Supporting information

Automated Spatially Targeted Optical Micro Proteomics (autoSTOMP) to Determine Protein Complexity of Subcellular Structures

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ABSTRACT:

Spatially Targeted Optical Micro Proteomics (STOMP) is a method to study region-specific protein complexity of a biological specimen. STOMP uses a confocal microscope to both visualize structures of interest and to tag the proteins within those structures by a photo-driven crosslinking reaction so that they can be affinity purified and identified by mass spectrometry[1]. STOMP has the potential to perform discovery proteomics on sub-cellular structures in a wide range of primary cells types and biopsy-scale tissue samples. However, two significant limitations have prevented the broad adoption of this technique by the scientific community. First, STOMP is performed across two software platforms written in different languages, which requires user operation at each field of view. Up to 48 hours of microscope time is necessary to tag sufficient protein (~1 µg) for mass spectrometry making STOMP prohibitively time and labor-consuming for many researchers. Second, the original STOMP protocol uses a custom photo-crosslinker that limits the accessibility of the technique for some users. To liberate the user, we developed a protocol that automates communication between Zeiss Zen Black imaging software and FIJI image processing software using a customizable code in SikuliX. To fully automate STOMP (autoSTOMP), this protocol includes a tool to make tile array, autofocus and capture images of fields of view across the sample; as well as a method to modify the file that guides photo-tagging so that subsets of the structures of interest can be targeted. To make this protocol broadly accessible, we implemented a commercially available biotin-benzophenone crosslinker as well as a procedure to block endogenous biotin and purify tagged proteins using magnetic streptavidin beads. Here we demonstrate that autoSTOMP can efficiently label, purify and identify proteins that belong to structures measuring 1-2 µm in diameter using human foreskin fibroblasts or mouse bone marrow-derived dendritic cells infected with the protozoan parasite *Toxoplasma gondii* (*Tg*). The autoSTOMP platform can easily be adapted to address a range of research questions using Zeiss Zen Black microscopy systems and LC-MS protocols that are standard in many institutional research cores.

Supporting Methods

Reagents and consumables
Unless otherwise noted chemical reagents and consumables were purchased from Thermo Fisher Scientific, USA, and used according to manufacturer instructions.

***Tg* infections and immunofluorescence staining**
Tg is cultured and stored under BSL2 conditions in accordance with the University of Virginia Environmental Health and Safety approved Biosafety Protocol. The Type II *Tg* parasite strain ME49 was used in all experiments. *Tg* was passaged in confluent human foreskin fibroblasts (HFFs) grown in 25cm^2 tissue culture flasks. Parasites and HFFs were cultured in complete DMEM media containing DMEM (11965118), 10% heat-shocked FBS (12303C-500ML, Sigma Aldrich), 1% Penicillin/Streptomycin solution (15140163, Fisher Scientific), 1% L-glutamine solution (25030164, Fisher Scientific), and 1% 100 mM sodium pyruvate solution (11360070, Life Technologies), 1% 1 M HEPES solution (15630080, Life Technologies). Media was stored at 4 °C and warmed to 37 °C before use. C57BL/6 mice were bred in the University of Virginia vivarium in accordance with ABSL-1 standards and AALAC approved protocol. Mouse bone marrow-derived dendritic cells (mBMDCs) were differentiated for 6-8 days in complete RPMI media supplemented with 10% mouse GMCSF supernatant derived from B16 cells stably expressing mouse GMCSF as previously described[9]. THP-1 cells were cultured in suspension in complete RPMI media containing RPMI (10-040-CV, Corning), 10% heat-shocked FBS, 1% Penicillin/Streptomycin solution, 1% L-glutamine solution, and 1% 100 mM sodium pyruvate solution, 1% 1 M HEPES.
For experimental infections, 0.5x10^6 THP-1 cells were differentiated with complete RPMI media containing 100 ng/mL PMA (AAJ63916MCR, Fisher Scientific) for 2 days on 12 mm cover glass (64-0712, Harvard Apparatus, USA) coated with poly-D-lysine (ICN15017550). 0.5x10^6 immortalized bone marrow derived macrophages (iBMDM, mouse) were grown in complete RPMI media for 1 day on 12 mm cover glass coated with poly-D-lysine. mBMDCs were plated 1.35x10^6 per well of a 12 well plate containing a single 18 mm round cover glass (64-0714, Harvard Apparatus, USA) coated with poly-D-lysine. HFFs were grown to confluency on 18mm round cover glass. *Tg* was grown in HFFs until large intracellular parasite vacuoles were observed. Intracellular *Tg* was harvested by scraping infected HFF monolayers with a rubber policeman and syringe lysing the HFFs through a 25G blunt-ended syringe needle. *Tg* was counted on a hemocytometer and added to host cells at a multiplicity of infection (MOI) of 10, or for HFF infections, 8.8x10^4 parasites per mm^2. The *Tg* infected HFFs on coverslips were harvested after 2 hours.

Slide Preparation for STOMP
Tg infected HFFs on coverslips were fixed with 100% methanol at -20 to -30 ˚C (prechilled) for 15 min. Methanol was decanted, and samples were washed three times with room temperature PBS. If staining did not take place immediately, PBS was aspirated, and coverslips were stored at -30 ˚C. To stain *Tg*, the slides were blocked with 2% BSA in TBS at room temperature for 1 hour, a rabbit polyclonal antibody specific to soluble tachyzoite antigen (STAG) directly conjugated to FITC (PA1-7253, Invitrogen) was diluted 1:300 in TBS-T (0.1% Tween20) and coverslips were stained at room temperature for 1 hour. Slides were washed three times with TBST. Endogenous biotin was blocked with Avidin/Biotin Blocking Kit (SP-2001, Vector Laboratories) following the manufacturer's protocol. The stock solution of 0.5 M biotin-dPEG®3-benzophenone (biotin-BP, 10267, Quanta BioDesign) in anhydrous DMSO (89139-666, VWR) is stable for 12 months when stored with desiccant in the dark at -30 ˚C. The biotin-BP mounting media must be prepared fresh (used within 4 hours) by diluting the biotin-BP stock solution in 50/50 (v/v) DMSO/water to a working concentration of 1 mM biotin-BP. Coverslips were mounted in 12 µL biotin-BP mounting media in a cold room (4 ˚C) with weak light and sealed with nail polish (Insta-Dri Fast Dry Nail and Double Duty Base and Topcoat, Sally Hansen). If multiple slides were necessary, coverslips were mounted the day of crosslinking and stored in the dark at room temperature before microscopy.

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Validation of biotin-benzophenone cross-linking by microscopy

Following biotin-BP cross-linking, each coverslip was soaked in DI water (RT, dark) for 30 minutes and the nail polish seal was gently pulled away. Excess mounting media was removed by three washes with 50/50 (v/v) DMSO/water followed by three washes with Mili-Q water. Slides were blocked in 2% BSA in TBST for 30 min, and then incubated in TBST (0.1% Tween20) containing 1: 500 dilution Alexa Fluor® 594 Streptavidin (#016-580-084, Jackson ImmunoResearch Lab) for 45 min. Coverslips were washed three times in TBST (0.1% Tween20) and mounted with mounting media containing DAPI (H-1000, Vector Laboratories) for imaging.

Image acquisition, mask generation and UV-biotinylation using autoSTOMP

All microscopy and cross-linking were performed on an LSM880 confocal microscope (Carl Zeiss, Inc., Germany) and a Chameleon multiphoton light source (Coherent Inc., USA) in the Ewald Lab at the University of Virginia. Images were acquired using Zen Black (Carl Zeiss). Image modification and MAP file generation were performed in FIJI (FIJI Is Just ImageJ)[12]. Structures of Interest (SOI, *Tg* here) were visualized using the 25x oil emersion lens (LD LCI Plan-Apochromat 25x/0.81 mm Korr DIC M27) with immersion oil (518 F for 30°C, refractive index = 1.518, 444970-9000-000, Carl Zeiss) and the argon laser source (488 nm) with 500-530 nm bandwidth. A 512 x 512 pixel^2 image was acquired for each field of view. Sikulix version 1.1.4 (http://sikulix.com/) was used to automate tasks between software platforms. Python (version 3.6, www.python.org) and Spyder (version 3.2.8, www.spyder-ide.org) were used to generate a tile array across the slide surface (~ 500 tiles). Using the SikuliX automation platform, each field of view was processed as follows. First, the SOI in each field of view were imaged. The .czi file was exported into FIJI where a binary image was created, thresholded and/or “erode”, “dilate” or “ROI manager” functions were used to create a “MAP file” of the regions to be photo crosslinked. The MAP file is converted to a .txt file which is imported by the STOMP macro in Zen Black. In Zen Black, the STOMP macro directs the Chameleon to deliver two-photon 720nm light to each pixel defined in the MAP file. Crosslinking four to five million pixels typically labeled sufficient protein (approximately 1 µg) for mass spectrometry analysis. **A step-by-step tutorial of the autoSTOMP protocol and all the source codes are deposited at GitHub (GitHub Inc.).**
https://github.com/boris2008/Sikulix-automates-a-workflow-performed-in-multiple-software-platforms-in-Windows.git

Streptavidin precipitation

Once the UV-cross linking is complete for each slide, the coverslip was soaked in DI water (RT, dark) for 30 min and the nail polish seal was gently pulled away. Excess mounting media was eliminated by three washes with 50/50 (v/v) DMSO/water followed by three washes with Mili-Q water. Excess water was aspirated and the coverslip was stored at -30 ˚C while photo-crosslinking was performed on additional slides.

Our purification protocol for Mass Spectrometry (MS) analysis is modified from *Hadley et al.* 2015[1]. Samples are dissociated from the coverslip in 8 M urea lysis buffer containing 100 mM NaCl, 25 mM Tris, 2% SDS, 0.1% tween 20, 2 mM EDTA, 0.2 mM PMSF, and 1x Roche cOmplete Protease inhibitor (11873580001). Coverslips were placed on a parafilm (Bemis) membrane with cells facing up and incubated with 50 µL urea lysis buffer at room temperature for 30 min. The coverslip was then rinsed with 100 µL Mili-Q water and followed by another incubation with 50 µL urea lysis buffer at room temperature for 30 min. All of the solutions after lysis were collected and combined as lysate in low protein binding 1.5 mL microcentrifuge tubes. To reduce the nucleic acid-protein complex formation and associated lysate viscosity, benzonase (E1014-25KU) and RNase A from bovine pancrease (10109142001) were used. Benzonase (0.1 µL per 5 x 10^6 cells) was added and incubated at 37 °C for 30 min followed by RNase (0.5 µL per 5 x 10^6 cells) treatment at 65 ˚C for 15 min. The lysate was cooled to room temperature and ready for affinity purification.

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Four different buffers were used for the affinity purification: TU (50 mM Tris-Cl, 2 M urea, and 150 mM NaCl at pH = 7.4), TUST (TU buffer spiked with 0.1% SDS and 0.1% Tween 20 at pH = 7.4), TUB (0.5 mM biotin in TU buffer) and 100 mM NH_4HCO_3 buffer. The volume of lysate of each sample was filled up to 1 mL with TUST. 10 µL Pierce™ Streptavidin (SA) Magnetic Beads (88817) 50% slurry was washing with 1 mL TUST once and then added to the lysate and mixed by vortexing. The mixture was incubated on a rotator at room temperature for 1 hour to bind the biotinylated proteins (longer incubation time is not recommended as it will increase unspecific binding). The magnet (DynaMag™-2, Invitrogen) was used to pellet the SA beads. To remove unspecific bound proteins, samples were washed three times with 1 mL TUST, five times with 1 mL TU, 1 time with 1 mL TUB, and three times with 1 mL 100 mM NH_4HCO_3 buffer. After each wash, SA beads were pelleted on the magnet for 3 min and the buffer was removed by pipetting while on the magnet. For each wash, the SA beads were mixed by vortexing and incubated for 5 minutes before the SA beads were applied to the magnet again. After all washing, the SA beads were resuspended in 100 µL 100 mM NH_4HCO_3 buffer.

Western blot

To validate *Tg* protein enrichment by western blot, total protein content for each sample was normalized using Pierce™ Micro BCA kit (#23235, Pierce). Proteins were separated by SDS-PAGE using Mini Gel Tank tool kit (25977, ThermoFisher) and were transferred to PVDF membrane (1704274, Bio-Rad) using Trans-Blot Turbo Transfer System (Bio-Rad). The PVDF membrane was blocked in 2% milk TBST (0.1% Tween 20) solution for 1 hour at room temperature followed by sequential incubation with antibody diluted in TBST (0.1% Tween 20) and alternating washing with TBST (0.1% Tween 20). Streptavidin-HRP (016-030-084, Jackson ImmunoResearch) was used to probe biotin. Proteins were visualized on ChemiDoc™Touch imaging system (Bio-Rad) with HRP substrate (WBLUF0100, Millipore).

Sample preparation for LC-MS

After affinity purification, on-bead reduction and alkylation of SA beads were performed in 1 mL 25 mM NH_4HCO_3 by adding 25 µL 10 mM DTT at room temperature for 30 min and 25 µL 15 mM iodoacetamide in dark at room temperature for 1h in sequence. To quench excess iodoacetamide, 25 µL 5 mM DDT solution was added and incubated for 10 min. Trypsin/Lys-C (V5072 Promega) was directly spiked into the protein lysate at a ratio of Trypsin/Protein = 1:25. Digestion was continued overnight (~12 h) at 37 ˚C. Formic acid was added to a final concentration of 1% (v/v) to stop the trypsinization. SA beads were removed by the magnet and the supernatant was saved. 7.5 nmol Angiotensin/Vasoactive Standard (A9650-1MG/V0131-.1MG, Sigma) was spiked into the peptide digest. The peptide digest was desalted using Pierce™ C18 Tips with binding, washing (with 0.1% formic acid in water), and eluting (with 60% acetonitrile in water) steps by following the manufacturer's instructions. The sample was dried completely on the SpeedVac concentrator (Model: SPD131DDA-115). The peptide digest was dissolved in 21 µL of LC-MS grade water containing 0.1% formic acid (F0507-100ML, Sigma). The digest was filtered with a 0.65 µm pore-size micro-centrifuge filter before it was loaded to an HPLC auto-injection sample.

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Peptide mass quantification by LC-MS

For the initial validation of autoSTOMP, LC-MS was performed on a linear ion trap mass spectrometer (Thermo Scientific LTQ, Thermo Fisher, USA) coupled with LC (LC-20AD, Shimadzu, USA) in the Hsu lab at the University of Virginia. 1 µL (5%) of the peptide digest was injected to a C18 capillary column (20 cm of 5 µm C18 (YMC*GEL) packed in 360 µm o.d. x 75 µm i.d. fused silica) and desalted with 0.05% acetic acid at a 185 nL/min for 30 min at room temperature. After desalting, a gradient of acetonitrile/0.1 M formic acid was applied for 200 min: solvent A (water + 0.05% acetic acid) and solvent B (80% acetonitrile + 0.05% acetic acid) were applied in the order of 0% B for 5 min, 0-25% B for 111 min, 25-45% B for 35 min, 45-95% B for 1 min, 95% B for 7 min, 95-0% B for 6 min, 0% B for 34 min, and re-equilibration with 0% B for 10 min. The separation column ran at room temperature. The eluted peptides were electro-sprayed into the LTQ MS, which was operated in the positive ion mode with a "top 3" data-dependent acquisition method that consisted of one MS scan (m/z: 350-1800) followed by three MS/MS scans of the most abundant ions recorded in the preceding MS scan. For mass assignment IP2 (the Integrated Proteomics Applications, Inc., USA) using the ProLuCID algorithm was used to search against human protein database (https://www.uniprot.org) and *Tg* protein database (released 41, https://toxodb.org/toxo/) with reversed sequence decoys. The peptide and proteins were identified with false discovery rate (FDR) smaller than 1%, separately. Differences in protein and peptide abundances between the samples were based on MS/MS spectral counting using the COMPARE function in the IP2-Integrated Proteomics Pipeline. The resulting MS2 spectra matches were assembled into protein identifications.

To compare protein enrichment in *Tg* with protein enrichment at the parasite vacuole membrane using the "donut" MAP file function, a Thermo Electron Q Exactive HF-X mass spectrometer system with an Easy Spray ion source connected to a Thermo 75 µm x 15 cm C18 Easy Spray column was used at the University of Virginia Biomolecular Analysis Facility Core. 7 µL (33.3%) of the extract was injected and the peptides eluted from the column by a gradient at a flow rate of 0.3 µL/min at 40 °C for more than 1 hour. Solvent A (water + 0.1% formic acid) and solvent B (80% acetonitrile + 0.1% formic acid) were applied in the order of 2% B for 1.5 min, 2-23% B for 51 min, 23-35% B for 10 min, 35-95% B for 1 min, and 95% B for 5 min. The nanospray ion source was operated at 1.9 kV. The digest was analyzed using a Top10 method with the MS scan set to 120K resolution and HCD scans set to 30K resolution. This mode of analysis produces approximately 25000 MS/MS spectra of ions ranging in abundance over several orders of magnitude. The data were analyzed by database searching using the Sequest search algorithm deployed in Proteome Discoverer™ (Thermo Fisher Scientific Inc. USA) against Uniprot mouse and *Tg* database from ToxoDB with reversed sequence decoys separately. Protein (FDR < 2%) and peptide identification (FDR< 0.2%) were organized and summarized by Scaffold (Proteome Software, Inc).

Data Analysis

Common contaminants listed on the MaxQuant website http://www.coxdocs.org/doku.php?id=maxquant:start_downloads.htm have been identified and excluded from our protein data. The proteins possess high similarly (> 90% proteins identification and >80% peptide sequence coverage) between human and *Tg* protein were excluded from downstream analysis using a custom python script "myBlast.py" running on the Rivanna computing server (https://arcs.virginia.edu/rivanna) at the University of Virginia. A Student's t-test was used to perform pairwise comparison across the three replicates of each sample with R scripts in Rstudio (version 1.1.456, www.rstudio.com/) with R packages (for examples, readr, xlsx, dplyr, tidyverse) and p-values for each protein were reported. Gene Ontology (GO) and KEGG pathway annotation were added using David Bioinformatics Resources 6.8[13]. The enriched proteins and the statistical summarization were made in R scripts with the table organization and statistical tool packages mentioned above. These custom scripts are deposited at GitHub (GitHub Inc.) https://github.com/boris2008/codes-for-validating-STOMP-with-MS.git. Plots were created with R package "ggplot2 and "ggrepel" in R or GraphPad Prism (version 8.2.1).

Supporting Figures

Figures	Note
S-1	Photo-crosslinking parameter optimization
S-2	Biotin-BP concentration optimization
S-3	Enriched *Tg* proteins by spectral count distribution model
S-4	Tile scan of the STOMP DONUT biotin targeting
S-5	Validation of biotin targeting *Tg* in infected mBMDCs
S-6	GO Term and KEGG enrichment analysis of STOMP DONUT protein enrichment in mBMDCs

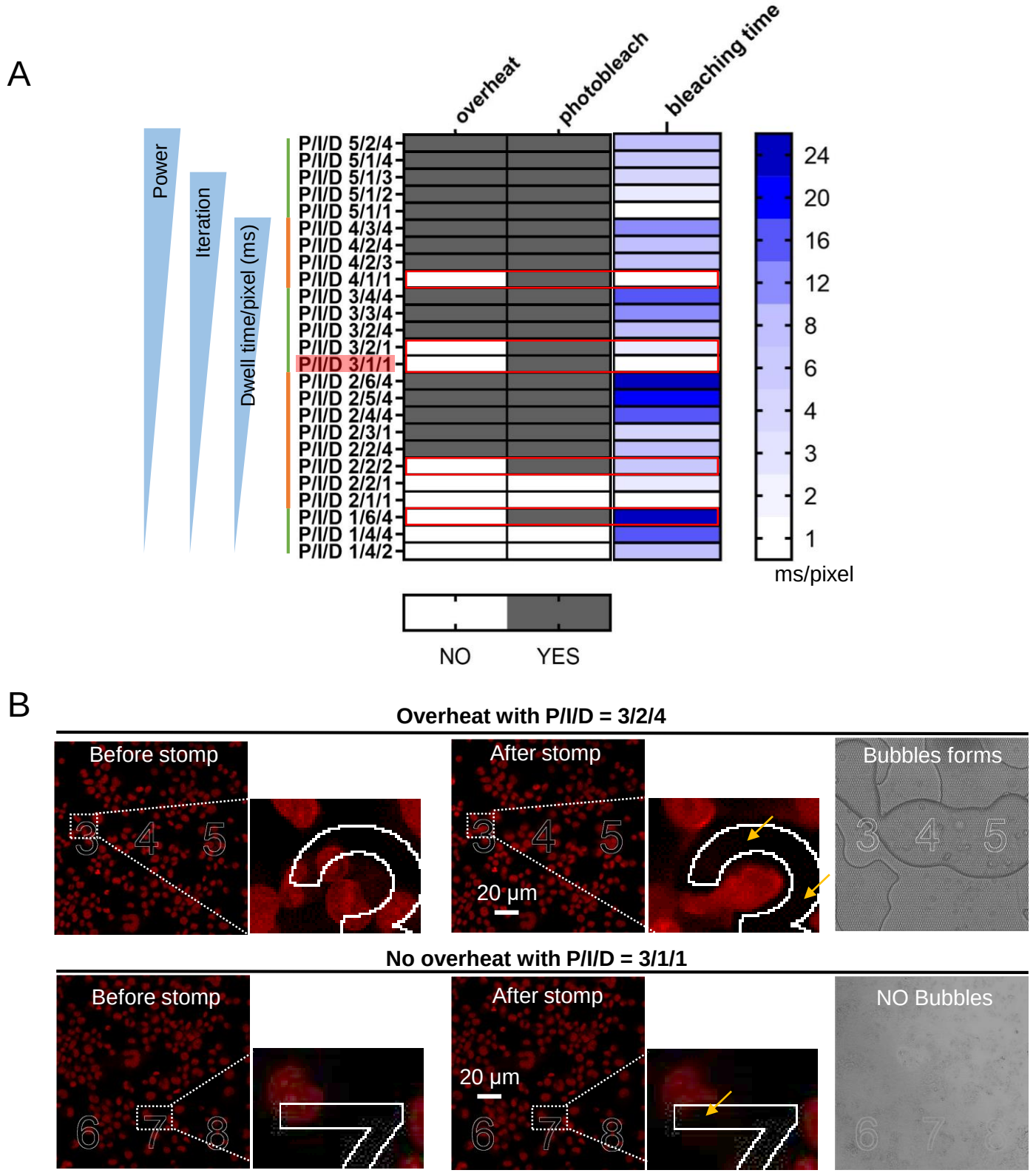

Figure S-1 The optimal photo-crosslinking condition was determined varying the laser power (P), the number of iterations each field of view was photo-excited (I) and the dwell time (D) represented as "P/I/D" using methanol fixed THP-1 cells stained with propidium iodide. **A**, Five conditions were identified with sufficient power for photo-bleaching that did not induce overheating (red box). P/I/D = 3/1/1 was chosen to minimized the time necessary to process each field of view. Samples are primarily ordered by the power (P), secondly ordered by the iteration time (I) and thirdly ordered by the Dwell time/pixel (ms). The bleaching time per pixel in milliseconds (ms) to excite the photo-crosslinking equals the product of the iteration time and Dwell time/pixel. **B**, Representative laser settings for photo-crosslinking that causes the overheating with P/I/D = 3/2/4 (Top) or no overheating with P/I/D = 3/1/1 (Bottom) inside the slide. The STOMP crosslinking was directed to the SOIs with the shapes of numbers "3", "4", and "5" (Top) or numbers "6","7","8" (Bottom). The photobleaching occurs in the autoSTOMP targeted SOIs (yellow arrow) while the outside area was not affected (outside the white outline). Bubbles formed were detected under the bright field indicating overheating.

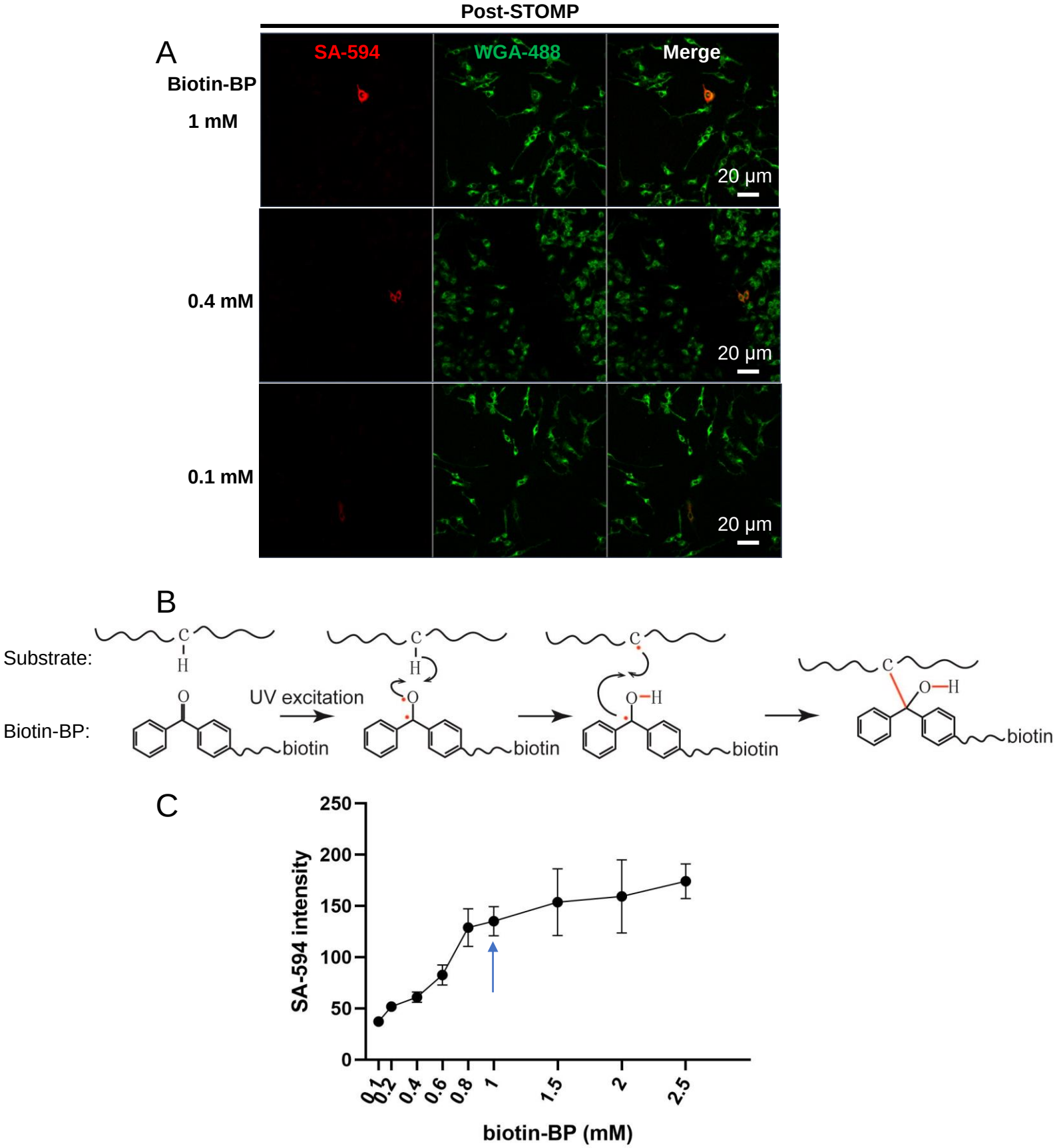

Figure S-2. Biotin-BP concentration was optimized by titration 0.1 mM to 2.5 mM biotin-BP in mounting media. Immortalized mouse bone marrow derived macrophages (iBMDMs) were methanol fixed and stained with fluorescein labeled wheat germ agglutinin (WGA-488) then mounted with biotin-BP at concentrations of 0.1 – 2.5 mM. Samples were photo-crosslinked at two-photon 720 nm at laser Power/Iteration/Dwell time = 3/1/1. **A**, Representative images of iBMDMs (green) and 0.1, 0.4 and 1mM biotin-BP showing an individual cell targeted with streptavidin-594 (SA-594, red). **B**, A diagram shows biotin-BP inserts into C-H or N-H bonds under UV. **C**, SA-594 staining relative to biotin-BP concentration. 1 mM biotin-BP (blue arrow) was selected as the lowest concentration needed for optimal SA-594 staining.

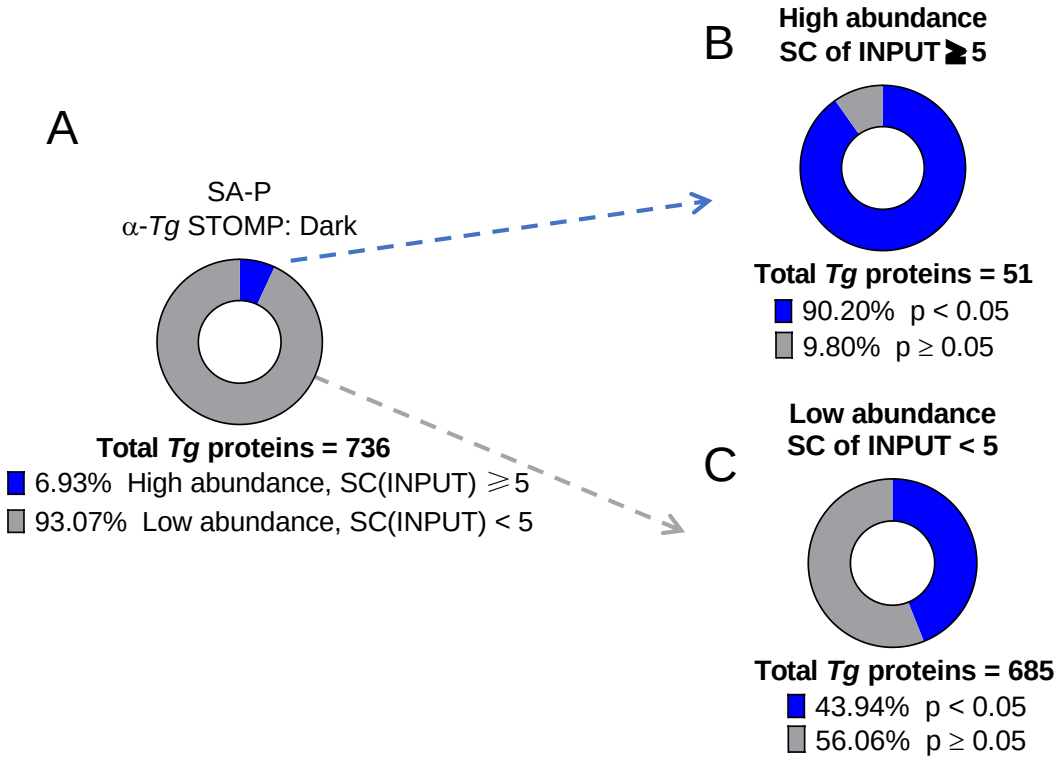

Figure S-3 Both high and low abundance *Tg* proteins are enriched by autoSTOMP using the spectral counts distribution analysis method. **A,** Of the 736 *Toxoplasma gondii (Tg)* proteins identified in Figure 4E, 6.93% of proteins are "high abundance" with spectral counts (SC) in the INPUT controls ≥ 5, and 93.07% of proteins were "low abundance" with SC in the INPUT controls < 5. p-values based on student's t-test comparing α-*Tg* STOMP SA-P and Dark SA-P samples. N=3 independent experiments (significant difference, $p < 0.05$; no significant difference, $p \geq 0.05$). **B,** 90.20% of the high abundance proteins are significantly enriched by autoSTOMP. **C,** Whereas, 43.94% of the low abundance proteins are enriched in the α-*Tg* STOMP compared to the Dark.

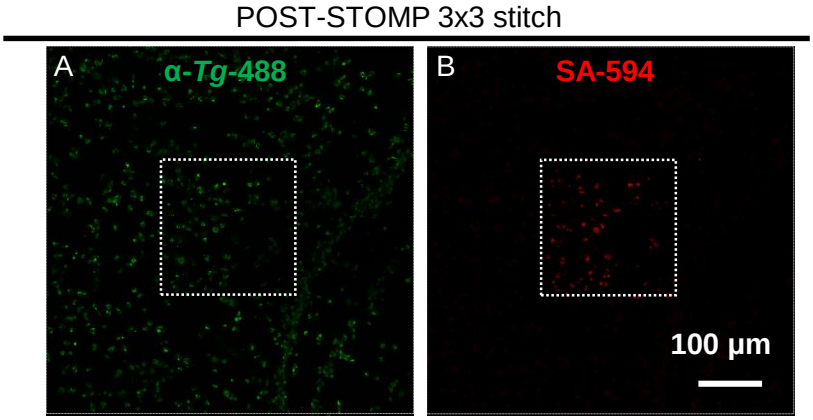

Figure S-4 AutoSTOMP selectively biotinylated *Toxoplasma gondii (Tg)* DONUT SOI without increasing background biotinylation. 3x3 tile array centered on autoSTOMP field of view shown in Figure 5 stained for *Tg* (**A**, green) or SA-594, (**B,** red). Representative of 3 independent experiments.

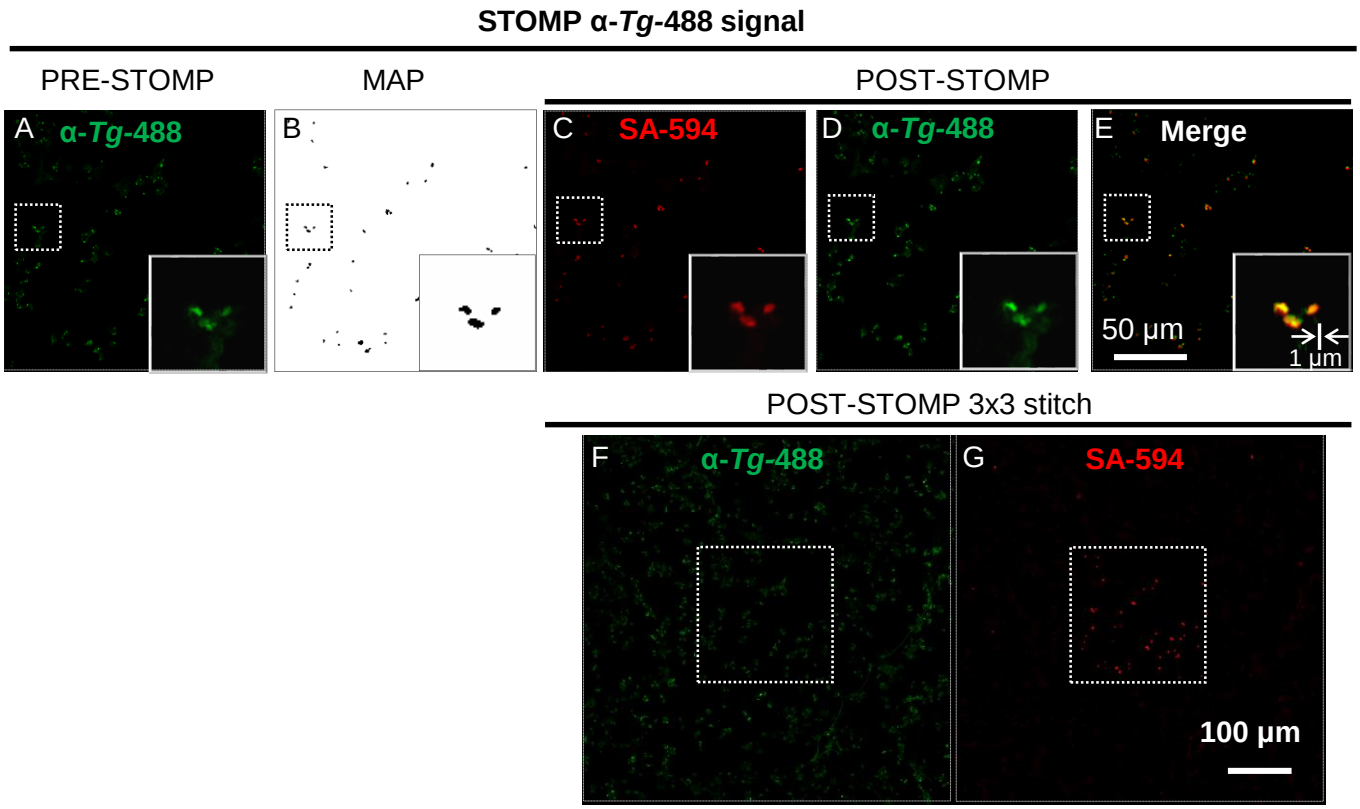

Figure S-5. *Tg* is selectively biotinylated by auto-STOMP UV-cross linking in infected mouse bone marrow derived dendritic cells (mBMDCs, control for DONUT staining in Figure 5). **A**, *Tg* in mBMDCs were stained with a *Toxoplasma*-specific antibody directly conjugated to alexafluor-488 (α-*Tg*-488, green) to identify *Tg* as the SOI. **B**, The MAP file generated in FIJI identifying the *Tg* SOI imaged in **A**. **C-E**, After UV-mediated biotin-BP tagging, biotinylated proteins were visualized using streptavidin-Alexafluor594 staining (**C**, SA-594, red), and directly compared to the *Tg* signal (**D,** α-*Tg*-488, green). **E**, merge of **C** and **D**. **F-G**, 3x3 tile array centered on the field of view targeted by the STOMP macro (white dotted line box):*Toxoplasma* staining (**F,** α-*Tg*-488, green) and biotinylation (**G**, SA-594, red). Representative of 3 independent experiments.

A

Tg Gene	*Tg* proteins	Mouse Gene	Mouse proteins
TGGT1_235470	Myosin A	Iqgap1	Ras GTPase-activating-like protein IQGAP1
TGVEG_208030	Microneme protein MIC4	Gdi2	Rab GDP dissociation inhibitor beta
TGGT1_295110	Rhoptry protein ROP7	Atp6v1b2	V-type proton ATPase subunit B, brain isoform
TGGT1_300100	Rhoptry neck protein RON2	Arhgap25	Rho GTPase-activating protein 25
TGGT1_227280	Dense granule protein GRA3	Rhog	Rho-related GTP-binding protein RhoG
		Gvin1	Interferon-induced very large GTPase 1
		Rab7a	Ras-related protein Rab-7a
		Ctsd	Cathepsin D
		Tcirg1	V-type proton ATPase subunit a
		Ap2a2	AP-2 complex subunit alpha-2
		Ap2a1	AP-2 complex subunit alpha-1
		Ctss	Cathepsin S
		Nsf	Vesicle-fusing ATPase
		Cyb5r3	NADH-cytochrome b5 reductase 3

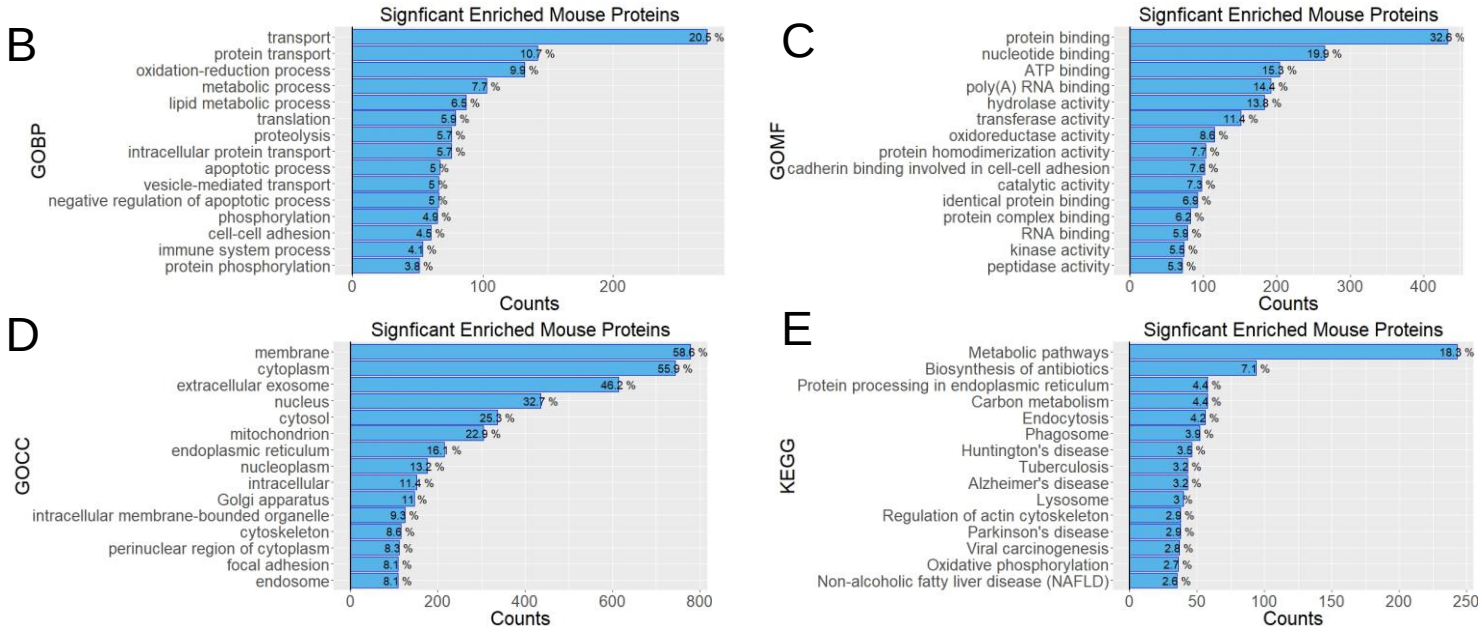

Figure S-6 Known PVM localized *Tg* and host proteins and infection-induced cell biology are identified by AutoSTOMP. **A.** Representative *Tg* proteins or mouse proteins with published localization to the PVM are enriched in the DONUT structure of *Tg* in mBMDCs. **B-E,** 72.5% or 1616 of mouse proteins identified in the STOMP DONUT (Figure 6D-E), are significantly enriched. These were searched against the Gene Ontology (GO) terms (Biological Process (BP, **B**), Molecular Function (MF, **C**), or Cellular Component (CC, **D**) or the KEGG pathway (**E**) using DAVID Bioinformatics Resource. Ranking is based on the number of proteins identified as percentage of all proteins in the term. The top 15 terms are shown ranked from high to low.