)). Hybridized arrays were incubated for 16h in a rotating hybridization oven (10rpm) at 65 $^{\circ}$ C. Prior to scanning, arrays were washed in 6X and 0.06X SSPE (both containing 0.005 % N-lauryl-sarcosine (Sigma-Aldrich, St. Louis, MO, USA), followed by an acetonitrile rinse.

Array scanning, data acquisition, and analysis. Arrays were scanned on an Agilent G2505B Microarray Scanner (Agilent Technologies) with 5 μ m resolution at wavelengths of 532nm (Cy3) and 633nm (Cy5) using the extended dynamic range (10–100%) setting. Normalized intensities were extracted using Agilent Feature Extractor Software Version 9.5 employing the GE2_1100_Jul11_no_spikein extraction protocol and the resultant text files were analyzed using the Rnits (v1.2.0) Bioconductor package in R (Sangurdekar 2014). All array intensity values were background subtracted and normalized within each timecourse and sample (ex: 4-TU labeled, unlabeled mRNA) and converted to $\text{Log}_2(\text{Cy5}/\text{Cy3})$ ratios. The same data are available on Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>) under study accession number

GSE72695. Probes excluded in this analysis were those representing the following: Agilent Controls, Cy3 or Cy5 signal below background (<200), *P. falciparum* surface variants (*rifin*, *stevor*, *var*, *pfmc-2tm*), and exogenous controls (Kafsack et al. 2012). All gene values (\log_2 ratios) were within-strain median centered and clustered based upon the K10 means of 3D7^{cam} and 3D7^{pfs16}. In total, 5168 genes are represented in the thiol-labeled mRNA pool (transcription) and 5175 genes are represented in the non-thiol-mRNA pool (stabilization) out of 5509.

Within-strain correlation analyses were carried out by determining the Pearson correlation coefficient of the \log_2 ratios of all genes over time between either *cam-fcu-gfp* or *pfs16-fcu-gfp* expressing lines. The Pearson correlation coefficients for both Transcription and Stabilization arrays were plotted over time for each strain.

The full dataset was K10 means clusters within 3D7^{cam} and 3D7^{pfs16} using Cluster 3.0. Subsequently, all genes in F12^{cam} and F12^{pfs16} were ordered based on the K10 means clustering from 3D7 and arranged in order of peak abundance from 0-36 hpi (Table S1). To determine fold changes of genes specific to parasites with an active *pfs16*-promoter (committed gametocytes), the \log_2 ratio from *cam-fcu-gfp* expressing lines were subtracted from the partner *pfs16-fcu-gfp* expressing lines (Fold change = $3D7^{pfs16}(\text{Log}_2(\text{Cy5}/\text{Cy3}))/3D7^{cam}(\text{Log}_2(\text{Cy5}/\text{Cy3}))$). Significant fold changes were determined based upon a \log_2 fold change ≥ 1.0 and percentile ranking of ≥ 95 within 3D7 (Table S2).

Comparison of total transcript abundance throughout the IDC. Data from cDNA microarrays that were hybridized with total RNA extracted from wild-type 3D7, 3D7^{cam} and 3D7^{pfs16} throughout each timecourse was extracted as described above. Next, the Pearson correlation was determined for each gene using the $\text{Log}_2(\text{Cy5}/\text{Cy3})$ ratios intensities over time between a previously published 3D7 mRNA abundance DNA microarray time course (Kafsack et al. 2012) and either 3D7^{cam} or 3D7^{pfs16}.

Motif and GO-term enrichment analysis. Identification of motifs enriched in genes identified as stabilized or transcribed (\log_2 ratio fold change ≥ 0.05 and p-value ≤ 0.01) in gametocytes was carried out using a regulatory element discovery algorithm (FIRE) (Elemento et al. 2007).

GO-term enrichment of select gene groups or clusters was carried out using the Analysis Tool at <http://www.PlasmoDB.org> (Aurrecochea et al. 2009).

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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'-CGTACGTAAACACAGTAGTATCTGTCCACC-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 Na₂HPO₄, 7.3mM NaH₂PO₄, 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 MgCl₂
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 can be introduced by electroporation using a well-established transfection protocol in *Plasmodium falciparum* (Fidock and Wellems 1997). Expression of the FCU transgene should be verified in each line via Western blot analysis using anti-GFP (1:2000) or anti-CD (1:500) antibodies (Figure 1). Visualization of the localization and cell-population expression of FCU can be carried out by immunofluorescent microscopy as per well-established protocols (Tonkin et al. 2004) using an Olympus BX61. Cells should be 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 malarial *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 (3000g 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 the 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 of 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 0.5 μ g/ μ l)
2. Wash 2 μ l of Dynabeads® MyOne™ Streptavidin C1 magnetic beads per μ g 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 manufacturer's 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 1 μ g yeast tRNA per 5 μ l 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 each individual microfuge tubes.
9. Incubate at 25°C 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: A₂₆₀/A₂₈₀ 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 A₂₈₀. 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://lilaslab.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://lilaslab.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 colorimetric 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.

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