Video Article

A Genetic Screen to Isolate *Toxoplasma gondii* Host-cell Egress Mutants

Bradley I. Coleman, Marc-Jan Gubbels
Department of Biology, Boston College

Correspondence to: Marc-Jan Gubbels at gubbelsj@bc.edu

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Abstract

The widespread, obligate intracellular, protozoan parasite *Toxoplasma gondii* causes opportunistic disease in immuno-compromised patients and causes birth defects upon congenital infection. The lytic replication cycle is characterized by three stages: 1. active invasion of a nucleated host cell; 2. replication inside the host cell; 3. active egress from the host cell. The mechanism of egress is increasingly being appreciated as a unique, highly regulated process, which is still poorly understood at the molecular level. The signaling pathways underlying egress have been characterized through the use of pharmacological agents acting on different aspects of the pathways[1-5]. As such, several independent triggers of egress have been identified which all converge on the release of intracellular Ca^{2+}, a signal that is also critical for host cell invasion[6-8]. This insight informed a candidate gene approach which led to the identification of plant like calcium dependent protein kinase (CDPK) involved in egress[9]. In addition, several recent breakthroughs in understanding egress have been made using (chemical) genetic approaches[10-12]. To combine the wealth of pharmacological information with the increasing genetic accessibility of *Toxoplasma* we recently established a screen permitting the enrichment for parasite mutants with a defect in host cell egress[13]. Although chemical mutagenesis using N-ethyl-N-nitrosourea (ENU) or ethyl methanesulfonate (EMS) has been used for decades in the study of *Toxoplasma* biology[11,14,15], only recently has genetic mapping of mutations underlying the phenotypes become routine[16-18]. Furthermore, by generating temperature-sensitive mutants, essential processes can be dissected and the underlying genes directly identified. These mutants behave as wild-type under the permissive temperature (35 °C), but fail to proliferate at the restrictive temperature (40 °C) as a result of the mutation in question. Here we illustrate a new phenotypic screening method to isolate mutants with a temperature-sensitive egress phenotype[13]. The challenge for egress screens is to separate egressed from non-egressed parasites, which is complicated by fast re-invasion and general stickiness of the parasites to host cells. A previously established egress screen was based on a cumbersome series of biotinylation steps to separate intracellular from extracellular parasites[11]. This method also did not generate conditional mutants resulting in weak phenotypes. The method described here overcomes the strong attachment of egressing parasites by including a glycan competitor, dextran sulfate (DS), that prevents parasites from sticking to the host cell[19]. Moreover, extracellular parasites are specifically killed off by pyrrolidine dithiocarbamate (PDTC), which leaves intracellular parasites unharmed[20]. Therefore, with a new phenotypic screen to specifically isolate parasite mutants with defects in induced egress, the power of genetics can now be fully deployed to unravel the molecular mechanisms underlying host cell egress.

Video Link

The video component of this article can be found at http://www.jove.com/video/3807/

Protocol

Overview

Protocols are provided to first define the dosage of the mutagen leading to a 70% killing of the parasites (protocol 1). The next procedure is provided to enrich the induced egress mutants from a mutagenized parasite pool (protocol 2, Figure 2). This is followed by a protocol to test the incidence of egress mutants in the enriched pool, or to validate the egress phenotype in individual mutants (protocol 3). Finally, a protocol is provided to generate single parasite clones from enriched populations by limiting dilution (protocol 4).

1. Titration of mutagen

Use special caution, such as double gloves, when working with highly mutagenic compounds and liquids. Collect liquid waste separately for proper disposal.

1. Inoculate T25 tissue culture flasks confluent with human foreskin fibroblast (HFF) cells with 1 ml of freshly lysed tachyzoites and grow 18-25 hr at 37 °C under 5% CO_2 in Ed1 medium (D-MEM supplemented with 1% heat-inactivated fetal bovine serum, 0.2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 0.25 µg/ml Amphotericin B). See Roos *et al*. for general growth and media of HFF cells and parasites[21].
2. Replace medium with 10 ml 0.1% Fetal Bovine Serum medium (dilute Ed1 1:10 in D-MEM) and leave in humidified 37 °C incubator under 5% CO_2 (called "37 °C incubator" from here on) for 10 min.
3. Add 0, 12.5, 25, 50, or 100 µl ENU (1 M Stock in DMSO) or EMS (1 M Stock in DMSO) per flask (mutagen working dilutions will be 1.25, 2.5, 5, and 10 mM, respectively). Add DMSO to 100 µl for each flask. Incubate for 4 hrs in a 37 °C incubator.
4. Wash three times for 10 seconds at room temperature by rinsing the monolayer with 10 ml cold PBS (pre-cooled at 4 °C).

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5. Add 5 ml PBS, scrape the monolayer loose with a rubber policeman (cell scraper), pass the scraped cells through a 26.5 G needle to physically remove the parasite from the fibroblasts (clip off the shaft outside the needle with heavy duty scissors to not expose the needle), and filter with 3.0 µm polycarbonate filter. Multiple needle passages will increase the efficiency of releasing parasites from the host cell.
6. Count the parasite concentration using a hemocytometer. Let the parasites settle for 5 min in the hemocytomer before counting.
7. Dilute parasites to 10,000 parasites per 3 ml in Ed1 (for 10 ml 33,333 parasites are needed).
8. Inoculate one well in a 6 well plate with 3 ml of the diluted parasites (containing 10,000 parasites). Serially dilute the parasites 10-fold over 3 wells in a 6-well plate confluent with HFF cells containing 2.7 ml Ed1 by transferring 300 µl out of the first well. Leave plates undisturbed in the 37 °C incubator for 7 days.
9. Aspirate medium, fix 15 min with 3 ml/well 100% ethanol, stain with 3 ml/well crystal violet solution (12.5 g crystal violet in 125 ml ethanol mixed with 500 ml 1% ammonium oxalate) for 15 min, rinse with 3 ml/well PBS (1 minute) and air dry. All at room temperature.
10. Count plaques and select concentration of mutagen needed to achieve survival of 30% of the exposed parasites (70% killing dosage: see Figure 1). A dosage of 70% killing has been used historically[14] and induced less than 100 point mutations per genome (Farrell, Marth, Gubbels *et al*., manuscript submitted).

2. Enrichment of egress mutants (Figure 2)

1. Perform mutagenesis as described above using a mutagen dosage inducing 70% killing. Grow up the mutagenized population for one passage in a new flask of host cells.
2. Infect a T25, HFF confluent tissue culture flask with 120,000 freshly lysed parasites from a mutagenized population in 10 ml Ed1. Incubate for 2 hours in a 35 °C incubator under 5% CO_2 and humidified (from hereon called "35 °C incubator").
3. Aspirate medium and rinse 10 seconds with 10 ml cold PBS and then add 10 ml Ed1 medium supplemented with 25 mg/ml dextran sulfate (DS)[13]. Incubate for 26 hours in a 40 °C incubator under 5% CO_2 and humidified (from hereon called "40 °C incubator").
4. Prepare working solution of egress enhancers. Dilute the egress inducer of choice at the working concentration in a 15 ml Falcon tube containing 10 ml HBSSc supplemented with 25 mg/ml DS. Pre-warm the dilutions for 30 min in a 37 °C waterbath. See Table 1 for working concentrations.
5. Aspirate medium from the parasite-infected flasks and add pre-warmed egress inducer solution. Incubate in the 37 °C incubator for the times indicated in Table 1.
6. Aspirate medium and rinse 10 seconds with 10 ml cold PBS and then add 10 ml Ed1 supplemented with 25 mg/ml DS and 50 µM pyrrolidine dithiocarbamate (PDTC: add 5 µl of 100 mM PDTC Stock in PBS)[13]. Incubate 5 hours in a 35 °C incubator.
7. Aspirate medium and rinse 10 seconds once with 10 ml PBS (room temperature) and then add 10 ml Ed1 medium. Put flasks back into the 35 °C incubator until parasites destroy the monolayer: shake flasks daily. This recovery takes around 7 days.

3. Validation of mutant phenotypes by egress assays

After performing the enrichment screen and growing up the enriched population for one passage in HFF cells, the phenotypes need to be confirmed by an egress assay [11,13]. This assay should also be used to validate single clones after protocol 4.

1. Inoculate 20,000 parasites per well into a 24-well plate containing confluent HFF cells grown on coverslips (1 ml Ed1 medium per well). Incubate 8 hrs in a 35 °C incubator then transfer into a 40 °C incubator for 24 hrs.
2. Wash with 1 ml/well PBS (10 seconds at room temperature). Add 1 ml egress inducers (include a DMSO only negative control), pre-warmed and diluted in HBSSc and incubate for the times described in Table 1.
3. Aspirate medium and fix with 1 ml/well 100% methanol for 15 min at room temperature.
4. If parent parasites expressing an autofluorescent protein were used for the mutagenesis, proceed to step #3.5[13]. If non-fluorescent protein expressing parasites were used as parent line, stain the fixed coverslips with Diff-Quick stain for 1 min at room temperature[11].
5. Wash 5 min with 1 ml/well PBS in the 24-well plate at room temperature.
6. For fluorescent protein expressing parasites: quickly rinse the coverslip in ddH_2O (dipping) and mount on slides under gelmount to protect the fluorescence signal. For Diff-Quick stained parasites, wash 10 seconds in 100% ethanol and let air-dry before mounting on slide.
7. Using a (fluorescence) microscope with a 40-60x objective count the percentage of vacuoles egressed versus the vacuoles that stayed intracellular (see Figure 4).

4. Clone egress mutants by limiting dilution

After validation of the phenotype of the enriched egress mutant population using protocol 3, the polyclonal population needs to be cloned to obtain single parasite clones.

1. Count parasite population in a hemocytometer and dilute to a concentration of 500 parasites per ml in Ed1 medium.
2. In a 384-well plate containing a confluent monolayer of HFF cells, replace the medium with 40 µl/well of Ed1 medium using a multi-channel pipet. As shown in Figure 5, four polyclonal populations can be cloned per plate as follows: pipet 40 µl/well of diluted mutant 1 into wells C3-C12, mutant 2 into wells C13-C22, mutant 3 into wells H3-H12, and mutant 4 into wells H13-H22 (end volume in wells is now 80 µl). Using a multi-channel pipet, pipet the solution up and down 5 times, then transfer 40 µl to the row below the starting row (from row C to D or row H to I). Continue these 2-fold serial dilutions through row G (mutants 1 and 2) or row N (mutants 3 and 4). Discard the extra 40 µl from the last row. Incubate in a 35 °C incubator for 7-10 days without disturbing the plate.
3. Check the wells on an inverted microscope with a 10-20x objective for the presence of single plaques, visible as 'holes' in the monolayer.
4. Pick 4 wells per mutant with a single plaque and transfer the parasites into a tissue culture flask confluent with HFF cells and filled with Ed1 medium. Grow the parasites up in a 35 °C incubator; shake the flasks daily to disperse the extracellular parasites. This typically takes 7 days.

5. Representative Results

The mutagens ENU and EMS are not stable when stored over long periods of time. Therefore, testing the mutagenic power of the stocks is critical to obtain reproducible results. Typical titration results and killing curves for both ENU and EMS are shown in Figure 1. However, it is recommended to perform plaque assays for every mutagenesis experiment to ascertain that the appropriate dosage was used. To maintain the diversity in the mutagenized parasite population, it is important to proceed with the egress mutant screen as quickly as possible. Typically, one passage of the parasites into a new flask is performed to let the surviving parasites recover before going forward with the screen. It should be

kept in mind that doing this will result in division of the mutants. Therefore it cannot be excluded that multiple clonal mutants isolated after completing the whole procedure contain exactly the same genotype. To avoid isolating the same mutant multiple times it is recommended to isolate only a single egress mutant per mutagenesis, unless their phenotypes (e.g. differential egress inducer sensitivities) are very different from each other. On the other hand, not every screen results in isolation of mutants with the desired phenotype. In particular the Ca^{2+}-ionophore A23187 resistant phenotype is rare and requires multiple mutagenesis experiments and screens to isolate a single egress mutant. Therefore it is recommended to perform 5-10 mutagenesis and screen experiments in parallel.

The enrichment power of the screen was tested by mixing a known egress mutant with wild-type parasites at different ratios. These mixes were subjected to the screen and the incidence of the mutant phenotype in the enriched population was assessed. By using wild-type parasites expressing cytoplasmic RFP and a mutant line expressing cytoplasmic YFP the incidences could quickly be established by flow cytometry (Figure 3)[13]. The results show that mutant phenotypes can be routinely enriched to 80% purity by starting with 1 egress mutant parasite per 10,000 wild-type parasites[13]. However, when the egress mutant: wild-type parasite start ratio is 1:100,000 parasites, the isolated population is only 1-2% (1:100) egress mutants. Therefore the enrichment power of the screen is 1,000-fold. Since the screen starts with inoculation of 120,000 parasites, of which typically 70% are viable, we typically perform two rounds of enrichment. The isolated parasites are grown up between the enrichment rounds. This procedure leads to a 100% mutant population even when starting with 1:1,000,000 egress mutant:wild-type parasites.

The egress assay to validate and characterize the phenotypes requires a multiplicity of host cell infection that allows differentiation of individual vacuoles. This is especially critical when analyzing conditions with high percentages of egress as the individual parasites are scattered around. As shown in Figure 4, if egressed populations are not well separated it is easy to underestimate the percentage of egress by counting two egressed vacuoles as one vacuole. Since egress and invasion are related processes, and some temperature-sensitive phenotypes display a mild phenotype at the lower temperature, not all mutants will have similar invasion efficiencies. Such mutants must therefore be inoculated at several fold higher dosages to obtain a high enough vacuole density for an accurate assessment of their egress phenotype. Furthermore, some egress inducers do not efficiently stimulate egress of vacuoles containing four parasites or less. As such, it is important that vacuoles contain eight parasites or more when starting the egress assay. In our lab we screen mutants isolated with a particular egress enhancer against other egress enhancers. By profiling their cross-reactivity the mutants can be grouped in different classes. Lastly, not all egress mutants will be temperature-sensitive. In particular mutants isolated with egress inducers triggering steps before the release of intracellular Ca^{2+} are prone to non-temperature sensitive phenotypes. This is due to the parallel pathways that can lead to egress before the signal converges on the release of intracellular Ca^{2+}.

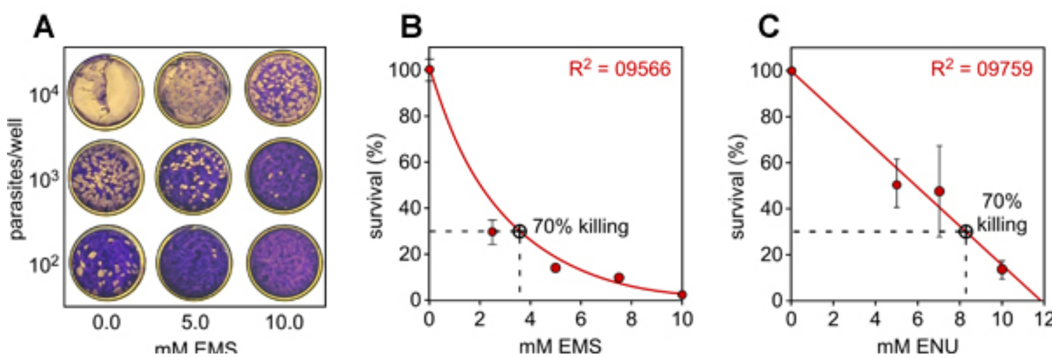

Figure 1. Chemical mutagen dosage titration. A. Plaque assays performed in a 6-well plate using various EMS concentrations and various numbers of parasites per well as indicated. The white spots are parasite plaques formed in the HFF monolayer. **B, C.** Survival curves of parasites upon exposure to various dosages of EMS (**B**) and ENU (**C**). Survival was assessed by plaque assays. A dosage inducing 70% killing is chosen for mutagenesis experiments. Averages of three independent experiments +/- standard deviation are shown.

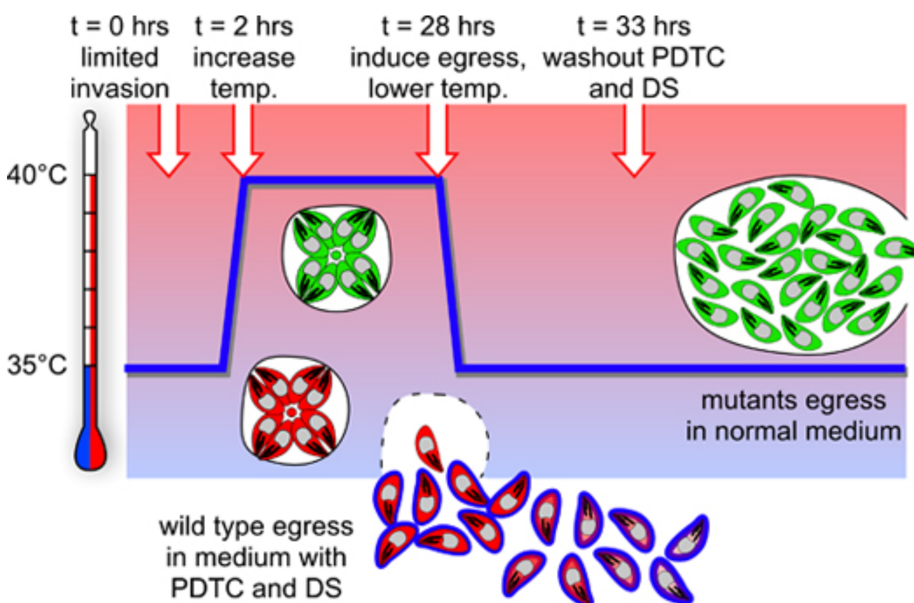

Figure 2. Schematic representation of the egress mutant enrichment screen. Temperature is indicated on the y-axis, whereas time progression is represented by the x-axis. Green parasites reflect egress mutant parasites, red parasites reflect wild-type parasites. The timing of

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changes in conditions and stimulation by egress inducer are indicated at the top and are marked by arrows on the blue line reflecting the temperature profile. Adopted from Eidell *et al.*[13].

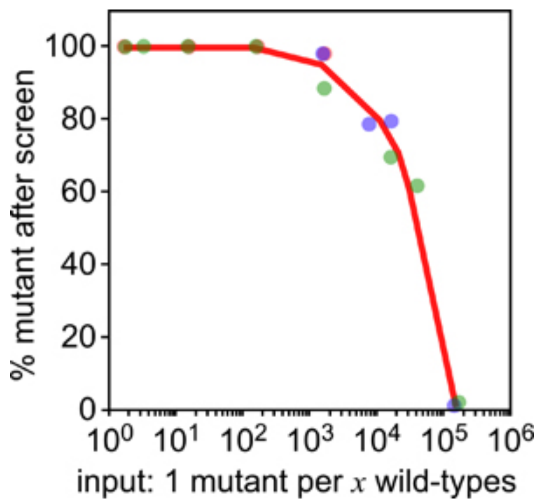

Figure 3. Typical enrichment of egress mutant phenotypes. Enrichment results of an egress mutant mixed into wild-type parasites at various ratios (x-axis). The percentage of egress mutant phenotypes in the population grown up after the screen is plotted on the y-axis and was assessed by flow cytometry (egress mutant parasites expressed cytoplasmic YFP, wild-type parasites expressed cytoplasmic RFP). Results of three independent experiments are represented by red, blue and green data points. Adopted from Eidell *et al.*[13].

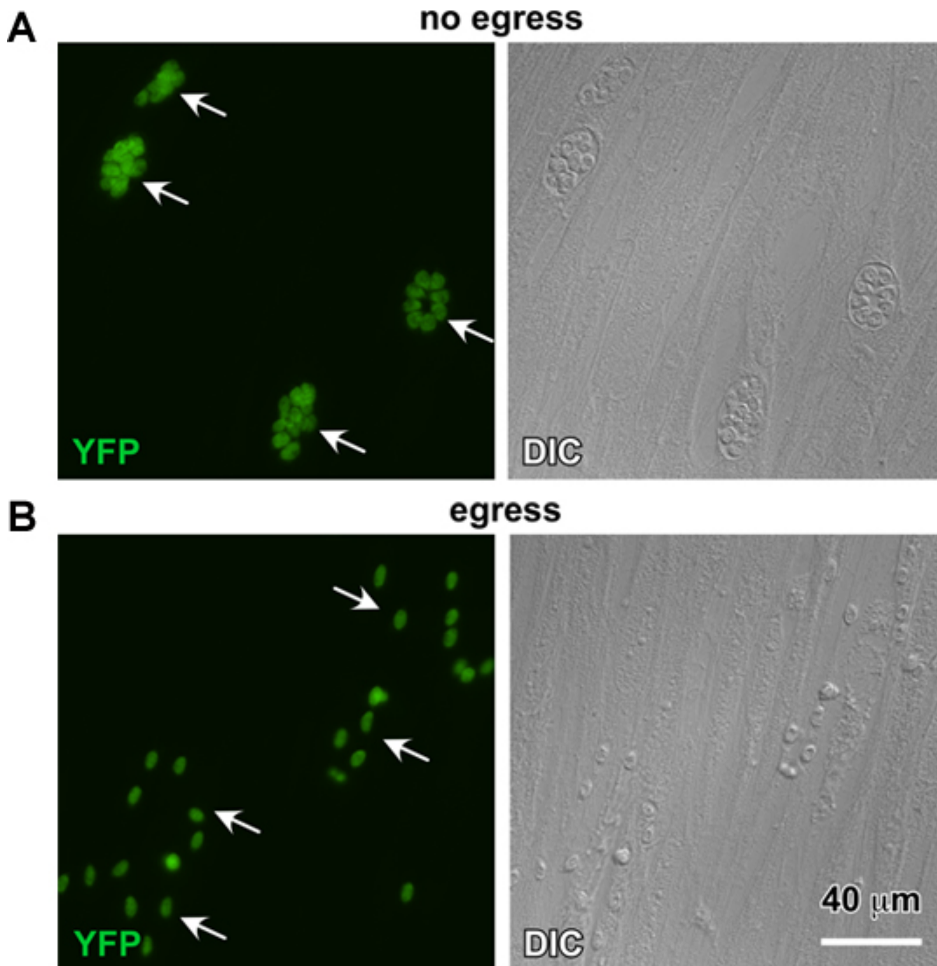

Figure 4. Typical results of an egress assay. A. Arrows mark four different intact vacuoles containing 4-8 parasites. **B**. Arrows mark four groups of scattered parasites reflecting four independent egressed vacuoles. Egress was induced by A23187 (**B**) or DMSO as a negative control (**A**). Parasites express cytoplasmic YFP[22].

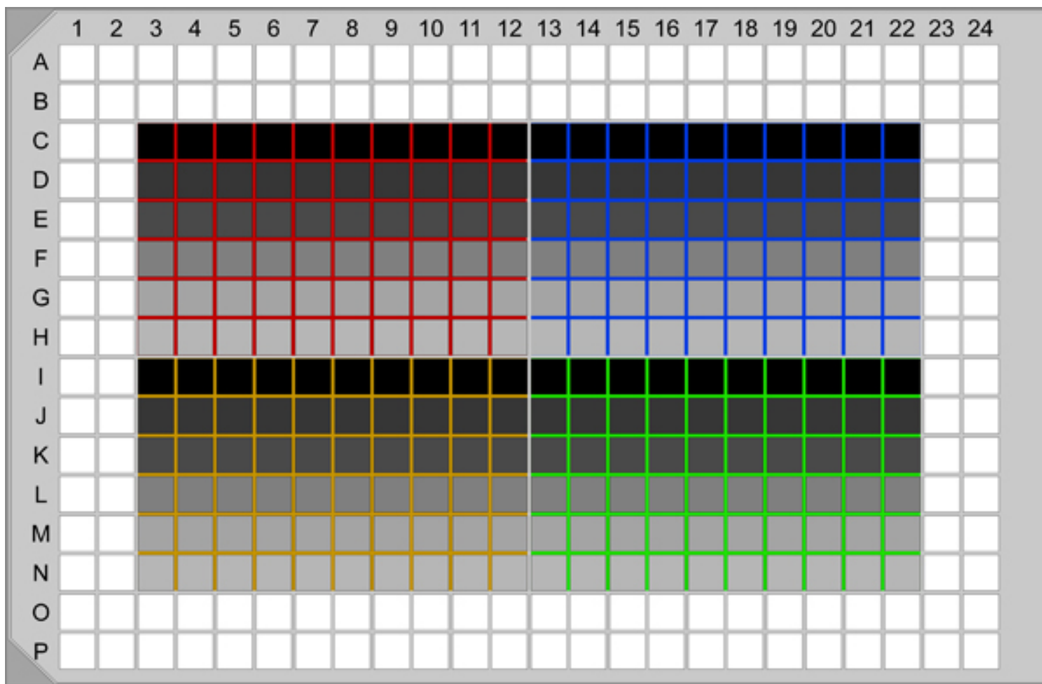

Figure 5. Serial dilution for clonal parasite lines. Four polyclonal populations (red, blue, yellow, green) are serially diluted in a single 384-well plate confluent with HFF cells. Row C and I receive 10 parasites per well and are 2-fold diluted (40 µl + 40 µl) until rows H and N, respectively. The shade of grey indicates the decreasing number of parasites per well. Typically, single clones are found in rows D-F and J-L.

Compound	Stock	Working concentration	µl needed for T25 (10 ml)	Incubation time (min)
DTT	1 M in DMSO	5 mM	50	15
ethanol	190 Proof	5%	500	30
A23187	2 mM in DMSO	1 µM	5	5
nigericin*	2 mM in DMSO	10 µM	50	30

*nigericin cannot be used in the enrichment screen as the parasites do not survive nigericin stimulation; only use nigericin in the validation of egress mutants.

Table 1. Egress inducers used in procedures. Dilute in 10 ml HBBSc containing 25 mg/ml DS for the screen, or no DS for mutant validation (egress assay). All compounds from Sigma-Aldrich.

Discussion

The described protocol provides an efficient method to isolate *Toxoplasma* mutants with an egress defect. We have successfully isolated mutants along various steps of the egress pathway, some of which have a dual invasion phenotype[13]. Potential effects on invasion can be determined using the so-called red-green assay, which differentiates invaded from non-invaded parasites by differential antibody staining[23,24]. For both invasion and egress assays it could be convenient to express an autofluorescent protein marker in the cytoplasm of the parasites[13,22]. However, if the parasites being mutagenized already express a fluorescent protein this could interfere with additional phenotypic characterization by immunofluorescence later on. It is always possible to add a fluorescent marker to a mutant after its isolation. As described, a simple, non-fluorescent alternative is Diff-Quick staining for egress.

Historically, the mutagen ENU has been applied to *Toxoplasma* genetics[14]. However, ENU preferentially targets AT base pairs[25], which we validated in *Toxoplasma* (Farrell, Marth, Gubbels *et al.*, manuscript submitted). Since the frequency of A/T is lower in amino acid coding sequence than non-coding sequence, the majority of mutations induced by ENU will be in non-coding sequence. However, EMS typically has a preference for GC base pairs, i.e. coding sequences. The vast majority of temperature-sensitive mutations originate in codon-changing mutations, which is a reason to use EMS over ENU.

Although a variety of egress inducers triggering different aspects of the signaling pathway are described in Table 1, there are other pharmacological agents known to act on processes required for egress. For instance, caffeine has been shown to induce microneme secretion in extracellular parasites, which is required for both egress and invasion[7,26,27]. However, caffeine cannot be used to trigger egress from infected host cells, likely because caffeine does not efficiently diffuse through the host cell toward the parasites residing in a vacuolar compartment. Similarly, one egress trigger is the accumulation of abscisic acid, but it is impossible to screen for mutants in this pathway since abscisic acid does not diffuse into the host cell[28]. And as indicated in Table 1, the K+-ionophore nigericin is an efficient egress inducer[5], however, parasites do not survive nigericin stimulation. Therefore this compound is also not suitable for the screen. In an alternative, more elaborate screening procedure for ionophore mutants it was reported that many mutants with a delay in ionophore induced egress survive prolonged extracellular exposure to ionophore[11]. However, no egress mutants resistant to ionophore induced egress were isolated in this screen, making the phenotypes weaker. It is at present unclear whether mutants with resistance to prolonged exposure to other egress inducers can be obtained.

Since there appears to be several parallel pathways that can lead to egress, the question arises as to which one would be the most relevant in an (experimental) infection. It appears that immune system attacks on the infected cell are the main trigger of egress[29]. It is possible to use such

triggers in the mutant screen. For instance, CD8 T-cells can stimulate egress through both a Fas-mediated and a perforin-mediated pathway[30]. Therefore, by using a FasL expressing human T-cells as a host cell in the egress screen, egress can be triggered by incubation with an anti-Fas antibody to enrich for mutants in this pathway.

To genetically map the mutations underlying the egress phenotypes, two different approaches are available in *Toxoplasma*. Unlike genetic model organisms, allelic segregation by crossing is not an option since the sexual cycle of the parasite can only be triggered in the gut of the cat. Aside from the impracticality and poor efficiency of this process, parasite strains grown in the lab very quickly lose the ability to infect cats. To bypass this problem an efficient wild-type cosmid library complementation approach has been developed, which complemented 90% of cell division mutants[18]. In addition, we recently established complete genome resequencing protocols to identify all mutations induced in the genome (Farrell, Marth, Gubbels *et al*., manuscript submitted). Typically we identify 20-70 single nucleotide polymorphisms in chemically induced mutants. Between these two approaches we have been able to identify the causative mutation in all chemically induced mutants characterized to date.

Taken together, the described screen provides a versatile tool that will allow forward genetic dissection of the egress pathways. We expect this will identify several components of the signaling pathway known to exist based on pharmacological data, but for which no good candidate genes could be identified in the *Toxoplasma* genome.

Disclosures

We have nothing to disclose.

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