Video Article

Biolistic Transformation of a Fluorescent Tagged Gene into the Opportunistic Fungal Pathogen *Cryptococcus neoformans*

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Abstract

The basidiomycete *Cryptococcus neoformans*, an invasive opportunistic pathogen of the central nervous system, is the most frequent cause of fungal meningitis worldwide resulting in more than 625,000 deaths per year worldwide. Although electroporation has been developed for the transformation of plasmids in *Cryptococcus*, only biolistic delivery provides an effective means to transform linear DNA that can be integrated into the genome by homologous recombination.

Acetate has been shown to be a major fermentation product during cryptococcal infection, but the significance of this is not yet known. A bacterial pathway composed of the enzymes xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (Xfp) and acetate kinase (Ack) is one of three potential pathways for acetate production in *C. neoformans*. Here, we demonstrate the biolistic transformation of a construct, which has the gene encoding Ack fused to the fluorescent tag mCherry, into *C. neoformans*. We then confirm integration of the *ACK*-mCherry fusion into the *ACK* locus.

Video Link

The video component of this article can be found at http://www.jove.com/video/52666/

Introduction

Cryptococcus neoformans, an invasive opportunistic pathogen of the central nervous system, is the most frequent cause of fungal meningitis resulting in more than 625,000 deaths per year worldwide [1]. Acetate has been shown to be a major fermentation product during cryptococcal infection [2,3,4], and genes encoding enzymes from three putative acetate-producing pathways have been shown to be upregulated during infection [5]. This suggests that acetate production and transport may be a necessary and required part of the pathogenic process; however, the significance of this is not yet understood. One possible pathway for acetate production is the xylulose 5-phosphate/fructose 6-phosphate phosphoketolase (Xfp) - acetate kinase (Ack), a pathway previously thought to be present only in bacteria but recently identified in both euascomycete as well as basidiomycete fungi, including *C. neoformans* [6].

To determine the localization of these enzymes of this pathway in the cell, a construct carrying a neomycin resistance gene downstream of an *ACK* gene fusion to the fluorescent tag mCherry (ACK:mCherry:Neo) will be introduced into *C. neoformans* using the well-established method of biolistic transformation [7,8]. Although electroporation is an efficient method for transformation of plasmids that will be maintained as episomes into *Cryptococcus* [9], it is not useful in creating stable homologous transformants [8]. Only biolistic delivery using a gene gun provides an effective means to transform linear DNAs that will be integrated into the genome by homologous recombination. For example, Edman *et al.* showed that of the transformants resulting from electroporation of a plasmid-borne *URA5* selectable marker into *C. neoformansura5* mutants, just 0.001 to 0.1% of transformants were stable [9]. Chang *et al.* achieved just a 0.25% stable transformation efficiency using electroporation to reconstitute capsule production in an acapsular mutant [10]. Unlike electroporation, biolistic transformation has been shown to result in stable transformation efficiency of 2-50% depending on the gene that is being altered [7,8,11].

This visual experiment will provide a step-by-step demonstration of biolistic transformation of the linear ACK:mCherry:Neo DNA construct into *C. neoformans*, and will describe how to confirm its proper integration via homologous recombination into the *ack* locus. The protocol demonstrated here is a modification of the method developed in the Perfect laboratory [8].

Protocol

NOTE: The overall scheme of this protocol is outlined in **Figure 1**.

1. *C. neoformans* Preparation

1. For each transformation reaction, grow a 2-3 ml O/N culture of *C. neoformans* in YPD medium at 30 °C shaking at 250 rpm.
2. Centrifuge the O/N culture for 5 min at 900 x g at 10 °C and discard the supernatant.
3. Resuspend each cell pellet in 300 µl of Yeast Peptone Dextrose (YPD) medium.
4. Using glass beads, gently spread 300 µl of the washed cell suspension onto YPD agar containing 1 M sorbitol.
5. Allow plates to dry at ambient temperature for 3-4 hr.

2. Gold Microcarrier Preparation

1. Resuspend 0.25 g of 0.6 µm gold beads in 1 ml of ddH_2O, centrifuge for 1 min at 900 x g to pellet the beads, and remove the supernatant.
2. Resuspend the gold beads in 1 ml of 100% ethanol.
3. Distribute the beads into 4 tubes, 250 µl each, and add 750 µl of 100% ethanol.
4. Store gold bead aliquots at 4 °C.

3. DNA Preparation

1. Prepare orange macrocarrier biolistic discs by submerging them in 100% ethanol using forceps. Place discs into a large petri dish containing drierite to dry (make sure the drierite does not touch the discs).
2. Once dry, press the macrocarrier discs into the silver disc holders (previously wiped down with 100% ethanol).
3. Vortex gold beads (prepared as in step 2) and aliquot 12 µl into a 1.5 ml microcentrifuge tube, one tube per transformation.
4. Add to each tube in order: 2 µg of DNA (preferably 2 µl of 1 µg/µl of DNA), 10 µl 2.5 M $CaCl_2$, and 2 µl 1 M spermidine free base.
5. Set up a negative control as in step 3.4 but with no DNA.
6. Vortex each tube and incubate at ambient temperature for 5 min. Gently flick each tube occasionally to resuspend the settled beads during this incubation.
7. Spin tubes at 225 x g for 30 sec to pellet the DNA-coated gold beads. Carefully remove the supernatant (by pipetting or aspiration) and discard.
8. Resuspend beads completely in 600 µl of 100% ethanol by slowly pipetting up and down.
9. Spin tubes at 225 x g for 30 sec to pellet the beads without packing. Carefully remove and discard the supernatant.
10. Resuspend the DNA-coated gold beads in 8 µl of 100% ethanol by slowly pipetting up and down.
11. Pipette the DNA-coated gold beads onto the center of the biolistic disc in a 1 cm diameter and allow to dry.
    NOTE: A dried gold circle visible on the center of the biolistic disc indicates that a sufficient concentration of gold beads is present.
    NOTE: The macrocarrier discs loaded with DNA-coated gold beads are now ready for use with the gene gun.

4. Operating the Gene Gun

1. Turn on the vacuum pump.
2. Turn on the helium gas by turning the knob counterclockwise until a pressure of approximately 2,200 psi is reached on the pressure gauge.
3. Turn on the gene gun by flipping the red switch on the left.
4. Be sure that the flow rates for the vacuum and the vent are adjusted so the vacuum will reach 28 inches Hg within 15 sec.
5. Be sure the distance between the rupture disc and macrocarrier is approximately 3/8 inch.
6. Clean the entire chamber by wiping down with ethanol.
7. Submerge the rupture discs in 100% ethanol. Allow to dry on a sterile surface (*e.g.*, Petri dish).
8. Use a torque wrench to loosen the rupture disc holder. Insert a clean rupture disc into the holder. Screw the rupture disc holder back into place and tighten with torque wrench by turning it once to the right.
    NOTE: Rupture discs will be replaced following each shoot.
9. Submerge the mesh screens in 100% ethanol. Allow to dry on a sterile surface (*e.g.*, Petri dish).
10. Once dry, place a washed mesh screen on the white plastic mounting plate. Place the macrocarrier disc holder DNA side down into the disc chamber. Screw on the silver cap, and place the mounting plate in the highest slot.
    NOTE: The mesh screen will be replaced after each shoot.
11. Place a YPD agar plate containing 1 M sorbitol on the bottom plate.
12. Shut chamber door and lock into place.
13. Push and hold the middle red switch up to engage vacuum and allow the vacuum to reach 28 inches Hg. Once proper vacuum level is reached, move this switch to the down position. When ready, hold down the red switch on the right to fire. When the rupture disc pops, immediately release the fire button and push the middle red switch to the middle position to vent the chamber to 0 psi.
14. Clean out rupture disc debris and turn off the gene gun. Then turn off the helium gas by turning the knob clockwise, and finally, turn off the vacuum pump.

5. Plating Transformed Cells

1. Allow the transformation plates to sit at RT for 4 hr to allow the cells to recover.
2. Pipette 700 µl of YPD onto the plate. Use a cell scraper to gently scrape the cells off of the agar and pipette liquid into a sterile 1.5 ml microfuge tube. Repeat this step to ensure all cells have been recovered from the plate.
3. Pellet cells at 225 x g for 30 sec. Remove and discard the supernatant.
4. Resuspend the pellet in 500 µl of YPD.
5. Pipette 100 µl of the cell suspension onto the center of the YPD + antibiotic plates and spread using glass beads.

6. Leave inverted plates at RT for 3-4 days. As colonies appear, patch onto new YPD + antibiotic plates.

6. Genomic DNA Isolation for PCR

NOTE: This is a modified version using reagents from a DNA purification kit (See Table of Materials).

1. Grow a 5 ml culture of each of the *C. neoformans* transformants in YPD liquid at 30 °C shaking at 250 rpm O/N.
2. Pellet 3 ml of cells at 900 x g, and resuspend in 600 µl of nuclei lysis solution.
3. Add the suspension to a new 1.5 ml microcentrifuge tube with 200 µl of 0.5 mm acid washed glass beads.
4. Homogenize for 45 sec in a mini beadbeater at ambient temperature, cool tube on ice, and repeat.
5. Allow sample to settle on ice for 2 min and transfer supernatant to a new 1.5 ml tube. Add 200 µl of protein precipitation solution to each tube, (100 µl for every 600 µl of supernatant recovered) and vortex vigorously for 20 sec.
6. Allow samples to settle on ice for 5 min, and centrifuge at 11,000 x g for 3 min.
7. Transfer the supernatant to a clean 1.5 ml tube containing 300 µl of RT isopropanol. Gently mix by inversion.
8. Centrifuge samples at 11,000 x g for 2 min, carefully remove the supernatant, and drain the tubes onto paper towels.
9. Add 300 µl of RT 70% ethanol to each tube, and gently invert to wash the pellet.
10. Centrifuge samples at 11,000 x g for 2 min, and carefully remove all of the ethanol.
11. Drain the tube onto clean paper towels, and allow the pellet to air dry for 10-15 min.
12. Add 50 µl of DNA rehydration solution and 1.5 µl of RNase solution to each pellet and vortex.
13. Centrifuge samples for 5 sec to remove all of the liquid from the cap.
14. Incubate samples at 37 °C for 15 min.
15. Rehydrate the DNA by incubating the samples at 65 °C for 1 hr.
16. Quantify DNA spectrophotometrically by measuring the absorbance at 260 nm (an A_{260} reading of 1.0 is equivalent to ~50 µg/ml double-stranded DNA), and use up to 200 ng in each PCR reaction.

7. RNA Isolation for Reverse Transcriptase-PCR.

1. Using a RNA purification kit (See Table of Materials), follow the manufacturer's instructions to isolate RNA from yeast cells using a minibeadbeater.
2. Quantify the concentration of the RNA by measuring the absorbance at 260 nm (an A_{260} reading of 1.0 is equivalent to ~40 µg/ml single-stranded RNA).
3. Using an RT-PCR kit (See Table of Materials), follow the manufacturer's instructions to set up RT-PCR reactions with approximately 1 µg of RNA. For the results obtained in this study, use the primers listed in **Table 1**.

Representative Results

A successful biolistic transformation of *C. neoformans* can be obtained by following this protocol scheme (**Figure 1**). With biolistic transformation, a successful shoot of the coated gold beads is indicated by a gold ring visible on the plate after the DNA is shot (**Figure 2A**). Colonies should appear within 4 to 5 days when left at room temperature after plating the recovered cells from the YPD + 1M sorbitol plates onto selective media. Transforming 2 µg of DNA should result in 20 to 30 colonies (**Figure 2B**). When colonies appear, they should be restreaked on selective media for individual colonies.

The individual colonies can be grown in YPD media, and both DNA and RNA can be isolated from these cells and analyzed through PCR and RT-PCR to confirm proper integration and expression. If this protocol is used for tagged gene fusion, as in this example, the primers would need to anneal within the coding region of the gene of interest (primer 2) and within the 3' noncoding region of the gene of interest (primer 4) (**Figure 3A**). With this construct, the DNA amplified from the PCR reaction was sequenced for another confirmation that the mCherry tag was fused in frame to the *ACK* gene. A positive PCR confirmation would be a larger PCR product from the DNA isolated from the transformed cells compared to the DNA isolated from the wild type cells. Another PCR reaction would also need to be conducted utilizing the primer set (primers 2 & 5) where one primer anneals outside of the construct and within the surrounding genome (primer 5) to confirm the correct recombination into the desired locus (primer 7 in **Table 1**) (**Figure 3B**). RT-PCR will be used to make sure that both the gene of interest and the tag are both being expressed (**Figure 3C**). Sequencing of the RT-PCR fusion product indicates that the tag is properly fused to the gene at the RNA level.

If this protocol is utilized to knock out a gene of interest, primer sets for PCR should be designed such that one primer anneals to a genome sequence outside of where the construct should recombine into the genome, and the other primer anneals either in the coding region of the gene or in the selective marker. A positive confirmation that the construct has successfully and correctly recombined into the genome would be the presence of the correct size product for the primer set that anneals within the marker but not with the primer set that anneals to the gene of interest. Another primer set should be made that has one primer that anneals outside of the designed construct, which is used with PCR to confirm that the recombination event occurred at the correct locus. In the same design to create a knockout, RNA is isolated from both the transformed cells and wild type (WT) cells, and RT-PCR is performed to confirm that no expression of the gene of interest is observed from the transformed cells.

Because a fluorescent tag was fused to the *ACK* gene, another confirmation that recombination was a success into the desired locus and that RNA is being translated into protein is through fluorescent microscopy (**Figure 4**). Ideally, conditions have already been established where it is known that the protein of interest is being expressed. However, if the fluorescent signal is too low to observe, there is a possibility that successful recombination still occurred, but growth conditions need to be altered in the chance that optimal conditions have not been met for sufficient expression, which would lead to a low fluorescent signal. This would need to be confirmed through other methods such as a western blot.

Protocol Scheme

1. Plate *C. neoformans* onto YPD + 1M sorbitol and allow to grow for 3-4 hours at ambient temperature.
2. Observe under a microscope for thorough distribution of cells.
3. Wash and aliquot gold beads.
4. Coat the gold beads with the DNA that is to be transformed.
5. Prepare macrocarriers with gold beads coated with DNA.
6. Using the gene gun, transform DNA into *C. neoformans*
7. Allow cells to recover by incubating plates for 3-4 hours at room temperature.
8. Scrape recovered *C. neoformans* cells onto selective media.
9. Allow cells to grow on the selective media until colonies appear.
10. Restreak each colony onto a freshly made YPD + antibiotic plate to ensure individual colonies.
11. Isolate DNA and RNA from those individual colonies, and perform PCR and RT-PCR analysis to confirm a successful recombination event.
12. Examine cells under a fluorescent microscope to confirm fluorescently tagged fusion protein is being expressed.

Figure 1. Protocol scheme.

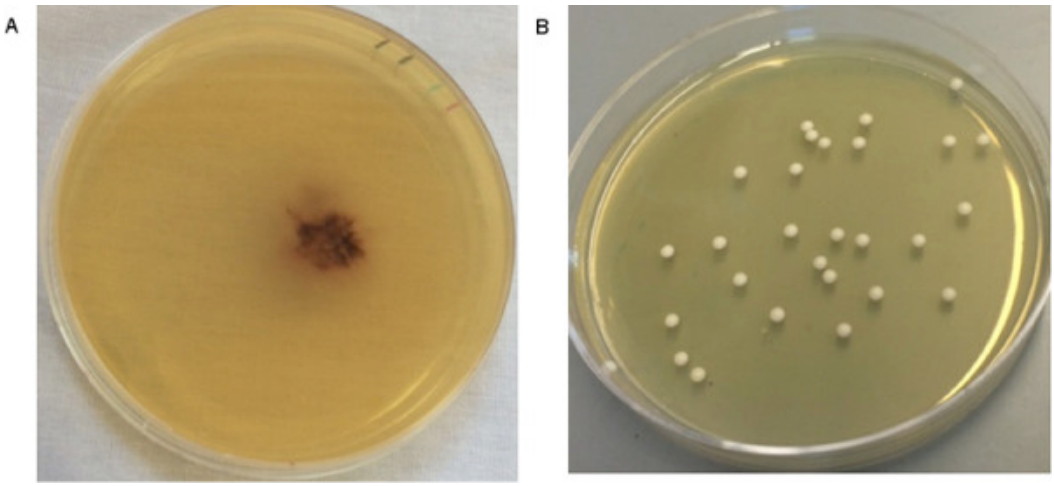

Figure 2A. DNA-coated gold beads successfully shot onto a YPD + 1M sorbitol plate. An orange patch seen in the center of the YPD + 1M sorbitol plate is due to the DNA-coated gold beads, indicating proper gold preparation, as well as a successful shoot. **Figure 2B.** Transforming 2 µg of DNA results in 20-30 colonies per plate. If the cells were diluted as mentioned in the protocol, approximately 20-30 colonies are expected prior to plating on selective media. Please click here to view a larger version of this figure.

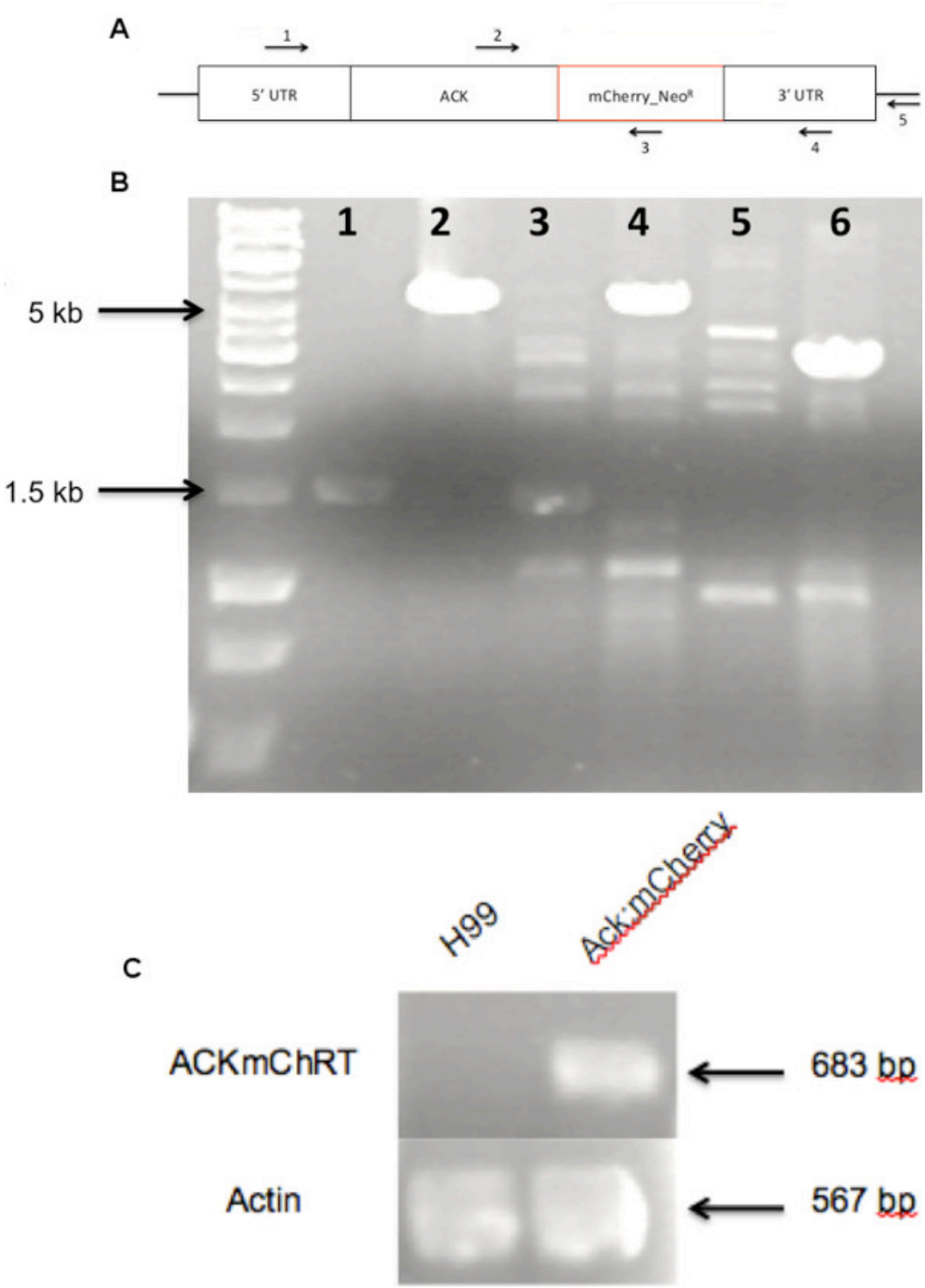

Figure 3A. Schematic of the ACK:mCherry:Neo construct and primer design. Figure 3B. PCR used to confirm successful homologous recombination. Lanes 1 and 2: PCR products obtained using primers 2 and 5 (Table 1) with genomic DNA from wild type *C. neoformans* H99 (lane 1) and the ACK:mCherry transformed strain, (lane 2). Expected sizes are 1511 and 5622 bp, respectively. Lanes 3 and 4 are the DNA products of the *C. neoformans* H99 (expected size 1443 bp) and the ACK:mCherry (expected size 5552 bp) strains, respectively, using primers 2 and 4 in Table 1. Lanes 5 and 6 are the DNA products of the *C. neoformans* H99 (should not anneal) and ACK:mCherry (expected size 3016 bp) strains, respectively, using primers 1 and 3. **Figure 3C.** RT-PCR confirmation of expression of the mCherry tag. The top lanes are the cDNA products of the ACKmCherry fusion product (expected size 683 bp) amplified from the *C. neoformans* H99 and the ACK:mCherry strains using primers 2 and 3 in **Table 1**. The actin gene was included as a control and was amplified under the same conditions as ACKmCherry (expected size 567 bp) using primers 6 and 7 in **Table 1**. Please click here to view a larger version of this figure.

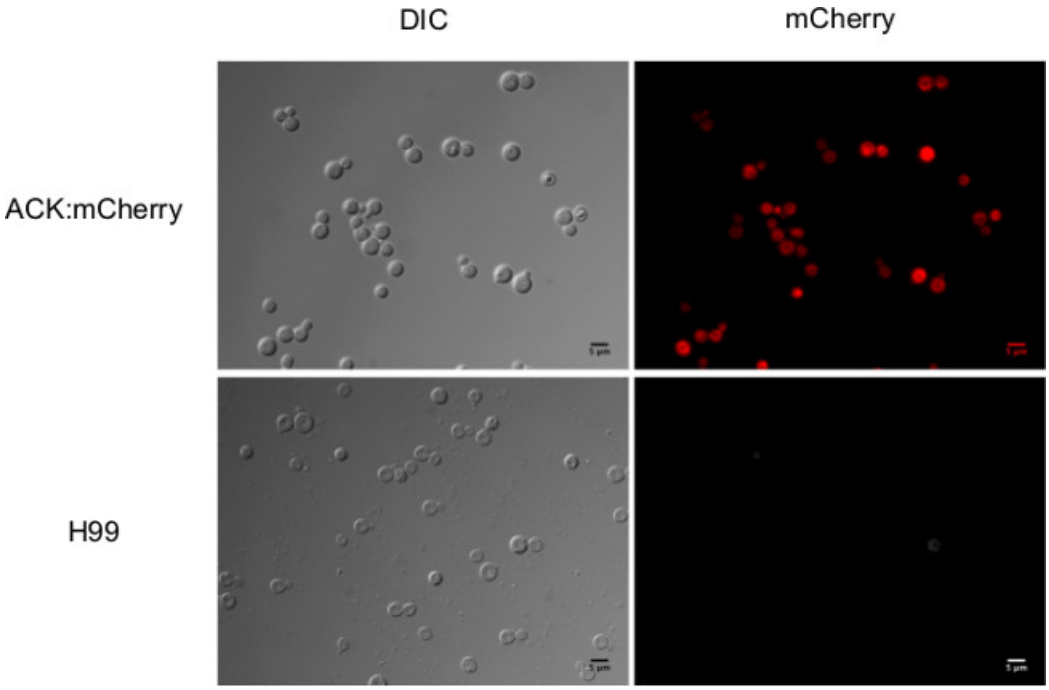

Figure 4. Fluorescence of the mCherry tagged Ack. Microscopic analysis of strains producing Ack fused to a mCherry tag with an excitation optimum at 587 nm and an emission optimum at 610 nm. Please click here to view a larger version of this figure.

1	KI003	5' – GTA GCG AGG TCT GGA AGC CAC – 3'
2	ACKmChRT-F	5'- GCT TTG GCC GGT ACT ACC AAC -3
3	ACKmChRT-R	5'- GAC AGC TTC AAG TAG TCG GGG -3'
4	KI004	5' – GAC TTG GGG AAG AGG AAT TC – 3'
5	KI0032	5' – CGG GGT ACC ATC AAT AAA AGC TTT CTT CAC TCC - 3'
6	Actin 1	5'- CGC TAT CCT CCG TAT CGA TCT TGC -3'
7	Actin 2	5'- CAG CTG GAA GGT AGA CAA AGA GGC -3'

Table 1. PCR and RT-PCR primers.

Discussion

Utilizing this protocol, biolistic transformation can be accomplished in which linear DNA is integrated into a desired locus in the *Cryptococcus neoformans* genome by homologous recombination. Certain steps in the protocol can have a dramatic effect on the effectiveness/efficiency of the transformation. For a successful transformation, it is imperative that the DNA utilized in the shoot has a concentration of at least 1 µg. However, the volume of DNA added to the gold beads can be increased in the chance the DNA yield is lower than 1 µg (Step 3.4).

Another important step is in the DNA coating of the gold beads. Insufficient numbers of beads in the DNA preparation sample, due to an error in the preparation, leads to a decreased amount of DNA shot onto the plate. After the DNA has been loaded onto the gold beads, they are pipetted onto the biolistic disc and allowed to dry. When dry, a visible gold circle about 1 cm in diameter should be present on the disc. The absence of this circle suggests that the concentration of the gold beads is not high enough. Another clue that the gold bead concentration is too low is following the shoot. There should be a gold ring visible on the plate (**Figure 2A**), and if no gold ring is visible and the rupture disc burst, this could indicate that the concentration of gold beads used in the preparation was not high enough.

The typical yield using the biolistic transformation method is 20-30 colonies. Fewer colonies may indicate that the technique or gene gun set-up is not 100% efficient. One reason for the fewer colonies may be the amount of cells scraped off from step 5.2. Depending on the size of the pellet in step 5.3 and the number of colonies that appear from the previous experiments, the volume the pellet is resuspended in, in step 5.4, may need to be altered. From these colonies, DNA should be isolated, and PCR conducted to confirm a larger size gene product compared to WT, indicating the presence of the tag. RNA should be isolated and RT-PCR performed to confirm that there are transcripts of the gene product being made, and if a fluorescent tag was inserted into the genome, then microscopy should be used to observe whether the tag is being expressed.

The main limitation to this protocol is the requirement for specialized equipment such as a gene gun and a fluorescent microscope. However, biolistic transformation is the best choice for introduction of linear DNAs for gene knockouts versus electroporation, which is used for introduction of episomes or *Agrobacterium tumefaciens* mediated transformation, which has been used for random insertional mutagenesis[12]. Biolistics

may also prove to be a suitable method for rapid introduction of a wide variety of vital dyes into *Cryptococcus*. Lipophilic dyes are used to stain extracellular vesicles and the capsule of *C. neoformans* [14]. Biolistic delivery of gold particles coated with lipophilic dye that imbed into the membranes of cells and organelles has been used to study the interconnection of neighboring cells [13]. Therefore, biolistics may be a less time-consuming technique to visualize extracellular vesicles and organelles.

Disclosures

We have nothing to disclose.

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