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Introducing a gene knockout directly into amastigote stage of Trypanosoma cruzi by CRISPR/Cas9 system

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March 7, 2019

Dear Editor Stephanie Weldon,

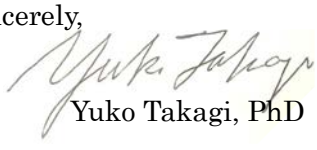
Thank you for giving us the opportunity to submit our manuscript entitled "**Introducing a gene knockout directly into amastigote stage of *Trypanosoma cruzi* by CRISPR/Cas9 system**" to JoVE.

The replication stage of *T. cruzi* in host cells, amastigote, is the least studied developmental stage in the parasite's life cycle, although it is the most relevant stage in terms of drug development research. This is because amastigote had been considered as an obligate intracellular parasite and could not be isolated for experimental use.

Our new methodology to utilize temporal axenic culture of *T. cruzi* amastigote enables investigation of drug target essentiality specifically in amastigote stage, without going through other developmental stages. Conventional method takes 1 - 2 months to generate a knockout amastigote, but our protocol only takes 2 - 3 days. We believe our method can be applied to variety of stage-specific studies and contribute to advance our understanding of this clinically important parasite.

Please address all the correspondent regarding this manuscript to me at [yuko-takagi@aist.go.jp]. I will be happy to respond to any further questions and comments.

Sincerely,



Yuko Takagi, PhD

TITLE:

Introducing a Gene Knockout Directly Into the Amastigote Stage of *Trypanosoma cruzi* Using the CRISPR/Cas9 System

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KEYWORDS:

CRISPR/Cas9, gene knockout, stage-specific, *Trypanosoma cruzi*, Chagas disease, kinetoplastid parasite, drug target, axenic culture, amastigote

SUMMARY:

Here, we describe a protocol to introduce a gene knockout into the extracellular amastigote of *Trypanosoma cruzi,* using the CRISPR/Cas9 system. The growth phenotype can be followed up either by cell counting of axenic amastigote culture or by proliferation of intracellular amastigotes after host cell invasion.

ABSTRACT:

Trypanosoma cruzi is a pathogenic protozoan parasite that causes Chagas' disease mainly in Latin America. In order to identify a novel drug target against *T. cruzi*, it is important to validate the essentiality of the target gene in the mammalian stage of the parasite, the amastigote. Amastigotes of *T. cruzi* replicate inside the host cell; thus, it is difficult to conduct a knockout experiment without going through other developmental stages. Recently, our group reported a growth condition in which the amastigote can replicate axenically for up to 10 days without losing its amastigote-like properties. By using this temporal axenic amastigote culture, we successfully introduced gRNAs directly into the Cas9-expressing amastigote to cause gene knockouts and analyzed their phenotypes exclusively in the amastigote stage. In this report, we describe a detailed protocol to produce in vitro derived extracellular amastigotes, and to utilize the axenic culture in a CRISPR/Cas9-mediated knockout experiment. The growth phenotype of knockout amastigotes can be evaluated either by cell counts of the axenic culture, or by replication of intracellular amastigote after host cell invasion. This method bypasses the parasite stage differentiation normally involved in producing a transgenic or a knockout amastigote. Utilization of the temporal axenic amastigote culture has the potential to expand the experimental freedom

of stage-specific studies in *T. cruzi*.

INTRODUCTION:

Trypanosoma cruzi is the causative agent of Chagas' disease, which is prevalent mainly in Latin America[1]. *T. cruzi* has distinctive life cycle stages as it travels between an insect vector and a mammalian host[2]. *T. cruzi* replicates as an epimastigote in the midgut of a blood-sucking triatomine bug and differentiates into an infectious metacyclic trypomastigote in its hindgut before being deposited on a human or animal host. Once the trypomastigote gets into the host body through the bite site or through a mucous membrane, the parasite invades a host cell and transforms into a flagella-less round form called an amastigote. The amastigote replicates within the host cell and eventually differentiates into trypomastigote, which bursts out of the host cell and enters the blood stream to infect another host cell.

Since currently available chemotherapeutic agents, benznidazole and nifurtimox, cause adverse side effects and are ineffective in the chronic phase of the disease[3], it is of a great interest to identify novel drug targets against *T. cruzi*. In recent years, the CRISPR/Cas9 system has become a powerful tool to effectively perform gene knockout in *T. cruzi*, either by transfection of separate or single plasmid(s) containing gRNA and Cas9[4], by stable expression of Cas9 and subsequent introduction of gRNA[5–7] or transcription template of gRNA[8], or by electroporation of the pre-formed gRNA/Cas9 RNP complex[7,9]. This technological advancement is highly anticipated to accelerate the drug target research in Chagas' disease.

To proceed with the drug development, it is crucial to validate the essentiality of the target gene or efficacy of drug candidate compounds in the amastigote of *T. cruzi*, as it is the replication stage of the parasite in the mammalian host. However, this is a challenging task, because amastigotes cannot be directly manipulated due to the presence of an obstructive host cell. In *Leishmania*, a closely related protozoan parasite to *T. cruzi*, an axenic amastigote culturing method was developed and has been utilized in drug screening assays[10–13]. Although there are some discrepancies in susceptibility to compounds between axenic amastigotes and intracellular amastigotes[14], the ability to maintain the axenic culture nonetheless provides valuable experimental tools to study the basic biology of the clinically relevant stage of *Leishmania*[15,16]. In the case of *T. cruzi*, literatures regarding the presence of naturally occurring extracellular amastigotes (EA)[17] and in vitro production of EA[17–19] date back to decades ago. In addition, EA is known to have an infectious capability[20], albeit less than that of trypomastigote, and the mechanism of amastigote host invasion has been elucidated in recent years (reviewed by Bonfim-Melo et al.[21]). However, unlike *Leishmania*, EA had not been utilized as an experimental tool in *T. cruzi*, primarily because EA had been regarded as an obligate intracellular parasite, and thus had not been considered as "replicative form" in a practical sense.

Recently, our group proposed to utilize EA of *T. cruzi* as a temporal axenic culture[22]. Amastigotes of *T. cruzi* Tulahuen strain can replicate free of host cells in LIT medium at 37 ˚C for up to 10 days without major deterioration or loss of amastigote-like properties. During the host-free growth period, EA was successfully utilized for exogenous gene expression by conventional electroporation, drug titration assay with trypanocidal compounds, and CRISPR/Cas9-mediated

knockout followed by growth phenotype monitoring. In this report, we describe the detailed protocol to produce in vitro derived EA and to utilize the axenic amastigote in knockout experiments.

PROTOCOL:

NOTE: An overview of the entire experimental flow is depicted in **Figure 1**.

[Place **Figure 1** here.]

1. Parasite culture preparations

1.1. Use the Tulahuen strain of *Trypanosoma cruzi* throughout this protocol. Maintain epimastigotes of *T. cruzi* in LIT medium (10% FCS)[23]. Securely close the cap and keep the culture flask at 28 ˚C.

1.2. Generate a transgenic strain of *T. cruzi* that harbors the Cas9 endonuclease. Examples of the expression plasmids that contain Cas9 coding sequence and G418 (neo^r) selection marker can be found in Lander et al.[4] and Peng et al.[5]

1.2.1. Transfect the above plasmid into epimastigote by electroporation, using the kit listed in the **Table of Materials**. For 1 cuvette, spin down 2 x 10^7 cells, discard the supernatant, and resuspend with 100 µL of electroporation buffer containing the provided supplement solution.

NOTE: The electroporation buffer can be substituted with EM buffer[24] (3:1 mixture of cytomix[25] and phosphate-sucrose buffer).

1.2.2. Add 20 – 40 µg of plasmid and transfer the mixture into a 2 mm gap electroporation cuvette. Apply the pulse with an electroporation device (see **Table of Materials**), using the X-14 program[26].

1.2.3. Transfer the cuvette contents into a T-25 flask containing 5 mL of LIT medium (10% FCS). Incubate the flask at 28 ˚C for 24 h.

1.2.4. Add G418 to a final concentration of 250 µg/mL and continue incubation at 28 ˚C. It takes about 1 week to kill off the non-transfected epimastigotes. Once the G418-resistant population starts to recover, passage the culture once or twice a week to avoid saturation and to dilute out the dead cells. Establishment of a stable cell line usually takes total of 4 weeks.

NOTE: If constitutive expression of Cas9 is a burden for the cell[5], make the expression specific to the amastigote stage by conjugating the 3'-UTR of amastin gene immediately downstream of the Cas9 open reading frame[27]. The detailed description of the amastigote-specific Cas9 expression plasmid can be found in Takagi et al.[22]

1.3. Establish a host-parasite co-culture of Cas9-expressing *T. cruzi* and mammalian host cells. In this report, use 3T3-Swiss Albino fibroblast cell as a host.

1.3.1. Differentiate *T. cruzi* epimastigotes into metacyclic trypomastigotes. Determine the cell density of the epimastigote culture using a hemocytometer and collect 5 x 10^7 cells by centrifugation for 15 min at 2,100 x *g*. Discard the supernatant and resuspend the cells with 10 mL of RPMI medium. Incubate the parasite at 28 ˚C for 1 week[28].

1.3.2. Collect metacyclic trypomastigotes in RPMI medium. Carefully tilt the flask and pipet out the solution without disturbing the parasites adhered to the bottom surface. Transfer the medium to a conical tube and centrifuge for 15 min at 2,100 x *g*. Discard the supernatant and resuspend the parasite with 5 mL of DMEM (10% FCS).

NOTE: The parasite population is a mixture of metacyclic trypomastigotes, epimastigotes, and some intermediate forms. Although not necessary, isolate trypomastigote by DEAE ion exchange chromatography[29]. Alternatively, epimastigotes can be eliminated by incubating the parasites with active serum to subject them to complement lysis[30].

1.3.3. Seed 3T3-Swiss Albino fibroblast cell to 60 – 70% confluency, or about 1.7 – 2.0 x 10^6 cells in 5 mL of DMEM (10% FCS) in T-25 culture flask. Remove growth medium and apply the parasite mixture from step 1.3.2. Incubate for 24 h at 37 ˚C under 5% CO_2 in a humidified incubator to establish infection.

1.3.4. Remove the parasites remaining outside of the host 3T3 cells by washing the co-culture with DMEM (10% FCS) twice.

1.3.5. Once the co-culture is saturated, passage amastigote-infected host cells by trypsinization. Aspirate the medium and rinse the culture once with PBS. Apply 1 mL of 0.05% trypsin solution to cover the entire culture surface, and incubate for few minutes at room temperature until the attached host cells become loose. Detach the cells from the flask surface by flushing 3 mL of DMEM (10% FCS) over the cells.

1.3.6. Transfer detached host cells into a conical tube, and centrifuge for 3 min at 300 x *g*. Aspirate the supernatant and resuspend the cells with 3 mL of fresh DMEM (10% FCS). This step helps to eliminate remaining epimastigotes. Transfer 1 mL into a clean T-75 flask containing 4 mL of DMEM (10% FCS). Continue culturing until trypomastigote is released into culture supernatant.

1.3.7. Maintain the co-culture by passaging with trypsinization twice a week, repeating steps 1.3.5 and 1.3.6. Once 70 – 80% of the host population become infected, regularly add uninfected host 3T3 cells at the ratio of 5:1 (carryover:fresh), in order to avoid culture deterioration. Trypomastigote egresses continuously if the balance between host cells and *T. cruzi* is properly maintained.

2. Differentiation of trypomastigotes into EA

2.1. On the day before this experiment, remove the growth medium of host-parasite co-culture and add fresh DMEM (10% FCS) to wash away EA and trypomastigotes that had already been released from the host cells in previous days. Regular experiments require at least two T-75 flasks of confluent co-culture.

2.2. Collect culture supernatant into a conical tube to harvest freshly emerged trypomastigotes. Check the sample under a microscope (10x or 20x objective lens) for the quality. If there is host cell debris, briefly centrifuge the sample and transfer the supernatant into a new tube. If there are significant number of EAs, isolate trypomastigotes by the following swim-out procedure.

2.2.1. Spin down the mixture of trypomastigotes and amastigotes for 15 min at 2,100 x *g*. Discard most of the supernatant, leaving 0.5 – 1.0 mL of medium in the tube.

NOTE: Reducing the volume is optional, but it makes the following step easier.

2.2.2. Incubate the pellet at 37 ˚C for 1 – 2 h, allowing active trypomastigotes to swim out of the pellet (**Figure 2**).

2.2.3. Transfer the supernatant containing trypomastigotes to a 1.5 mL microcentrifuge tube.

2.3. Centrifuge the conical tube for 15 min at 2,100 x *g* to collect trypomastigotes. If the swim-out procedure was performed above, centrifuge the 1.5 mL tube for 2 min at 4,000 x *g* to pellet the trypomastigote. Discard the supernatant.

2.4. Resuspend the pellet with 5 mL of DMEM buffered with 20 mM MES (pH 5.0), supplemented with 0.4% BSA[19]. Transfer the parasite to a T-25 culture flask. Leave the cap loose. The cell density must be around or below 1 x 10^7 cells/mL, since oversaturation increases the chance of cell death.

NOTE: The color of the DMEM must be yellow, not orange. If the original DMEM had high buffering capacity, 20 mM MES (pH 5.0) is not enough to lower the pH. The pH of the medium must be adjusted by addition of HCl in that case. The acidic medium can be kept at 4 ˚C, but no longer than 1 month.

2.5. Incubate the culture flask at 37 ˚C under 5% CO_2 in a humidified incubator. Around 95% of parasites differentiate into amastigotes after 24 h.

3. Electroporation of EA

3.1. Prepare gRNA for electroporation. This can be done by in vitro transcription, or simply by purchasing synthetic RNA oligonucleotides from a manufacturer. In this report, crRNA and tracrRNA from Integrated DNA Technologies, Inc. are used.

3.2. Centrifuge the culture of EAs for 15 min at 2,100 x *g*. Discard supernatant.

3.3. Resuspend the pellet with electroporation buffer containing provided supplement solution to the final cell density of 1 x 10^8 cells/mL.

NOTE: EM buffer causes more cell deaths compared to electroporation buffer; thus, it is not recommended for amastigote transfection (**Supplemental Figure 1**).

3.4. Aliquot 100 µL of resuspended parasites (1 x 10^7 cells) into 1.5 mL microcentrifuge tubes. Add 5 – 10 µg of gRNA and gently mix by pipetting.

3.5. Transfer the mixture to a 2 mm gap electroporation cuvette. Apply the pulse with the electroporation device, using X-14 program.

3.6. Transfer the cuvette contents into a T-25 flask containing 5 mL of pre-warmed LIT medium (10% FCS). Leave the cap loose and incubate the flask at 37 ˚C under 5% CO_2.

3.7. Monitor the cell growth either by continuation of axenic culturing (section 4) or as intracellular amastigotes after host cell infection (section 5).

4. Monitoring the growth of knockout cells as axenic amastigotes

4.1. EAs settle at the bottom of the culture medium, so gently shake the flask to resuspend them into the solution. Washing the flask surface by pipetting helps, as some cells are adhered to the flask.

4.2. Mix 1 µL of propidium iodide (PI) solution (20 µg/mL) with 20 µL of amastigote culture.

NOTE: Do not leave the culture flask outside of incubator for longer than necessary. Temperature is one of factors that enables axenic proliferation[22].

4.3. Apply the sample onto hemocytometer and observe under a fluorescence microscope. PI is permeant to damaged cell membrane but is excluded from live cells. Count the number of viable amastigotes that are not stained by PI (ex/em 570 nm/602 nm).

5. Monitoring the growth of knockout cells as intracellular amastigotes

5.1. Seed host 3T3 cells in a 12-well plate with DMEM (10% FCS). Adjust the cell density to 70 – 80% confluency, or about 3 x 10^5 cells per well.

NOTE: Since amastigotes are not motile, covering most of the growth surface by the host cells improves infection efficiency.

5.2. Collect knockout amastigotes from step 3.6 by centrifugation one day after electroporation. Discard the supernatant, and resuspend the parasite with 2 mL of DMEM (10% FCS).

5.3. Remove medium from the host cell culture and apply resuspended amastigotes. Multiplicity of infection should be 20 or higher. Incubate the plate at 37 ˚C under 5% CO_2 for 2 days to allow amastigotes to establish infection.

NOTE: Infection period can be 1 day, depending on the purpose.

5.4. Wash away EAs remained outside of the host cells twice with DMEM (10% FCS).

5.5. Add fresh DMEM (10% FCS) to the host-parasite co-culture and continue the incubation at 37 ˚C for additional 2 days.

5.6. To evaluate infection efficiency, visualize nuclei of the host cells and intracellular amastigote.

NOTE: Nuclei tend to be overlapped in saturated co-culture. Re-plating the cells at lower cell density (such as 1:5 dilution) prior to fixation and staining helps to count nuclei more easily.

5.6.1. Remove culture medium and apply 1 mL of 4% formalin solution in PBS to fix the cells. Incubate for 10 min at room temperature.

5.6.2. Replace the formalin solution with 1 mL of PBS containing 1 µg/mL Hoechst 33342 and 0.1% Triton X-100. Incubate for 5 min at room temperature.

5.6.3. Remove the Hoechst solution and rinse the cells once with PBS. Add 1 mL of fresh PBS.

5.7. Observe under fluorescence microscope and identify host cell nuclei that are associated with smaller parasite nuclei (**Figure 4**). Host cells that contain more than 2 amastigotes should be considered as infected, not to include nonproductive initial infection or unwashed EAs.

REPRESENTATIVE RESULTS:

Isolation of trypomastigotes by the swim-out procedure

To harvest fresh trypomastigotes from contaminating old EAs by swim-out procedure, cell pellets need to be incubated at least for 1 h. Incubating the pellets for more than 2 h does not significantly increase the number of trypomastigotes swimming in the solution (**Figure 2B**). In this particular experiment, the percentage of trypomastigote in the initial mixture was 38%, and the percentage after the swim-out was above 98% at any given time points. From two T-75 flasks of confluent co-culture, we routinely obtain 3 – 4 x 10^7 cells of pure trypomastigote by this swim-out protocol.

Growth monitoring of knockout amastigote as axenic culture

Unlike other developmental stages of *T. cruzi*, flagellar-less amastigotes are practically static. Thus, staining with PI helps to distinguish live amastigotes from dead ones. PI is impermeable to the intact cell membrane but easily penetrates dead cells (**Figure 3A**). Amastigotes transfected with gRNA against essential gene, *TcCGM1* (TcCLB.511807.80), showed a significant growth

defect comparing to the control group that received gRNA not homologous to the *T. cruzi* genome (**Figure 3B**).

Growth monitoring of knockout parasites as intracellular amastigotes

The essentiality of the target gene in the amastigote stage can also be demonstrated by evaluation of the growth phenotype of knockout *T. cruzi* as intracellular amastigote (**Figure 4**). The fraction of host nuclei that is associated with smaller *T. cruzi* nuclei was significantly lower in *TcCGM1*-KO group comparing to the control group.

Knockout of a stage-specific gene causes phenotypic difference in amastigote and trypomastigote stages

Paraflagellar rod component PAR1 is highly expressed in trypomastigote stage, but is downregulated in the amastigote stage of *T. cruzi*[22, 31]. Indeed, transfection of gRNA against *TcPAR1* (TcCLB.506755.20) did not significantly affect the growth of EA after 4 days of axenic culturing (**Figure 5B**). gRNA transfection followed by host cell invasion also showed that *TcPAR1*-KO does not inhibit the growth of intracellular amastigotes, in terms of the fraction of infected host cells at 4 days post infection (**Figure 5C**). The number of parasites within individual host cell did not seem to be affected by the knockout, either (**Supplemental Figure 2**). These results indicate that TcPAR1 is not essential in the amastigote stage of *T. cruzi*.

However, the number of trypomastigotes emerged out of host cells at 5 days post infection was significantly lower in *TcPAR1*-KO co-culture, comparing to the control co-culture (**Figure 5D**). Also, differentiated trypomastigotes within host 3T3 cell before egression appeared to be quite active in control group (**Supplemental Movie 1**) but seemed sluggish in *TcPAR1*-KO group (**Supplemental Movies 2**). These results suggest that TcPAR1 plays an important role in the trypomastigote stage of *T. cruzi*, presumably by providing motility to help the egression, despite its non-essentiality in amastigote stage.

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of the knockout experiment using EA. Tissue culture-derived trypomastigotes are harvested and differentiated into EA. gRNA is transfected into Cas9-expressing amastigotes by electroporation, and growth phenotype of the knockout amastigote is evaluated either by axenic replication or by intracellular replication after host cell invasion.

Figure 2: Isolation of trypomastigote by swim-out procedure. (**A**) Schematic representation of the protocol. (**B**) The number of trypomastigotes that have swum out of the pellet at indicated time points. Initial pellets contained total of 3×10^7 parasites, 38% of which were trypomastigotes and the rest of which were amastigotes. Experiments were performed in triplicate and mean values (±SD) are plotted. The purity of trypomastigotes in solution was above 98% at any given time points. (**C**) Microscopy images of parasites before swim-out procedure (left), trypomastigotes that have swum out of a pellet (middle), and parasites remaining in a pellet (right) after 1 hour of incubation. Scale bars: 10 μm.

Figure 3: Growth monitoring of axenic amastigote. (**A**) Propidium iodide (PI) exclusion assay.

Microscopy images of EA on a hemocytometer in bright field (left), fluorescence channel (middle), and overlaid image (right). Yellow arrows indicate PI-stained dead cells. Scale bars: 20 μm. (**B**) Cell count of Cas9-expressing amastigotes after transfection with gRNAs against *TcCGM1* (open circle) and control gRNA with no homology to *T. cruzi* (closed circle). Electroporations were performed in triplicates, and mean values (±SD) are plotted. (**p<0.01 by Welch's t-test). This figure has been modified from Takagi et al.[22]

Figure 4: Growth of knockout amastigotes after host invasion. (**A**) Nuclear staining of the host-parasite co-culture after intracellular replication of amastigotes transfected with gRNAs against *TcCGM1* and control gRNA. gRNA-transfected EAs were applied onto 3T3 cells 1 day after electroporation and allowed to infect host cells for 2 days. Amastigotes remaining outside of the host cells were washed away, and the host-parasite co-culture was incubated for an additional 2 days. Scale bars: 20 μm. This figure has been modified from Takagi et al.[22] (**B**) Percentage of host 3T3 cells infected by control amastigotes (black bar) and *TcCGM1*-KO amastigotes (gray bar). The mean (±SD) of three infection experiments are plotted (**p<0.01, Welch's t-test). This figure has been modified from Takagi et al.[22]

Figure 5: Phenotypic difference between knockout amastigote and differentiated trypomastigote. (**A**) Schematic representation of experimental time line. (**B**) Cell counts of axenic amastigote at 4 days post transfection for control amastigotes (black bar) and *TcPAR1*-KO amastigotes (gray bar). Mean values (±SD) of three electroporation experiments are plotted (n.s.: not significant, Mann-Whitney *U* test). (**C**) Percentage of infected host 3T3 cells at 4 days post infection by control amastigote (black bar) and *TcPAR1*-KO amastigote (gray bar). Mean values (±SD) of three culture wells are plotted (n.s.: not significant, Mann-Whitney *U* test). (**D**) Number of trypomastigote released into culture medium at 5 days post infection for control co-culture (black bar) and *TcPAR1*-KO co-culture (gray bar). Mean values (±SD) of three culture wells are plotted (*p<0.05, Mann-Whitney *U* test).

Supplemental Figure 1: Effect of electroporation buffer on amastigote cell viability. EA was electroporated either in electroporation buffer or in EM buffer. Amastigote was stained with PI after 24 h to count the number of live and dead amastigotes. Percentages of dead cells in each group and in the no pulse group are plotted. The mean (±SD) of three electroporation experiments are shown (*p<0.05, n.s.: not significant, Kruskal-Wallis test).

Supplemental Figure 2: Microscopy images of nuclear-stained *TcPAR1*-KO and control intracellular amastigotes. Co-culture of host 3T3 cells and transfected amastigotes were fixed and stained at 4 days post infection. Scale bars: 20 μm.

Supplemental Figure 3: Growth phenotype of Cas9-expressing epimastigote. Cell counts of wildtype epimastigotes (WT), epimastigotes that constitutively expresses Cas9-EGFP (Cas9-EGFP), and epimastigotes that expresses Cas9-EGFP under the regulation of amastigote-specific 3'-UTR (Cas9-EGFP-AmaUTR) are plotted in log scale. Epimastigotes were cultivated in LIT medium (10% FCS) at 28˚C. Parasite cell density was adjusted to 1 x 10^6 cells/mL on Day 0. Cumulative cell counts are calculated as cell density multiplied by the total dilution factor. The mean (±SD) of

three culture flasks are shown (*p<0.05, n.s.: not significant, Kruskal-Wallis test).

Supplemental Movie 1: Microscopy video image of control co-culture. Number and activeness of trypomastigotes that have differentiated from control amastigotes. Video image at 5 days post infection is shown.

Supplemental Movie 2: Microscopy video image of *TcPAR1*-KO co-culture. Number and activeness of trypomastigotes that have differentiated from *TcPAR1*-KO amastigotes. Video image at 5 days post infection is shown.

DISCUSSION:

We demonstrated that the axenic culture of *T. cruzi* amastigotes can be utilized in CRISPR/Cas9-mediated gene knockout, by electroporating gRNA directly into Cas9-expressing EA. This way, the essentiality of the target gene specifically in amastigote stage can be evaluated without going through other developmental stages.

Another beneficial aspect of amastigote transfection is the convenience in testing for a large number of target genes. Once the co-culture of Cas9-expressing *T. cruzi* and host mammalian cell is established, it takes only few days to transform the tissue-derived trypomastigotes into EA and transfect gRNA to obtain knockout amastigotes. gRNA is the only material that needs to be tailored to each target gene, but synthetic gRNA is available from number of manufacturers, so it can simply be purchased. An alternative method to analyze the stage specific essentiality of target genes would be to establish an inducible knockout system[32]. In that case, plasmids must be constructed for each target gene and transfected to the parasite in the epimastigote stage to allow drug selection of the transfectants, since transfection efficiency of the plasmid is much lower than that of gRNA (<15% for plasmid[26] as supposed to >96% for gRNA[22]). Selected transfectants must be differentiated into metacyclic trypomastigotes to infect host cells to finally induce a knockout in intracellular amastigote. This whole process would take 1 – 2 months.

In this report, we used PI to distinguish dead cells in axenic amastigote culture to quantitate the cell density with a hemocytometer. Alternatively, metabolic assays such as the resazurin viability assay can be employed to estimate the number of live amastigotes, which is more suited for a high throughput format. In our hands, 1 x 10^5 amastigotes per well yield sufficient redox activity to be detected by a resazurin assay after 5 h of incubation.

One drawback of establishing a Cas9-expressing cell line is the potential cellular burden and non-specific cleavage due to Cas9 overexpression[5,33]. There are several reports indicating that constitutive expression of Cas9 has no effect on the growth rate of *T. cruzi*[4,6–8]. However, in one instance[5] and also in our hands, Cas9-expressing parasite showed slow growth comparing to the wildtype. In order to overcome this issue, we employed the amastigote-specific 3'-UTR to restrict the expression of Cas9 to amastigote stage[22]. Conjugation of amastin 3'-UTR to the Cas9 open reading frame restored the replication rate of transgenic parasite (**Supplemental Figure 3**), thereby producing robust host-parasite co-culture to continuously supply trypomastigotes for in vitro amastigogenesis. Recently, other groups have demonstrated that introduction of

gRNA/Cas9 RNP complex to wildtype parasite can cause genome editing in *T. cruzi*[7,9]. This method should be explored as a less stressful approach to the parasite[9], since study in mammalian cells suggests that the use of RNP reduces unwanted off-target genome editing, owing to the short half-life of Cas9 protein[34].

Another optional modification to the protocol would be the co-transfection of donor DNA as a template for homologous recombination. It has been reported that the donor DNA containing homologous sequences to upstream and downstream of the cleavage site, in a form of either double-stranded DNA[4,5,8,35] or single-stranded oligonucleotide[7,9], facilitates the repair process of double-stranded break caused by Cas9 nuclease. Although some reports indicate that microhomology mediated end joining without donor template can repair the cleavage and introduce a deletion mutation[5], introduction of the defined sequence to the mutation site nonetheless helps to confirm successful genome editing, because it is difficult to show the DNA evidence of gene knockout in some cases, even though the target protein reduction and phenotypic outcome suggest the target gene was mutated[4].

Cas9/gRNA RNP transfection and co-transfection of a donor DNA are not demonstrated in this report to simplify the description of the protocol and to focus on the preparation of EA and the use of axenic culture thereafter. If one wishes to perform those experiments, it can be done by simply replacing the components of electroporation.

Our method of utilizing EA of *T. cruzi* as an experimental tool potentially enables a variety of stage-specific studies, including transient gene expression by plasmid transfection and drug efficacy test[22]. However, the effectiveness of this approach has been tested only in the Tulahuen strain thus far. Since strains of *T. cruzi* are quite diverse, applicability of this protocol to other strains must be investigated.

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The authors have no conflict of interest to disclose.

REFERENCES

1. World Health Organization (WHO) Fact sheet: Chagas disease (American trypanosomiasis). *Washington: World Health Organization International*. [updated 2017 Mar; cited 2017 Aug], at <http://www.who.int/mediacentre/factsheets/fs340/en/> (2017).
2. Clayton, J. Chagas disease 101. *Nature*. **465** (n7301_supp), S4–S5, doi: 10.1038/nature09220 (2010).
3. Apt, W. Current and developing therapeutic agents in the treatment of Chagas disease. *Drug Design, Development and Therapy*. **4**, 243–53, at <http://www.ncbi.nlm.nih.gov/pubmed/20957215> (2010).
4. Lander, N., Li, Z.-H.H., Niyogi, S., Docampo, R. CRISPR/Cas9-induced disruption of

paraflagellar rod protein 1 and 2 genes in Trypanosoma cruzi reveals their role in flagellar attachment. *mBio*. **6** (4), e01012, doi: 10.1128/mBio.01012-15 (2015).
5. Peng, D., Kurup, S.P., Yao, P.Y., Minning, T.A., Tarleton, R.L. CRISPR-Cas9-mediated single-gene and gene family disruption in Trypanosoma cruzi. *mBio*. **6** (1), e02097-14, doi: 10.1128/mBio.02097-14 (2015).
6. Romagnoli, B.A.A., Picchi, G.F.A., Hiraiwa, P.M., Borges, B.S., Alves, L.R., Goldenberg, S. Improvements in the CRISPR/Cas9 system for high efficiency gene disruption in Trypanosoma cruzi. *Acta Tropica*. **178**, 190–195, doi: 10.1016/j.actatropica.2017.11.013 (2018).
7. Burle-Caldas, G.A., Soares-Simões, M., Lemos-Pechnicki, L., DaRocha, W.D., Teixeira, S.M.R. Assessment of two CRISPR-Cas9 genome editing protocols for rapid generation of Trypanosoma cruzi gene knockout mutants. *International Journal for Parasitology*. **48** (8), 591–596, doi: 10.1016/j.ijpara.2018.02.002 (2018).
8. Costa, F.C. et al. Expanding the toolbox for Trypanosoma cruzi: A parasite line incorporating a bioluminescence-fluorescence dual reporter and streamlined CRISPR/Cas9 functionality for rapid in vivo localisation and phenotyping. *PLoS Neglected Tropical Diseases*. **12** (4), e0006388, doi: 10.1371/journal.pntd.0006388 (2018).
9. Soares Medeiros, L.C. et al. Rapid, Selection-Free, High-Efficiency Genome Editing in Protozoan Parasites Using CRISPR-Cas9 Ribonucleoproteins. *mBio*. **8** (6), doi: 10.1128/mBio.01788-17 (2017).
10. Callahan, H.L., Portal, A.C., Devereaux, R., Grogl, M. An axenic amastigote system for drug screening. *Antimicrobial Agents and Chemotherapy*. **41** (4), 818–22, at <http://www.ncbi.nlm.nih.gov/pubmed/9087496> (1997).
11. Bates, P.A. Axenic culture of Leishmania amastigotes. *Parasitology Today*. **9** (4), 143–6, doi: 10.1016/0169-4758(93)90181-E (1993).
12. Ravinder, Bhaskar, Gangwar, S., Goyal, N. Development of luciferase expressing Leishmania donovani axenic amastigotes as primary model for in vitro screening of antileishmanial compounds. *Current Microbiology*. **65** (6), 696–700, doi: 10.1007/s00284-012-0209-1 (2012).
13. Nühs, A. et al. Development and Validation of a Novel Leishmania donovani Screening Cascade for High-Throughput Screening Using a Novel Axenic Assay with High Predictivity of Leishmanicidal Intracellular Activity. *PLoS Neglected Tropical Diseases*. **9** (9), 1–17, doi: 10.1371/journal.pntd.0004094 (2015).
14. De Rycker, M. et al. Comparison of a high-throughput high-content intracellular Leishmania donovani assay with an axenic amastigote assay. *Antimicrobial Agents and Chemotherapy*. **57** (7), 2913–2922, doi: 10.1128/aac.02398-12 (2013).
15. Rochette, A., Raymond, F., Corbeil, J., Ouellette, M., Papadopoulou, B. Whole-genome comparative RNA expression profiling of axenic and intracellular amastigote forms of Leishmania infantum. *Molecular and Biochemical Parasitology*. **165** (1), 32–47, doi: 10.1016/j.molbiopara.2008.12.012 (2009).
16. Pescher, P., Blisnick, T., Bastin, P., Späth, G.F. Quantitative proteome profiling informs on phenotypic traits that adapt Leishmania donovani for axenic and intracellular proliferation. *Cellular Microbiology*. **13** (7), 978–91, doi: 10.1111/j.1462-5822.2011.01593.x (2011).

17. Andrews, N.W., Hong, K. su, Robbins, E.S., Nussenzweig, V. Stage-specific surface antigens expressed during the morphogenesis of vertebrate forms of Trypanosoma cruzi. *Experimental Parasitology*. **64** (3), 474–484, doi: 10.1016/0014-4894(87)90062-2 (1987).
18. Pan, S.C. Trypanosoma cruzi: intracellular stages grown in a cell-free medium at 37 C. *Experimental Parasitology*. **45** (2), 215–24, at <http://www.ncbi.nlm.nih.gov/pubmed/354956> (1978).
19. Tomlinson, S., Vandekerckhove, F., Frevert, U., Nussenzweig, V. The induction of Trypanosoma cruzi trypomastigote to amastigote transformation by low pH. *Parasitology*. **110** (05), 547, doi: 10.1017/S0031182000065264 (1995).
20. Ley, V., Andrews, N.W., Robbins, E.S., Nussenzweig, V. Amastigotes of Trypanosoma cruzi sustain an infective cycle in mammalian cells. *Journal of Experimental Medicine.* **168** (0022–1007 (Print)), 649–659, doi: 10.1084/jem.168.2.649 (1988).
21. Bonfim-Melo, A., Ferreira, E.R., Florentino, P.T. V, Mortara, R.A. Amastigote Synapse: The Tricks of Trypanosoma cruzi Extracellular Amastigotes. *Frontiers in Microbiology*. **9**, 1341, doi: 10.3389/fmicb.2018.01341 (2018).
22. Takagi, Y., Akutsu, Y., Doi, M., Furukawa, K. Utilization of proliferable extracellular amastigotes for transient gene expression, drug sensitivity assay, and CRISPR/Cas9-mediated gene knockout in Trypanosoma cruzi. *PLOS Neglected Tropical Diseases*. **13** (1), e0007088, doi: 10.1371/journal.pntd.0007088 (2019).
23. Fernandes, J.F., Castellani, O. Growth characteristics and chemical composition of Trypanosoma cruzi. *Experimental Parasitology*. **18** (2), 195–202, doi: 10.1016/0014-4894(66)90016-6 (1966).
24. Oberholzer, M., Lopez, M.A., Ralston, K.S., Hill, K.L. Approaches for Functional Analysis of Flagellar Proteins in African Trypanosomes. *Methods in Cell Biology*. **93**, 21–57, doi: 10.1016/S0091-679X(08)93002-8 (2009).
25. van den Hoff, M.J.B., Moorman, A.F.M., Lamers, W.H. Electroporation in 'intracellular' buffer increases cell survival. *Nucleic Acids Research*. **20** (11), 2902–2902, doi: 10.1093/nar/20.11.2902 (1992).
26. Lisandro Pacheco-Lugo, Yirys Díaz-Olmos, José Sáenz-García, Christian Macagnan Probst, W.D.D. Effective gene delivery to Trypanosoma cruzi epimastigotes through nucleofection. *Parasitology International*. **66** (3), 236–239, doi: 10.1016/j.parint.2017.01.019 (2017).
27. Coughlin, B.C., Teixeira, S.M., Kirchhoff, L. V, Donelson, J.E. Amastin mRNA abundance in Trypanosoma cruzi is controlled by a 3'-untranslated region position-dependent cis-element and an untranslated region-binding protein. *The Journal of Biological Chemistry*. **275** (16), 12051–60, at <http://www.ncbi.nlm.nih.gov/pubmed/10766837> (2000).
28. Shaw, A.K., Kalem, M.C., Zimmer, S.L. Mitochondrial Gene Expression Is Responsive to Starvation Stress and Developmental Transition in Trypanosoma cruzi. *mSphere*. **1** (2), doi: 10.1128/mSphere.00051-16.
29. Chao, D., Dusanic, D.G. Comparative studies of the isolation of metacyclic trypomastigotes of Trypanosoma cruzi by DEAE ion exchange chromatography. *Zhonghua Minguo wei sheng wu ji mian yi xue za zhi (Chinese Journal of Microbiology and Immunology)*. **17** (3), 146–52, at <http://www.ncbi.nlm.nih.gov/pubmed/6391853> (1984).

30. Nogueira, N., Bianco, C., Cohn, Z. Studies on the selective lysis and purification of Trypanosoma cruzi. *The Journal of Experimental Medicine*. **142** (1), 224–9, doi: 10.1084/JEM.142.1.224 (1975).
31. Minning, T.A., Weatherly, D.B., Atwood, J., Orlando, R., Tarleton, R.L., Tarleton, R.L. The steady-state transcriptome of the four major life-cycle stages of Trypanosoma cruzi. *BMC Genomics*. **10**, 370, doi: 10.1186/1471-2164-10-370 (2009).
32. Rico, E., Jeacock, L., Kovářová, J., Horn, D. Inducible high-efficiency CRISPR-Cas9-targeted gene editing and precision base editing in African trypanosomes. *Scientific Reports*. **8** (1), 7960, doi: 10.1038/s41598-018-26303-w (2018).
33. Fu, Y. et al. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nature Biotechnology*. **31** (9), 822–826, doi: 10.1038/nbt.2623 (2013).
34. Kim, S., Kim, D., Cho, S.W., Kim, J., Kim, J.-S. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Research*. **24** (6), 1012–1019, doi: 10.1101/GR.171322.113 (2014).
35. Chiurillo, M.A., Lander, N., Bertolini, M.S., Storey, M., Vercesi, A.E., Docampo, R. Different roles of mitochondrial calcium uniporter complex subunits in growth and infectivity of Trypanosoma cruzi. *mBio*. **8** (3), e00574-17, doi: 10.1128/mBio.00574-17 (2017).

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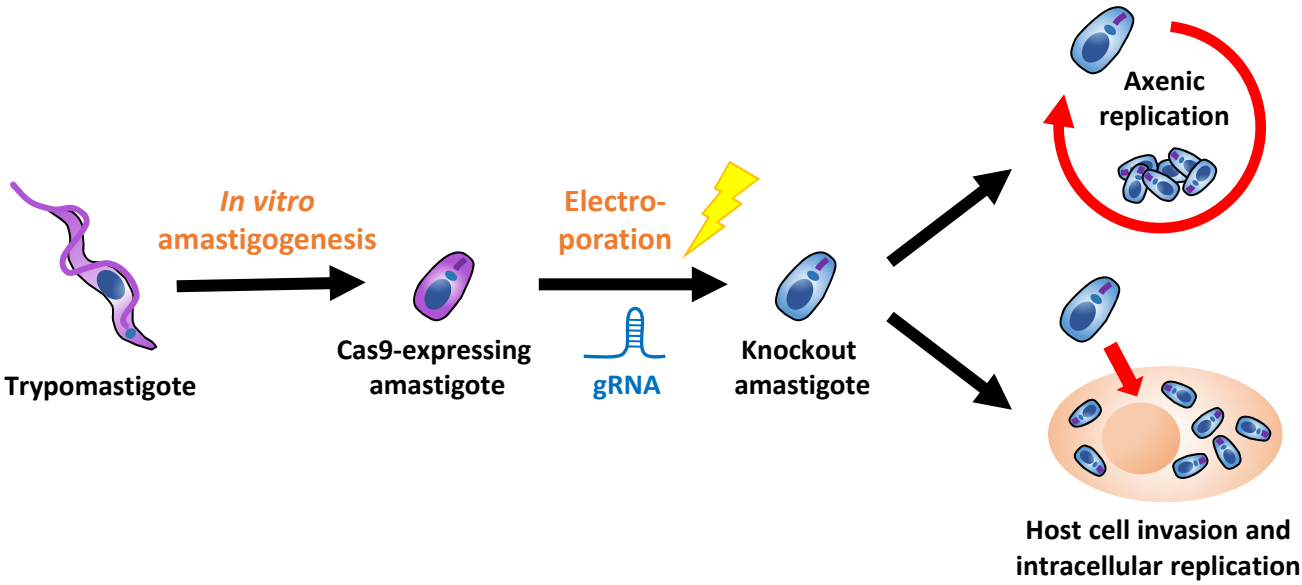

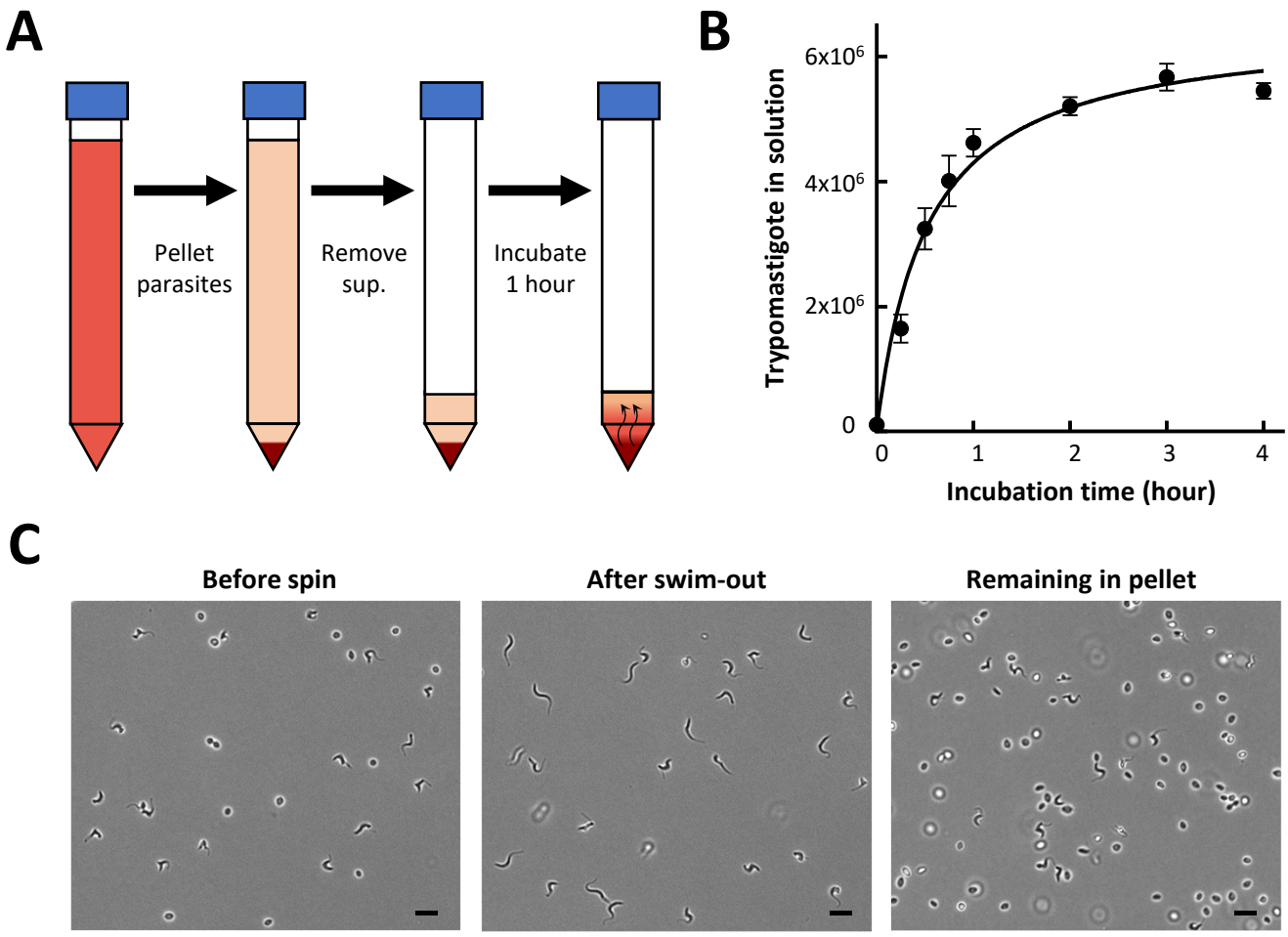

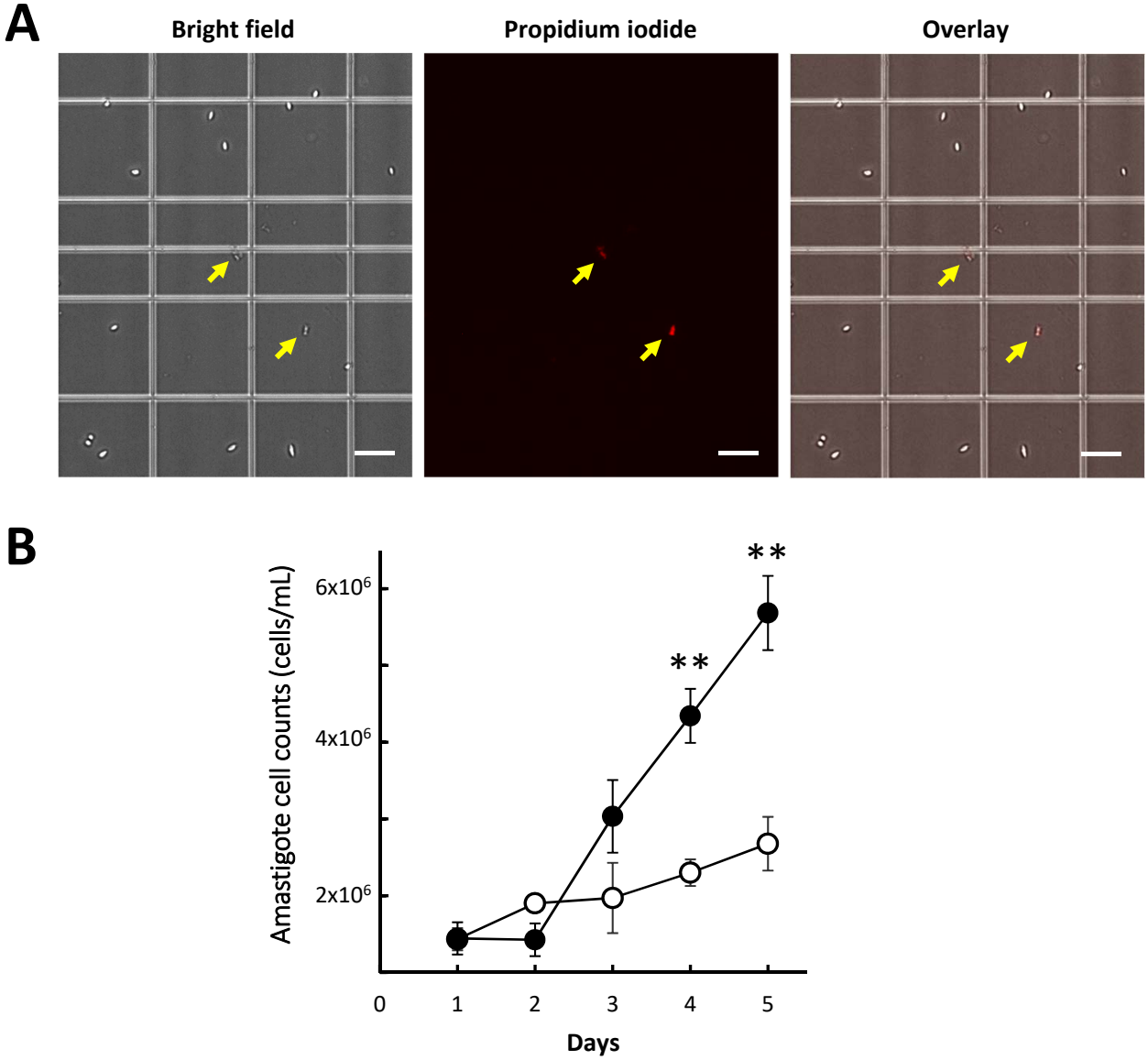

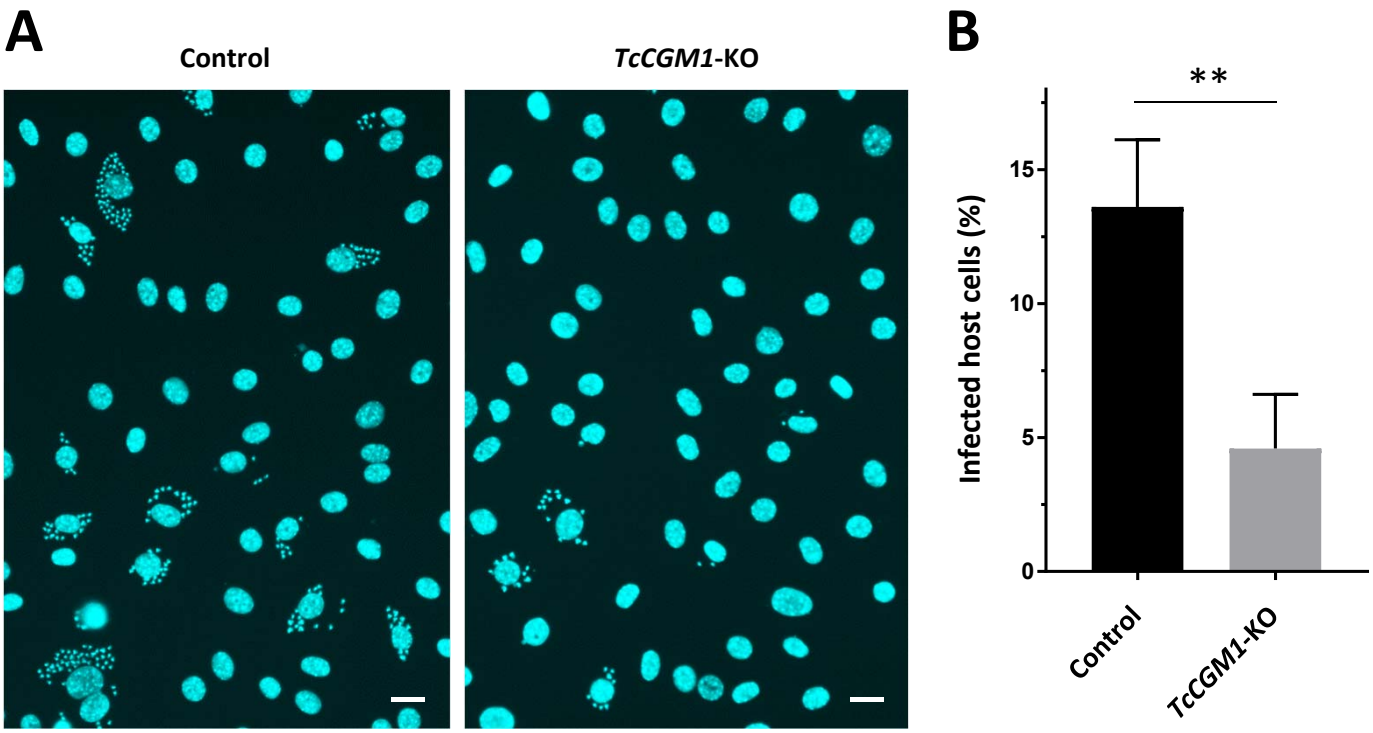

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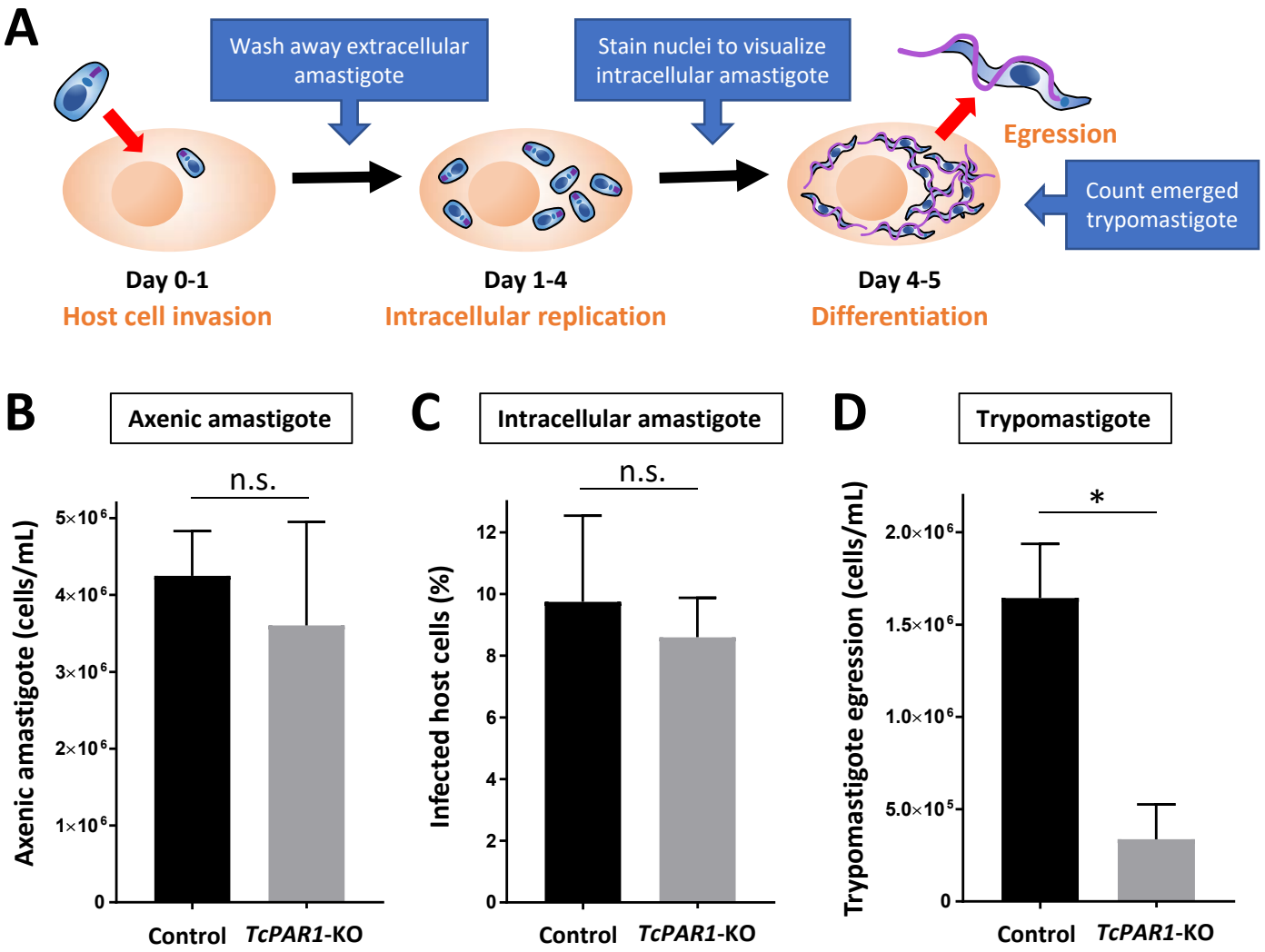

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25 cm^2 double seal cap culture flask	AGC Techno Glass	3100-025
75 cm^2 double seal cap culture flask	AGC Techno Glass	3110-075
All-in One Fluorescence Microscope	Keyence	BZ-X710
Alt-R CRISPR-Cas9 crRNA (for Control)	IDT	custom made
Alt-R CRISPR-Cas9 crRNA (for TcCGM1)	IDT	custom made
Alt-R CRISPR-Cas9 crRNA (for TcPAR1)	IDT	custom made
Alt-R CRISPR-Cas9 tracrRNA	IDT	1072532
Amaxa Nucleofector device	LONZA	AAN-1001
Basic Parasite Nucleofector Kit 2	LONZA	VMI-1021
BSA	Sigma-Aldrich	A3294
Burker-Turk disposable hemocytometer	Watson	177-212C
Coster 12-well Clear TC-Treated Multiple Well Plates	Corning	3513
DMEM	FUJIFILM Wako Pure Chemical	044-29765
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G-418 Sulfate Solution	FUJIFILM Wako Pure Chemical	077-06433
Hemin chloride	Sigma-Aldrich	H-5533
Hoechst 33342	Thermo Fisher Scientific	H3570
Liver infusion broth, Difco	Becton Dickinson	226920
MES	FUJIFILM Wako Pure Chemical	349-01623
PBS (–)	FUJIFILM Wako Pure Chemical	166-23555
Propidium Iodide	Sigma-Aldrich	P4864-10ML
RPMI 1646	Sigma-Aldrich	R8758

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fixing cells

target sequence = GGACGGCACCTTCATCTACAAGG
target sequence = TAGCCGCGATGGAGAGTTTATGG
target sequence = CGTGGAGAACGCCATTGCCACGG
to anneal with crRNA
electroporation
electroporation
component of the medium for in-vitro amastigogenesis
cell counting

culture medium

heat-inactivate before use

selection of transformant

component of LIT medium
staining of nuclei
component of LIT medium

component of the medium for in-vitro amastigogenesis

staining of dead cells
medium for metacyclogenesis

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A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Yuko Takagi
Department:	Biomedical Research Institute
Institution:	National Institute of Advanced Industrial Science and Technology (AIST)
Title:	Research Scientist
Signature:	
Date:	March 7, 2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

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Yuko Takagi
National Institute of Advanced Industrial Science and Technology
1-1-1 Higashi, AIST Central 6
Tsukuba, Ibaraki, Japan 305-8566

April 29, 2019

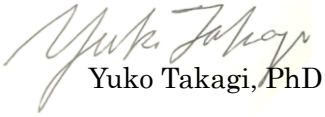
Dear Dr. Steindel,

Thank you for giving us the opportunity to revise our manuscript, JoVE59962 "Introducing a gene knockout directly into amastigote stage of Trypanosoma cruzi by CRISPR/Cas9 system." We greatly appreciate you and anonymous reviewers for your time and expertise to give us constructive feedback to improve the quality of our work.

We have reflected your comments in the updated version of our manuscript. Also, detailed description of changes and response to reviewers are listed below. We very much hope that the revised manuscript is acceptable for publication in JoVE.

Please address all the correspondent to me at [yuko-takagi@aist.go.jp]. I will be happy to respond to any further questions and comments.

Sincerely,



Yuko Takagi, PhD

List of Changes and Responses

Comments from editor and reviewers are in bold. Please note that comments are paraphrased or shortened for simplicity.

Editorial comments:

1. Thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have proofread our manuscript and corrected errors.

2. Please remove all commercial language from your manuscript and use generic terms instead.

We have deleted company names from the body text as much as possible.

3. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

We added more details based on your following comments.

4. 1.2.2: What are the program parameters?

Exact pulse condition of the Nucleofector is proprietary to the manufacturer. This electroporation device is commonly used in the field, and program X-14 has been used by other groups as well. We added a reference that reports X-14 is the best suited pulsing condition for Trypanosoma.

5. What are the culture conditions?

We clarified that cultivation was continued at 28 ˚C.

6. 1.3.1: How are the cells counted?

We specified to use hemocytometer for cell counting.

7. 1.3.2: What happens after centrifugation?

We added the description to discard the supernatant.

8. 1.3.3: Seed at confluency? Please provide cells/mL or cells/well. How much mixture is applied?

We added the equivalent cell numbers.

9. 1.3.5: How is the trypsinization done? How much of what concentration of trypsin is used?

We added more details on trypsinization step.

10. 1.3.6: How many fresh cells are added?

We added the instruction to mix the cells at the ratio of 5 : 1 (carryover : fresh).

11. Please specify what happens after each centrifugation, presumably aspiration.

We added the line to discard supernatant after each centrifugation step.

12. Please specify the microscope parameters used: magnification, etc.

We specified that the objective lenses are 10X or 20X.

13. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video.

We highlighted major steps in yellow.

14. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

We checked the flow of highlighted steps.

15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Sub-steps are highlighted when necessary.

16. Please obtain explicit copyright permission to reuse any figures from a previous publication. Please upload this information as a .doc or .docx file to your Editorial Manager account.

We will upload the e-mail correspondence between the author and journal staff regarding the re-use of published data.

Reviewer #1:

1. It is essential that the authors show that the knockout really worked, either by qPCR or Western blot.

We already published the concept of CRISPR/Cas9-mediated amastigote knockout elsewhere. In there, we validated the target protein reduction by GFP signal loss and enzymatic assay of the target protein. Evidence of genome editing was also

observed by T7 endonuclease cleavage of PCR product.

2. Line 100 - Since constitutive expression of Cas9 is a burden for the cell. This is not necessarily true for all T. cruzi strains.

We moved the description of amastigote-specific 3'UTR to the "NOTE," and made it optional. We also added a supplemental figure to show that Cas9-expressing *T. cruzi* (Tulahuen strain) grows slower than wildtype parasite in our hand, but growth kinetics can be restored by conjugating amastigote-specific 3-'UTR to Cas9 ORF.

Reviewer #2:

1- The authors did not mention that protocols to produce in vitro derived extracellular amastigote have been published since 1988. Similarly, the authors also failed to mention that several protocols for disrupting T. cruzi genes using CRISPR/Cas9 have been described since 2014.

We expanded the introduction section to include those points.

2- On Line 100, the authors stated that "constitutive expression of Cas9 is a burden for the cell" without showing any evidence for that or indicating any reference describing these findings (even though they mentioned this point as "data not shown" in line 411 in the discussion).

We moved the description of amastigote-specific 3'UTR to the "NOTE," and made it optional. We also added a supplemental figure to show that Cas9-expressing *T. cruzi* (Tulahuen strain) grows slower than wildtype parasite in our hand, but growth kinetics can be restored by conjugating amastigote-specific 3-'UTR to Cas9 ORF.

3- On line 287 and figure 2, the authors should indicate the percentages of trypomastigotes and amastigotes obtained before and after the "swim out" procedure.

We added the information to the corresponding sections.

4- On line 118, the authors should indicate how to maintain the cultures "until transformants are selected to homogeneity". It should be indicated whether, during the 4 weeks period, the medium needs to be changed, etc.

We indicated that culture needs to be split to avoid saturation and to dilute out the dead cells.

5- On line 141, it can be mentioned that epimastigotes can be eliminated from the culture by culturing for 24 hours in RPMI medium containing 10% non inactivated horse serum.

We included complement-mediated lysis as an alternative way to remove epimastigote in the NOTE section. We prefer to keep the wash-away method as a default, since susceptibility to complement-mediated lysis varies among strains.

6- Because the authors used only sgRNA during transfection and did not included a donor DNA template to promote DSB repair and gene knockout by homologous recombination, it can not be concluded that the observed growth defect is due exclusively to the TcCGM1 knockout. The same question needs to be addressed regarding the experiment shown in Figure 5.

We reflected this concern in the discussion section.

7- Fig 4 should indicate the time point post-infection.

We indicated the time frame of infection and intracellular growth.

8- On line 389, please eliminate the word "temporal"

We deleted the word.

9- In the discussion, the authors failed to mention two highly relevant points: (1) published results showing distinct protocols including the use of recombinant Cas9, which may the best strategy to avoid any possible off target effect of the constitutive expression of Cas9 and, (2) important discussions regarding the need to include a donor DNA template to promote DSB repair to assure that only the target gene has been affected.

We included these points in the discussion section.

End of list

Supplemental Figure 1

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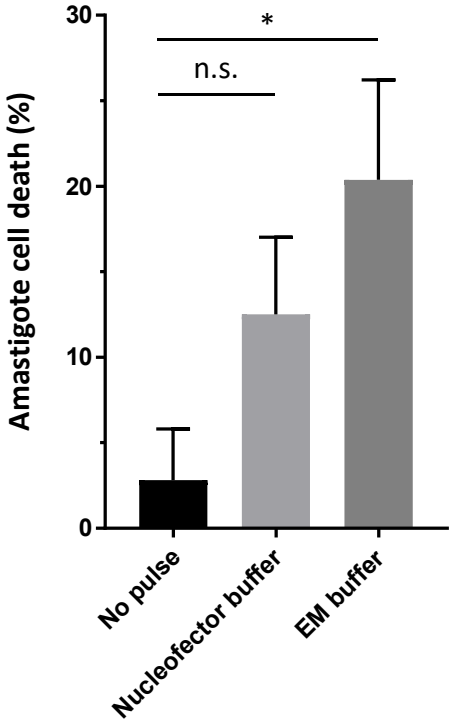

Supplemental Figure 2

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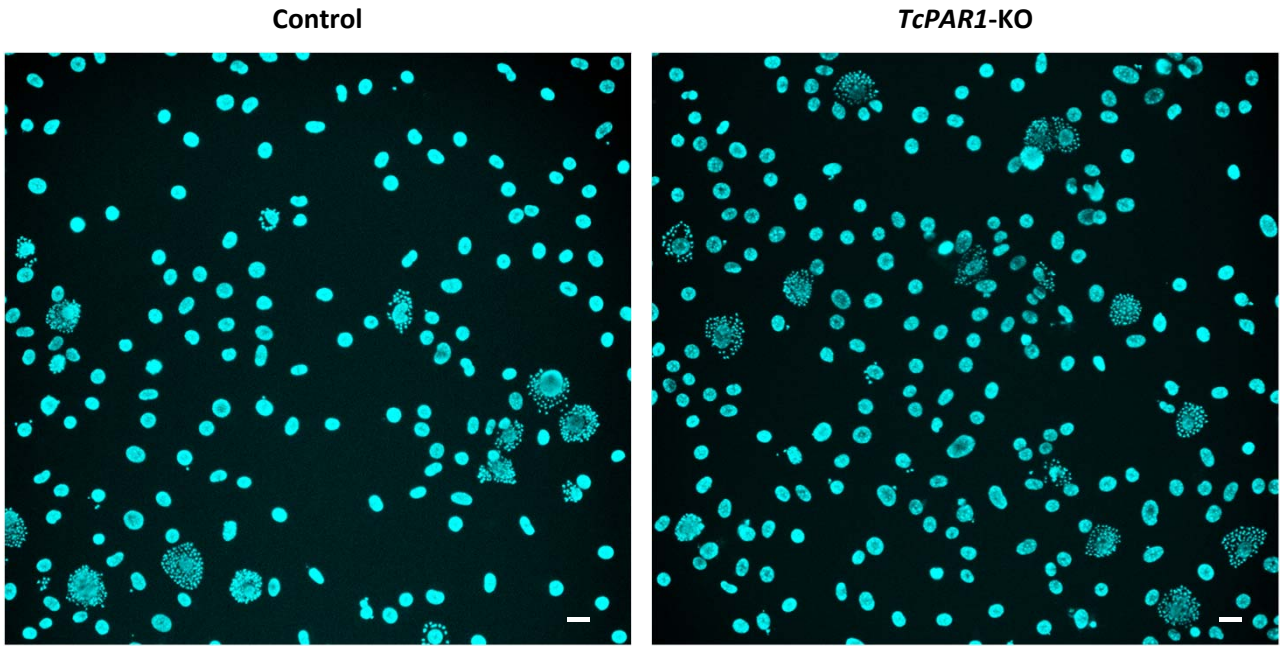

Supplemental Figure 3

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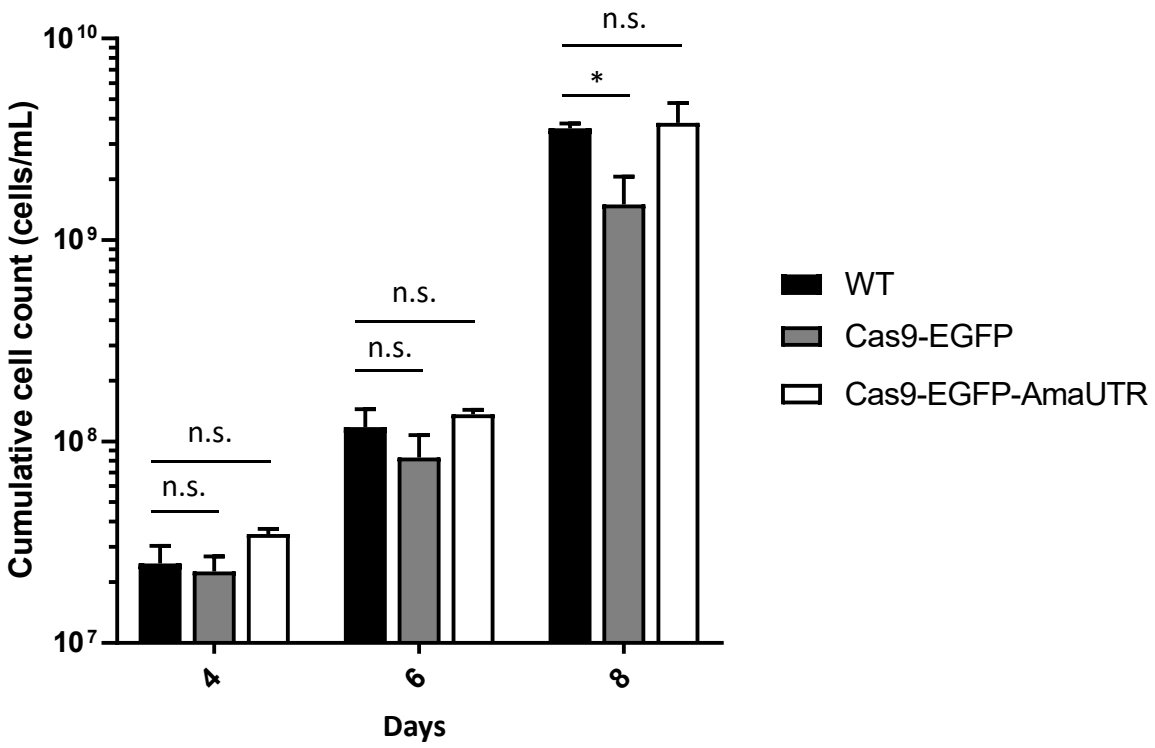

E-mail correspondence between the author and PLoS NTDs journal staff.

差出人: 高木悠友子
送信日時: 2019 年 2 月 9 日 15:05
宛先: PLOS Neglected Tropical Diseases
件名: RE: Question regarding re-use of contents

Dear Audrey,

Thank you for your reply.
In that case, I will indicate the figures as “modified from XXX” only when I use exactly the same data, and not when I use different versions of the images from the same experiment.

Thank you very much for your help.

Best regards,
Yuko Takagi

---------- Original Message ----------
From: noreply@salesforce.com <noreply@salesforce.com> **On Behalf Of** PLOS Neglected Tropical Diseases
Sent: Saturday, February 9, 2019 4:11 AM
To: 高木悠友子 <yuko-takagi@aist.go.jp>
Subject: Re: Question regarding re-use of contents

Dear Dr. Takagi,

Thank you for your email. I've consulted with our journal staff and they've advised that citing the new figures is not necessary, and they are not considered "modification of figure" according to our policy.

Thank you for your diligence and please be in touch if you require any further assistance.

All the best,
Audrey
PLOS | OPEN FOR DISCOVERY
Audrey Snider | Publications Assistant, PLOS Neglected Tropical Diseases
1160 Battery Street, Suite 225, San Francisco, CA 94111
plosntds@plos.org

Case Number: 06160889
ref:_00DU0Ifis._5000Br9kC5:ref

---------- Original Message ----------
From: ????? [yuko-takagi@aist.go.jp]
Sent: 2/7/2019 6:36 PM
To: plosntds@plos.org
Subject: Question regarding re-use of contents

Dear PLOS NTDs editorial staff,

I am currently writing a method paper based on a work I recently published with PLOS NTDs.
I would like to include some of my work in that paper as representative results.

According to your website on CC BY, it seems that I do not need any permission to re-use my own figures, as long as I reference the original paper.
My question is that do I still need to reference the paper if I use different figure but the same concept?

For example, if I perform another experiment and obtain new data and microscopy images, using exactly the same materials and methods as PLOS paper, I definitely reference the original paper for the method part, but I don't think the figures would be considered as "modification from original."

But, if I did not repeat the experiment and use the microscopy images I took in the previous experiment, I am not sure if the image would be considered as "re-use of the original figure."
The images were taken during the same experiment as PLOS paper, but in different magnification and was not used in the original paper.
Should I still consider this as "modification of figure" and reference my PLOS paper in figure section, not only in the method section?

Thank you in advance for your help.

Best regards,
Yuko Takagi