Chapter 15

Genetic manipulation of *Toxoplasma gondii*

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1. INTRODUCTION

The first genetic manipulations applied to *Toxoplasma* were performed by using chemical mutagenesis. These studies were pioneered in the 1970s by Elmer Pfefferkorn (Pfefferkorn and Pfefferkorn, 1976, Pfefferkorn, 1988) who perfected protocols to reproducibly cultivate tachyzoites in a tissue culture system and to mutagenize, select and finally clone parasites by limiting dilution. Based on these protocols a series of chemically induced mutants were used to map out the parasite's nucleotide biosynthetic pathways. These studies were critical for the establishment of protocols for genetic crosses in the cat (Pfefferkorn and Pfefferkorn, 1980). Crosses can be used to map a given phenotype to a single or multiple genome loci. This classical forward genetic approach has been instrumental to map virulence factors and to analyze *Toxoplasma* population structure and evolution. See chapters 3 and 14 for further discussion of these topics.

The reverse genetics approach, which introduces foreign DNA into parasites was achieved using electroporation. Initially the transient transfection of plasmid DNA containing reporter genes flanked by *T. gondii* 5' and 3' flanking sequences allowed the expression of reporter genes used for the characterization of the elements controlling transcription. This methodology was rapidly utilized to identify and validate several selectable marker genes, which then opened an avenue for stable transformation and the development of an invaluable panoply of tools associated with DNA transfection. A wide range of positive and negative selectable markers have been tailored for homologous recombination leading to allelic replacement and gene knockouts. In addition non-

homologous random integration vectors have been designed to express transgenes and as a strategy for random insertional mutagenesis.

The recent completion of the *Toxoplasma* genome sequencing project (www. Toxodb.org) and the availability of other apicomplexan genomes for comparison are delivering an unprecedented amount of exciting information. In this new area of post-genomics, the accessibility of *T. gondii* to multiple genetic manipulations approaches and to high throughput studies makes it a very attractive and powerful system to improve our understanding of the basic biology of the apicomplexan parasites. Figure 1 summarizes the available sources of information and experimental approaches. There is no limitation to the identification of relevant genes and little or no barrier to experimentally unravel their biological function at a relative large scale.

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The purpose of this chapter is to recapitulate and describe the strategies associated with DNA transfection including the most recent acquisitions and to provide a list of the most useful protocols, reagents and strains available to the researchers.

2. THE MECHANICS OF MAKING TRANSGENIC PARASITES

2.1 Transient transfection

Successful manipulation of the Toxoplasma genome is critically dependent on the efficiency of DNA transfection. Electroporation was and still remains the method of choice to introduce DNA into tachyzoites. Importantly, the combination of this method

with media mimicking the cytosolic ion composition of the cells (cytomix) confers the best survival rate (Van den Hoff et al., 1992). The protocol, initially established using a BTX electroporator, led to an efficiency of transient expression that oscillated between 30 and 50% (Soldati and Boothroyd, 1993). The optimal settings chosen on the BTX Electroporator were fixed for the RH strain (Type I, virulent strain) and were slightly modified for the cyst forming strains (ME49 and Prugniaud; Type II strains). It has been frequently observed that the cyst forming strains are less amenable to genetic manipulation probably due to several factors.

To monitor transfection efficiency chloramphenicol acetyl transferase (CAT) and β-galactosidase were originally used as reporter genes and subsequently the β-lactamase, alkaline phosphatase and fire-fly luciferase (LUC). These enzymes are classically used as reporters because their activities can be monitored with great sensitivity and in a quantitative fashion. Additionally, these enzymes are absent in eukaryotic cells, leading to virtually no background activity.

Interestingly, the β-lactamase and alkaline phosphatase exhibit no activity within the parasite probably due to presence of inhibitors and can be exploited to study the secretory pathway and quantify parasite secretion (Chaturvedi et al., 1999, Karsten et al., 1998). LacZ activity can be measured using a colorimetric assay that transforms yellow chlorophenol red-β-D-galactopyranoside (CPRG) substrate into a red product using an absorbance spectrophotometer at 570 nm (Seeber and Boothroyd, 1996). This colorimetric readout assay can be monitored in live parasites using culture medium

without phenol red and in multiwell plates allowing (at a high throughput level) the screening of the efficacy of a drug against the parasite (McFadden et al., 1997). Faithful expression of a reporter gene requires adequate 5' and 3' flanking sequences that are derived from *T. gondii* genes. The flanking sequences must contain the control elements necessary to drive an optimal level of transcription. The monocistronic nature of transcription in *T. gondii* facilitated the identification of promoter elements that are usually in close proximity to the transcription start site. Numerous vectors suitable for transfection are currently available, and as they exhibit different range of promoter strength and stage specificities, they can be chosen appropriately according to the purpose of the experiment.

It has been frequently observed that the strength, and probably also the timing of expression with respect to the cell cycle, critically influence the outcome of an experiment, especially when studying the subcellular localization of a given gene product. For example, the overexpression of microneme proteins often results in accumulation in the early compartment of the secretory pathway or leakiness into the parasitophorous vacuole (Soldati et al., 2001). The use of the native promoter is highly recommended if transgenic gene expression results in toxicity or mistargeting (Gubbels et al., 2006).

A constitutive level of expression can be obtained by using vectors derived from the *TUB1* (α-tubulin), DHFR (dihydrofolate reductase), *ROP1* (rhoptry protein 1), *MIC2* (microneme protein 2) several *GRA* (dense granules proteins) and *HXGPRT*

(hypoxanthine-guanine phosphoribosyl transferase) genes. The strength of these and other promoters have not been very systematically compared but the *GRAs* and *MIC2* promoters are the strongest, *TUB1* and *ROP1* promoters are intermediate while *DHFR-TS* is a weak promoter.

Stage-specific expression can be achieved using the 5-flanking sequences of stage-specific genes and so far no stage specific regulatory elements have been mapped in the 3' UTR sequences. Tachyzoite-specific expression is conferred by vectors derived from *SAG1* (surface antigen 1) *ENO1* (enolase 1) and *LDH1* (lactate dehydrogenase 1) genes. In contrast, vectors constructed from *BAG1* (bradyzoite antigen 1), *ENO2* (enolase 2) or *SAG4* genes confer expression in the bradyzoite stage exclusively. Detailed promoter analyses and identification of cis-acting elements have only been undertaken for a limited number of genes (Bohne et al., 1997, Kibe et al., 2005, Mercier et al., 1996, Matrajt et al., 2004, Soldati and Boothroyd, 1995, Yang and Parmley, 1997). See chapter 16 for a discussion of regulation of gene expression.

In addition to the promoter elements, sequence features carried on the mRNAs also contribute to the success of transfection. Sequence information derived from the 5' and 3' untranslated regions likely affects gene expression, but this level of regulation has not been rigorously investigated to date. The 3'UTR is an important element as transcription drops to less than 10% when such element is not included. In *Plasmodium* partial deletion of 3'UTR regions have been exploited to modulate the level expression of essential genes offering a way to analyze their function (Thathy et al., 2002)

At the start codon, a consensus sequence termed the "Kozak sequence" is recognized by the ribosome as a favorable sequence to initiate translation. A compilation of abundant expressed genes in *T. gondii* was used to establish a consensus translational initiation sequence *gNCAAa*ATGg, which is similar but not identical to the Kozak sequence found in higher eukaryotes (Seeber, 1997). Several genes including GFP were initially very difficult to express using their native sequence but the lack of expression was solved by the generation of fusions at the N-terminus (Striepen et al., 1998). These observations suggested a significant influence of the N-terminal amino acid sequences in recombinant protein expression. A systematic analysis aiming at the evaluation of the importance of the by the amino acid following the initiation methionine confirmed the existence of an N-end rule in *T. gondii* (Matrajt et al., 2002c). Amino acids such as Ala, Glu and Asp confer high level of expression of the transgene.

2.2 Stable transformation and positive and negative selectable markers

Most of the selectable marker genes commonly used for eukaryotic cells are not suitable for selection of stable transformants in *T. gondii* (*T. gondii* is an obligate intracellular parasite). Only drugs selectively affecting the parasite while keeping the host cells intact could be considered. In spite of this restriction, various selection protocols have been developed and are listed in Table 1.

<Table 1 near here>

Chloramphenicol shows a potent but delayed parasiticidal effect, allowing the use of *E. coli* chloramphenicol acetyl transferase (CAT) not only as reporter enzyme but also as a tight selectable marker gene (Kim et al., 1993). Parasite must complete up to three cycles of host cell lysis (up to 7 days) before an effect of the drug is evident. At this point parasite are cloned in 96 well plates for about five days, starting in presence of drug selection.

Another selection strategy based on the resistance to a drug can be achieved by exploiting the protective effect of the *ble* gene product of *Streptoalloteichus* or Tn5 against the DNA breaking activity of phleomycin (Soldati et al., 1995 and Messina et al., 1995). Parasites expressing *ble* become resistant to the drug, however, this selection needs to be applied on extracellular parasites to be effective. Phleomycin selection has been used successfully for the random insertion of transgenes (Soldati et al., 1995) and to disrupt genes by homologous recombination (Mercier et al., 1998). As an alternative to drug resistance, stable selection can be achieved by complementation of the naturally occurring tryptophan auxotrophy of Toxoplasma by addition of indole to the culture medium (Sibley et al., 1994) following the introduction of the bacterial tryptophan synthase (trpB) gene.

Two genes coding for non-essential nucleotide salvage pathway enzymes have been expoited as negative selectable markers. Loss of uracil phosphoribosyl transferase (*UPRT*) activity confers resistance to the pro-drug 5'-fluo-2'-deoxyuridine (FUDR) (Donald and Roos, 1995) and in absence of hypoxanthine-xanthine-guanine

phosphoribosyl transferase (*HXGPRT*) activity, 6-thioxanthine (6-Tx) can not be converted into an inhibitor of GMP synthase (Donald et al., 1996a). In *HXGPRT* deficient mutants, this gene can also be used for positive selection strategies since mycophenolic acid efficiently kills parasites lacking the enzyme.

The frequency of stable transformation fluctuates significantly depending on the type of selectable marker used. The conformation of the transfection plasmid (circular versus linearized by restriction) can also affect transfection efficiency. A much high frequency of stable transformation is achievable using pyrimethamine resistance vectors derived from the parasite's bifunctional dihydrofolate reductase-thymidylate synthase *DHFR–TS*. An artificially mutated *dhfr-ts* gene from *T. gondii* was used to design an expression vector pDHFR*–TSc3 (No. 2854) that confers pyrimethamine resistance (Donald and Roos, 1993b). The *DHFR-TS* based selection is unique and shows an exceptional frequency of chromosomal integration of up to 5% (Donald and Roos, 1993b). The flanking sequences of the *DHFR-TS* genes are responsible for this unusual property, which can be partially conferred to other selectable marker genes such as the *HXGPRT* if this latter is controlled by the *DHFR-TS* flanking sequences.

Furthermore, restriction enzyme-mediated integration (REMI) can be used to further enhance the frequency of transformation up to 400-fold (Black et al., 1995b) and enables co-transfection of several unselected constructs together with a single selectable marker. Any of the selectable markers genes listed above can, if needed, be efficiently recycled by the action of the site-specific Cre recombinase. The adaptation of the *cre lox*P system

from bacteriophage P1 to *T. gondii* enables the specific *in vivo* excision of any introduced sequence which was flanked by *lox*P sequences (Brecht et al., 1999).

2. 3 Homologous recombination and random integration

Unlike the situation in many protozoans, where integration into chromosomes occurs exclusively by homologous recombination and requires only a short segment of homology, homologous recombination is not favoured in *T. gondii*. Vectors lacking long stretches of contiguous genomic DNA typically integrate into chromosomal DNA at random. The high frequency of transformation and random integration throughout the small genome size of haploid *T. gondi* tachyzoites was developed as an efficient strategy to mutagenize the entire genome of *T. gondii* within one single electroporation cuvette (Roos et al., 1997). Such genomic scale tagging allows identification of any gene whose inactivation is not lethal to tachyzoites and for which a suitable functional selection or screen is available.

<figure 2 near here>

Homologous recombination leading to gene replacement (Fig. 2) is instrumental to study gene function and can be accomplished in *T. gondii* provided that sufficient contiguous homologous DNA (several kilobases) is used to the target the locus (Donald and Roos, 1994). Nevertheless, the efficiency of homologous recombination remains very low and a counter selection strategy has been developed to raise the yield. A gene targeting system based on *HXGPRT* as a positive/negative selectable marker enables the generation of genetic knock-outs or allelic replacement by ”hit and run” mutagenesis for

loci where no direct selection is available. A positive selection produces first a duplication of the target gene at the endogenous locus by single-site homologous recombination. Subsequently, a negative selection resolves the pseudo diploid to produce either wild type (revertant) or allelic replacement (gene knock-out).

Alternatively, a second negatively selectable marker can be inserted into a knockout construct outside of the homologous flanking regions in order to eliminate all the transformants originating from random integration or single homologous recombination. The herpes simplex virus thymidilate kinase (*TK*) and the bacterial cytosine deaminase (*CD* which confer sensitivity to ganciclovir and to 5-fluorocytosine respectively (Fox et al., 2001; Fox et al., 1999; Radke et al., 1998) have been successfully used as negative selectable markers in *T. gondii*. The fusion of these genes with *CAT* or *DHFR-TS* elegantly creates additional positive-negative selectable markers. Finally, counter selection by FACS using fluorescent protein markers has also been applied successfully.

Positive/negative selection be employed for knock-out experiments or be used to devise selection schemes for mutants and promoter traps. The *HXGPRT* gene has been exploited to identify genes that are expressed in a stage-specific fashion (Knoll and Boothroyd 1998). Parasites expressing *HXGPRT* under the control of a bradyzoite-specific promoter were mutagenized by random insertion of a plasmid and subjected to in vitro tachyzoite to bradyzoite conversion under 6-thioxanthine selection to isolate mutants deficient in differentiation (Matrajt et al., 2002b)

3. USING TRANSGENIC PARASITES TO STUDY THE FUNCTION OF PARASITE GENES

3.1 Tagging subcellular compartments

Visualizing different subcellular compartments is an essential tool for cell biological analysis. Specific antibodies raised against subcellular fractions or individual proteins are widely used for this purpose at the light and electron microscopic level. This approach, however, requires the production of antigen, either by purification from the parasite or by recombinant expression and subsequent immunization, which is time-consuming and not always technically feasible. Through transfection experiments, proteins can be tagged by gene fusion using a generic epitope (for which antibodies are already available) or using an autofluorescent protein. Autofluorescent proteins can be detected in vivo with minimal manipulation providing a unique tool to follow biological processes over time (see Fig. 3, and (Gubbels and Striepen, 2004) for an in depth review).

<figure 3 near here>

Numerous versions of green fluorescent protein (GFP) and related autofluorescent proteins have been successfully expressed in *T. gondii* (Kim et al., 2001, Striepen et al., 1998) and a range of colors is available now for the simultaneous use of multiple markers. Cyan (CFP) and yellow fluorescent protein (YFP) are a suitable pair for double labeling experiments and have been used in *in vivo* microscopic studies of *Toxoplasma* organelle biogenesis (Striepen et al., 2000, Pelletier et al., 2002, Joiner and Roos, 2002). A tandem repeat of the YFP gene yields exceptionally bright fluorescent transgenics which are now widely used to track parasites in tissue culture and in infected animals

(Gubbels et al., 2004b, Gubbels et al., 2003, Gubbels et al., 2005, Egan et al., 2005). Red fluorescent proteins (RFP) further extend the options. DsRed produces brightly fluorescent parasites (Striepen et al., 2001), however, the requirement of tetramerization of this marked can be problematic if the tagged protein is part of a complex or structure. Monomeric variants of RFP (e.g. mRFP (Campbell et al., 2002)) can help overcome these problems but suffer from considerably weaker fluorescence. The new 'cherry' and 'tomato' variants (Shaner et al., 2004) provide a reasonable compromise and a tandem tomato marker produces exceptionally bright fluorescence when expressed in *T. gondii* (Giel vanDooren and BS, unpublished).

A large number of organelle specific fluorescent protein markers is now available (see Fig. 3 for examples). However, not all proteins can be studied in this way, as the bulky GFP tag can affect targeting, maturation or function of its fusion partner. In such a case epitope tags can provide an alternative approach. These tags can be inserted internally or placed at the N- and C-terminus. Due to their short length, epitope tags cause limited steric hindrance. Epitope tags require fixation and staining with a specific antibody before visualization. While not suitable for live cell imaging they can be used for subcellular and ultrastructural localization, immunoprecipitation experiments or to monitor protein processing during targeting or maturation. A number of epitope tags have been used successfully in *Toxoplasma* (e.g. cMyc (Delbac et al., 2001), HA (Karsten et al., 1997) , FLAG (Sullivan et al., 2005) or Ty-1 (Herm-Gotz et al., 2002)).

Parasites expressing fluorescent proteins can also be analyzed and sorted by flow cytometry. Cell sorting is amenable for positive and negative selectable markers. To obtain clonal parasite lines stably expressing fluorescent protein, we routinely use two rounds of fluorescence activated cell sorting (FACS) and expansion of sorted parasites in culture (Gubbels et al., 2003). Multiple fluorescent proteins can be used and sorted simultaneously, however, an instrument with multiple lasers might be required (see protocol section). Fluorescent protein expression can also be detected using a plate reader. This provides a convenient growth assay for drug screening and genetic selections (Gubbels et al., 2003).

3.2 Genetic analysis of essential genes

In order to study the function of essential genes in a haploid organism, tools need to be developed to ectopically and selectively control gene expression while avoiding pleiotropic effects. One widely used approach is based on the *E coli* tetracycline-repressor system, which controls gene expression at the transcriptional level. The original tetracycline-repressor system interferes with transcription and has been optimised and coupled to T7 polymerase to tightly regulate gene expression in *Trypanosoma brucei* (Wirtz et al., 1999). The tet-Repressor system has also been developed for other protozoan parasites including *T. gondii* (Meissner et al., 2001). Gene fusion of the tet-repressor as recently reported (van Poppel et al., 2006) has led to higher transgene expression and tighter regulation.

Although suitable for the expression of toxic genes and dominant negative mutants, this system proved not to be appropriate for the generation of conditional knockouts in *T. gondii*. Indeed, the necessity to keep the parasites in presence of drug (anhydrotetracycline, ATc) during a prolonged period in order to maintain the expression of an essential gene led to generation of revertants that lost regulation.

To improve the system, a genetic screen based on random insertion was designed to identify a functional transcriptional activating domain in *T. gondii* and to establish a tetracycline transactivator-based inducible system (Meissner et al., 2002b). This screen led to the isolation of two artificial transactivators that were not functional in HeLa cells, illustrating the differences between the transcription machinery in the parasite and its higher eukaryotic hosts. Interestingly, these transactivators corresponding to short stretches of rather hydrophobic amino acids were also active in *P.falciparum* and allowed to establishment of an inducible system for the malaria parasite (Meissner and Soldati, 2005). This system is suitable for the conditional disruption of essential genes with no apparent reversion effect and operates on the parasites in the animal model. In addition to TgMyoA, the tet system has implemented to functionally analyse TgAMA-1 (Mital et al., 2005) and to determine the importantance of several other genes including TgMIC2 (Huynh and Carruthers, personal communication), TgACP (Mazumdar et al., submitted) and profilin (Platnner and Soldati, unpublished).

So far, the tet-inducible system has been relatively laborious, requiring two steps of selection. The first step is the construction of a stable line expressing an inducible copy

of the gene of interest. The second step is the actual knockout of the target gene (see protocol section for details). It is also conceivable to generate inducible knockouts by direct targeting of the inducible construct into the gene of interest by knock-in, replacing the endogenous promoter with the tet-inducible promoter. This could be achieved either by single-homologous recombination or preferably by double homologous recombination to avoid potential reversion of the phenotype by excision of the plasmid and restoration of the wild type locus. Once an essential gene has been conditionally disrupted by the tet-system, it is possible to use ATc as a positive selectable drug to complement the knockout mutants with either a wild type or modified form of the gene of interest.

Approaches that specifically lower the level of mRNA and consequently the level of the corresponding protein are powerful genetic tools available in many organisms. Various technical solutions relying on antisense RNA/oligonucleotides, ribozymes, or dsRNA interference (RNAi) have been developed. Such knock-down of genes usually is easier and faster to accomplish than the generation of conditional knockouts using a tet-regulated system. In *T. brucei*, a combination of efficient RNAi and tight tet-regulated transcription is routinely applied in large-scale functional genomic screens (Ullu et al., 2004).

In contrast, the efficiency of RNAi in apicomplexans is still a matter of debate. Previous studies have reported the successful use of antisense/ribozyme in *T. gondii* (Nakaar et al., 2000, Nakaar et al., 1999). More recent studies suggest that RNAi can operate in T.

gondii (Al-Anouti and Ananvoranich, 2002b, Al-Anouti et al., 2003). Unfortunately, these approaches are currently not sufficiently robust to be used in broad screens.

3.3 Insertional mutagenesis and promoter trapping as tools of functional genetic analysis

Random high frequency integration of a genetic element into the parasite genome can be used to disrupt loci and produce pools of insertional mutants. The integrated sequence can subsequently be exploited to identify the targeted gene with modest effort (Fig. 2). The exceptionally high frequency of non-homologous recombination of transgenes in *T. gondii* allows the use of simple plasmid constructs similar to the way transposons are used in other organisms (Donald et al., 1996b). Several non-essential genes have been identified using random insertion of a DHFR-TS or HXGPRT element (Sullivan et al., 1999a, Chiang et al., 1999, Donald and Roos, 1995, Arrizabalaga et al., 2004). The genomic locus tagged by the insertion can be identified by plasmid rescue or inverse PCR strategies (Roos et al., 1997).

The insertional strategy is not limited to gene disruption but can also be used to trap promoters and genes. Bradyzoite specific genes (Bohne and Roos, 1997, Knoll and Boothroyd, 1998) as well as genes controlling differentiation (Matrajt et al., 2002a, Vanchinathan et al., 2005) have been identified using differential HXGPRT selection under culture conditions that favor differentiation to bradyzoites followed by counter-selection under 'tachyzoite' conditions. The trapping of native *T. gondii* transcription factors might also be achievable. For this a recipient strain harbouring a YFP-YFP

marker under the control of a tet-regulated promoter would be randomly inserted. The tagging plasmid would harbor a Tet-repressor gene lacking a stop codon and 3'UTR sequences. Translational fusion of this marker with a transcription factor should result in transactivation and hence green fluorescence.

The fact that tachyzoites are haploid precludes the identification of essential genes by insertional mutagenesis. Nevertheless is it possible to generate a library of parasite mutants for essential genes by coupling random insertion to the tet-inducible system (M.J. Gubbels & BS, unpublished).

Signature-tagged mutagenesis is another strategy that has been used to identify essential genes by insertional tagging. In this case screening is performed in a different life-cycle stage or under different growth conditions to permit the identification of 'differentially essential' genes. This approach has recently been adapted for *Toxoplasma* (Knoll et al., 2001). Wild-type parasite clones are first tagged with unique oligonucleotide insertions (the signature-tag). These clones are then mutagenized (chemical or insertional) followed by another cloning step. Pools of mutants, which are distinguishable by their tag, are subsequently exposed to a selective condition e.g. infection into an animal. Tagging of genes that are essential in this condition will result in loss of the mutant. 'Missing' mutants are then identified by comparing the tags present in pools before and after selection. Several candidate genes important for parasite persistence in the mouse have been identified using this approach (Knoll, personal communication).

3.4 Forward genetic analysis using chemical mutagenesis and complementation cloning

Genetic analysis of pathways essential for growth in culture requires conditional mutants. Temperature sensitivity (ts) due to chemically induced point mutations can be exploited to obtain strains that are viable at the permissive temperature and display a mutant phenotype at the restrictive temperature. For *Toxoplasma* heat-sensitive (Pfefferkorn and Pfefferkorn, 1976, Radke et al., 2000) Gubbels and BS, unpublished) and cold-sensitive (Uyetake et al., 2001) mutants have been isolated. ENU (N-ethyl-N-nitrosourea) induces random point mutations and has been the mutagen of choice in most *T. gondii* studies. Chemical mutagenesis has been successfully used in *T. gondii* to produce mutants with defects in stage differentiation (Singh et al., 2002), invasion and egress (Black et al., 2000, Uyetake et al., 2001) and cell division and cell-cycle progression (Radke et al., 2000, White et al., 2005).

While generating chemical mutants is straightforward, identifying the mutated gene responsible for the phenotype is not. The two avenues most commonly used to accomplish this goal are physical mapping through crosses, and phenotypic complementation by transfection with a wild type DNA library. While crosses are feasible in *T. gondii,* their limited throughput makes them less practical as a general tool for mutant analysis (also the RH strain used as the molecular biology work horse for *T. gondii* is unable to complete the sexual life cycle). The second approach to identify the gene affected in a given mutant is phenotypic complementation using a library of wild type DNA. This strategy faces two technical challenges: full representation of the

genome (or transcriptome) in the complementation library, and efficient recovery of the complementing sequence. Black and colleagues identified a genetic elements that maintains stable episomes in *T. gondii* (Black and Boothroyd, 1998) allowing convenient rescue by hirt lysis and transformation of bacteria. A library harboring an episomal maintenance sequence on the backbone successfully complemented the HXGPRT locus in the knock-out mutant under mycophenolic acid selection. Analysis of the recovered plasmids however suggested that they might undergo extensive recombination, potentially decreasing their stability and usefulness (Black and Boothroyd, 1998).

The second effort to generate a complementation system was built on high frequency integration of library plasmids (Striepen et al., 2002). Mutants are transfected with a plasmid library and subjected to selection. Subsequently complementing DNA sequences (carried as stable chromosomal insets) are rescued into plasmid using an in vitro recombination protocol (Invitrogen Gateway system (Hartley et al., 2000)). Rescued library inserts can be shuttled back into a parasite expression plasmid through a second recombination step to confirm their complementation capacity. A cDNA library build on this model successfully complemented the *Toxoplasma* HXGPRT locus at high efficiency (Striepen et al., 2002) and was used to identify a phenotypic suppressor of the *T. gondii* ts cell cycle mutant C9-11 (Radke et al., 2000, White et al., 2005). An analogous library carrying *Cryptosporidium parvum* genomic DNA was used for heterologous complementation resulting in the identification of a *Cryptosporidium* gene encoding the purine salvage enzyme IMPDH (Striepen et al., 2002, Umejiego et al., 2004).

Several ts mutants could not be complemented using the cDNA libraries described above (Gubbels, White and BS unpublished). Genes encoding large mRNAs and/or transcribed at low levels are typically under-represented in cDNA libraries. To overcome these problems a large insert (40-50 kb) genomic cosmid library build on a DHFR-TS containing super-cos vector was constructed. This library provides sufficient coverage and transformation efficiency to complement the lack of HXGPRT in every transfection reaction attempted. In addition, we recently complemented a mutant with a ts cell division defect. Three overlapping cosmids were identified from independent transfections which all harbored the gene for a kinase known to be involved in cell cycle regulation (Gubbels and BS, unpublished; cosmid complementation was observed in four additional ts mutants, M.White, personal communication).

4. PERSPECTIVES

T. gondii has proven itself as an excellent experimental model and reverse genetic approaches were key to build a detailed molecular picture of apicomplexan biology. The reverse genetic tool-box has seen constant extension and refinement, however several technological challenges remain. One of these challenges is the now completed genome sequence. The genome is obviously a tremendous resource, but the number of 'candidate' genes produced by computational screens easily overwhelms the throughput of many of the functional genetic assays currently available. Knock-out and conditional knock-outs are powerful genetic experiments, but currently very time-consuming and not successful in all cases.

Protocols that would increase the frequency of homologous over non-homologous recombination could be very helpful to streamline the process. A better mechanistic understanding of apicomplexan DNA recombination and the enzymes involved in the process is needed (Dendouga et al., 2002). Based on such knowledge and following the example of several fungal systems, the recombination pathway could be remodeled by the disruption of non-homologous recombination pathways (Nayak et al., 2005), or conversely the overexpression of elements of the homologous recombination pathway (Shaked et al., 2005) could improve knockout frequency. RNA mediated knockdown presents an attractive alternative to gene targeting. While promising, RNAi in *T. gondii* currently is not sufficiently robust for large scale screens (Al-Anouti and Ananvoranich, 2002a, Al-Anouti et al., 2003, Sheng et al., 2004). Improvements could be derived from optimized protocols to choose and deliver interfering RNAs. It might also be feasible to genetically screen for strains that show higher responsiveness to dsRNA.

Forward genetic approaches have seen considerable progress as well. These approaches could hold the key to mechanistic analysis of phenomena for which the genome does not immediately present an obvious list of candidate genes and proteins. While the tools to complement mutants have improved and may now be at a level to permit robust analysis, the ways to generate and select such mutants still lag behind. Robust screens that reduce a complex cell biological phenomenon to a phenotype that can be easily scored in thousands of mutants with limited effort are needed. The success of visual screens using automated microscopic detection (Carey et al., 2004) points to one avenue to reach this goal. The past decade has seen tremendous progress driven by the ability to transfect and

genetically manipulate the parasites. The next decade will require a set of tools with sufficient throughput to take full advantage of the genome sequence.

5. THE TOXOPLASMA MANIATIS: A SELECTION OF DETAILED PROTOCOLS FOR PARASITE CULTURE, GENETIC MANIPULATION AND PHENOTYPIC CHARACTERIZATION

5.1 Propagation of *Toxoplasma* tachyzoites in tissue culture

T. gondii is promiscuous in its choice of host cell and will infect almost any mammalian cell commonly used in tissue culture work. In general large spread-out cells like fibroblasts or Vero cells are most suitable. Infection of these cells results in distinctive rosettes, which makes it easy to monitor parasite development by microscopy. Many laboratories use transformed cell lines like Vero or 3T3, which produce high parasite yields. Immortal lines grow fast, are easy to culture and can be obtained from many sources.

Primary cell lines like human foreskin fibroblasts (HFF) are also widely used. Their strong contact inhibition and slow growth makes them the cell of choice for plaque assays, bradyzoite induction experiments, genetic selections or any experiment in which cultures have to be maintained for longer periods of time. They also provide excellent microscopy for cell biological analysis. The disadvantage of primary lines is that they have to be managed more carefully as they will die at higher passage number due to senescence. A sufficient amount of early passage cells has to be cryopreserved to reinitiate the culture at that point. HTERT cells (BD Biosciences) have emerged as a

compromise, these cells are immortal but retain many characteristics of primary fibroblasts. We have found these cells to be equivalent to HFF cells in almost all applications. The protocol s below are based on HFF cells but can be used for HTERT cells as well (note difference in glutamine concentration). Many companies supply reagents for tissue culture, the suppliers mentioned in the following are the ones we have used, products from other sources might work just as well.

5.1.1 Maintenance of HFF cells

- T25 flask tissue cultures typically yield 4-7 10^7 parasites (yields are typically lower for the Type II and III cyst-forming strains). The protocols below are based on this scale. If more material is needed, larger flasks (e.g. T175), roller bottles and cell factories have been used successfully with appropriately scaled protocols.
- Warm media and trypsin solution in a 37°C water bath.
- Aspirate medium from a confluent culture and add 2.5 ml of trypsin solution to the flask (0.25% trypsin and 0.2 g/l EDTA in HBSS, Hyclone, store this solution in smaller 5 ml aliquots at –20°C for convenience). Carefully 'wash' monolayer by tilting flask several times, aspirate most of the solution and leave enough to just cover the cells (~ 0.5 ml). Incubate at 37°C for 2 min. Inspect cells for rounding and detachment using an inverted microscope equipped with phase or interference contrast optics. If cells are still attached after 2 min, tap flask with flat hand and/or prolong incubation. HFF are relatively fragile so take care to not over-trypsinize.
- Immediately take up detached cells in a defined volume of Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, if large batches are used medium can be prepared

from powder otherwise use ready made medium) supplemented with 10% new born calf serum (NBCS, Hyclone, cosmic calf serum), Penicillin and Streptomycin (1: 200 of a 10,000 unit/ml of antibiotic stock, Hyclone) and glutamine (1:100 of a 200 mM stock in water, note: for HTERT cells do not add glutamine to avoid overgrowing of cultures) and split 1:8 into new flasks. If fungal contaminations are a frequent problem use 1:100 Fungizone (250 μg/ml amphothericin B, Invitrogen). Move to incubators gassed to 5% CO_2 at 37°C. Allow gas exchange by loosening caps. Confluent cultures can be kept for several weeks prior to *T. gondii* infection.

5.1.2 Maintenance of tachyzoites

- Aspirate medium from a confluent HFF culture.
- Add 10 ml of infection medium (DMEM supplemented with 1% fetal calf serum (FCS, Invitrogen. For experiments which require tight control over the small molecule composition use dialyzed fetal calf serum), Penicillin and Streptomycin as above).
- Infect a new flask with culture supernatant of a freshly lysed culture. As a rule of thumb, passing 0.5 - 1 ml into a T25 culture will result in complete lysis within 2-3 days for RH derived strains. A high inoculum is preferable if parasites are to be use e.g. in a transfection experiment as the majority of the tachyzoites will egress synchronously resulting in high overall parasite viability. To maintain strains pass smaller number of parasites (e.g. 100 μl of a lysed culture). Transfection efficiency and invasion efficiency are greatly enhanced by using freshly lysed parasites. Host cells should not be over-infected. Ideally every host cell should be infected with one parasite.

5.1.3 Cryopreservation of host cells and parasites

- In general the aim is to freeze slowly and to thaw quickly. Wear a lab coat, face protection and appropriately insulated gloves when handling liquid nitrogen. For best results have all tubes and reagents prepared and labeled, chill them on ice and work quickly (if you have to freeze many vials at a time divide them into smaller batches).
- Label 2 ml cryo vials (fitted with silicone o-ring, Nalgene) using a pen dispensing ink that resists liquid nitrogen and chill on ice.
- Prepare an isopropanol/water containing freezing container (VWR, using this simple and inexpensive device will result in about 1°C/min cooling in a –80°C freezer, alternatively use a thin walled foam container to slow cooling)
- Use 'freshly' confluent (T175) HFF cultures for freezing. Trypsinize cells as described above and recover detached cells in DMEM 10% new born calf serum into a 15 ml sterile centrifuge tube. Pellet cells in a table-top centrifuge at 900 g for 10 min at 4°C using a swing bucket rotor.
- Discard the supernatant and resuspended cells in 1.8 ml chilled DMEM (no serum). Add 1.8 ml freezing medium (25% tissue culture grade DMSO and 20% FBS in DMEM) and mix quickly. Immediately dispense 0.5 ml aliquots into chilled freezing tubes, tightly cap tubes and move into chilled (ice) freezing container and place into a -80°C freezer.
- Thaw one vial the next day to insure that your stocks are viable and move the remaining vials into a liquid nitrogen storage container. Solid book keeping which keeps track of rack, box and vial position is essential as it is not easy to 'search' for

vials in liquid nitrogen stocks.

- Parasites are preserved as extracellular tachyzoites. Pellet a freshly lysed culture (1500 g, 20 min, 4°C) and then proceed as described for host cells above. Plan to freeze 2 10^8 per vial which means that you will produce 3 vials from a single T25 culture using 0.8 ml of DMEM and 0.8 ml of freezing medium. Test for viability by thawing before you discontinue the culture of the given line.
- Parasites can also be cryopreserved in host cells at the rosette stage in DMEM with 50% FBS/10% DMSO.
- To thaw HFF cells prepare a flask with medium warmed to 37°C. Remove one vial at a time from liquid nitrogen with thongs and immediately immerse into a beaker filled with water warmed to 37°C gently shaking the vial. Once the medium is thawed transfer cells to the flask and incubate as described for standard culture. Replace medium after 12 hours.
- To thaw parasites use above procedure and inoculate a confluent T25 culture.

5.1.4 Mycoplasma detection and removal

- Mycoplasma contamination is a frequent plague of tissue cultures. Heavy infection can affect the growth of host cells, mycoplasma DNA can produce unwanted background in genetic experiments, and bacterial contamination is a severe problem for immunological experiments as mycoplasma derived molecules potently stimulate a variety of immune cells and functions.
- A simple test for contamination can be performed by DNA staining. Culture cells (and/or parasites) for two passages in the absence of antibiotics (which will lead to

massive amplification of the bacteria) then transfer to 6 well plates with coverslips.

- Stain coverslip cultures for bacterial DNA using DAPI using the standard IFA protocol provided below (more sensitive staining can be obtaining by acid/alcohol fixation and Hoechst staining (see (Chen, 1977) for a detailed protocol).
- In contaminated cultures you will observe numerous small dots of DNA staining (about the size of the typical apicoplast genome staining) throughout the cytoplasm of the host cell.
- More sensitive PCR (ATCC, Stratagene) or luciferase-based (MycoAlert from Cambrex) assays are also available.
- If you suspect a recent contamination, discard your cultures, thaw fresh vial from liquid nitrogen and retest. Protocols to screen strains obtained from other laboratories should be routine.
- If you have to 'rescue' your particular strain treat with Mycoplasma Removal Agent according to the manufacturer's guidelines (an inhibitor of bacterial gyrase, e.g. MP Pharmaceuticals) for 3 passages and then retest (this antibiotic is reasonably tolerated by *T. gondii* at the suggested concentration). Other commercial agents kill *T. gondii* and should be screened prior to use as mycoplasma elimination agents.
- Alternatively, passage of the strain through a mouse and re-isolation into tissue culture will remove mycoplasma.

5.1.5 Passaging Toxoplasma tachyzoites in mice

- Tachyzoites of any strain can be maintained by passage in the peritoneal cavities of mice. 10^4 (Type I strain, i.e. RH) or 10^5 (Type II or III strain, i.e. ME49 or Prugniaud)

are injected intraperitoneally into the mouse.

- Replicating *T. gondii* can be harvested from the peritoneal cavity 3 days later (for Type I strains) and 5 days later (Type II or III strain) by peritoneal lavage with 4 ml of sterile saline or PBS.
- This material can be used to serially passage the strain in the peritoneal cavities of mice or to infect tissue culture cells. Murine inflammatory cells (macrophages and neutrophiles) will also be seen in this lavage material.
- Passage through mice can be useful to remove microorganisms which have contaminated *T.gondii* tissue culture, provided that they cannot replicate in murine peritoneum. Anecdotal data indicates that periodic murine passage of a *T. gondii* strain passaged continuously in tissue culture helps to maintain the vigor and biologic characteristics of the strain.

5.1.6 Passaging Toxoplasma bradyzoite cysts in mice

- Persistent cyst forming strains such as 76K, ME49(PLK) and Prugniaud can be passed by infection of mice with cysts collected from the brains of infected animals or by infection with tissue culture derived organisms (for these strains tissue cultures produce a mixture of tachyzoites and bradyzoites).
- Cysts or tissue culture derived organism can be administered orally or injected subcutaneously or intraperitoneally. Oral infection with cysts has anecdotally been reported to produce the highest number of brain cysts. In general from 10 to 100 tissue cysts are usually sufficient for oral infection.
- To prepare tissue cysts for infection 3 ml of sterile 0.9% NaCl or PBS are added to 1 to

2 infected murine brain(s) containing tissue cysts and this tissue is disrupted by passage through a syringe with an 18 gauge needle.

- The number of tissue cysts is determined by examining a 25 to 50µl aliquot of this material under a tissue culture microscope. Maximal reported yields of tissue cysts from the brain occur from 4 to 8 weeks following infection; however, tissue cysts will persist for the life of the infected animals. Anecdotally, repeated serial passage of a tissue cyst forming strain every 4 weeks in mice will increase the number of brain cysts formed by a strain.
- Cysts can be purified from the brains of infected mice by centrifugation using the method of Cornelissen et. al. 1981. Mouse brains are homogenized a ratio of 1 brain to 1.5 ml of 0.9% NaCl by 7 to 10 strokes in a Potter tube (using the loose fitting pestle).
- An isopycnic percoll gradient is formed by centrifugation of 30 ml of 45% (v/v) Percoll in PBS pH 7.2 for 20 min at 27,138 x g. Five ml of the brain suspension is layered on this preformed gradient and the material is centrifuged for 15 min at 1250 x g.
- After centrifugation the bottom 4 ml containing red blood cells is discarded and the next 20 ml is collected. This 20 ml fraction (specific gravity 1.056) contains the tissue cysts. This material is diluted with 20 ml of PBS and centrifuged for 10 min at 1000 x g to concentrate the cysts as a pellet free from Percoll. Recovery of cysts has been estimated at 80% with this technique.
- It is also possible to directly mix 5 ml of brain suspension with 30 ml of 45% (v/v) Percoll in PBS pH 7.2 followed by centrifugation for 20 min at 27.138 x g. Cysts are

found in this method in the same 20 ml fraction as when a preformed gradient is used. Recovery of cysts with this alternative method has been estimated to be 40%.

5.2 Transfection and stable transformation protocols

5.2.1 Transient Transfection

- Cytomix (120 mM KCl, 0.15 mM $CaCl_2$ 10 mM K_2HPO_4/KH_2PO_4 pH 7.6, 25 mM HEPES pH 7.6, 2 mM EGTA, 5 mM $MgCl_2$) can be prepared in larger batches, filter sterilized and stored in aliquots at –20°C or 4°C Van den Hoof, et al., 1992)
- Weigh 12 mg ATP and 15.2 mg glutathione, add to 10 ml of cytomix and sterilize by passing through a 0.22 um filter.
- Sterilize DNA by ethanol precipitation. Adjust 50 µg of plasmid DNA (typically in ~10 µl and purified using a commercial plasmid purification kit e.g. Qiagen) to 100 µl with TE (pH 8.0). Add 11 µl 3M NaOAc, and 250 µl ethanol. Precipitate DNA for 5 min at -20°C and spin at full speed in a micro-centrifuge.
- Wash the pellet with 1 ml cold 70% ethanol by gently inverting the tube and spin for 2 min in a micro-centrifuge.
- Move tubes into the laminar flow hood and discard the ethanol (keep an eye on the pellet).
- Let ethanol evaporate for 5-10 minutes (be careful not to 'over-dry' as it can be hard to redissolve DNA). Resuspend DNA in 100µl cytomix.
- Filter parasites from a freshly lysed T175 flask into a 50 ml polypropylene tube and count t in a hemocytometer (dilute sample 1:10 in PBS for counting). Pellet parasites at 1500 g, 20 min, 4°C and resuspend in complete cytomix to 3.3 10^7 parasites per ml (if

required the parasite concentration can be increased up to 8 times).

- Mix 100 µl plasmid DNA and 300 µl parasites in a 2 mm gap electroporation cuvette (genetronix) and electroporate parasites with a single 1.5 kV pulse, a resistance setting of 25 Ω, and a capacitor setting of 25 µF using a BTX ECM 630. If you use a BioRad electroporator set to 1.5 kV, 25 µF and square wave, employing an Amaxa system use the T-cell solution instead of cytomix and set the electroporation conditions to program U33.
- Transfer parasites immediately into a confluent T25 HFF culture (for selection and biochemical experiments) or onto coverslips for microscopy (see below).
- Expression of the transgene can be detected beginning 8 hours after transfection (depending on the transgene and the sensitivity of the assay employed) and peaks around 36 hours after electroporation. To measure transient transfection efficiency electroporate with a robust and easy to score visual marker (e.g. plasmid tubYFP-YFPsagCAT (Gubbels et al., 2003)). Inoculate coverslips and count total number of vacuoles and number of fluorescent vacuoles for several fields. All three electroporators yield transient efficiencies of 30-50% 24 hours after electroporation.

5.2.2 Selection of stable transformants

CAT: Selection for chloramphenicol acetyl transferase (CAT) can start immediately after electroporation in presence of 20 µM chloramphenicol (34 mg/ml stock in ethanol). Since the effect of the drug is delayed, it is important to passage the parasites every two days by inoculating at least 10^6 parasites to keep the pool of parasites as heterogeneous as possible. The minimal amount of plasmid required to generate stable transformants

depend on the vector used but 10 to 50 μg of linearized plasmid will usually yield stable transformants.

DHFR-TS: Electroporate parasites with 50 μg of a plasmid encoding the drug resistant dihydrofolate reductase-thymidylate synthase allele (Donald and Roos, 1993a) e.g. plasmid pDHFR*-TScABP (Sullivan et al., 1999b). After electroporation culture in the presence of 1μM pyrimethamine (1 μl of a 10mM stock in ethanol). This plasmid results in the highest frequency of transformation (up to 1-5%). Be careful handling transgenic strains as pyrimethamine is used in the treatment of human toxoplasmosis.

HXGPRT: This selection requires a hypoxanthine-xanthine-guanine phosphoribosyltransferase null mutant (such mutants are available now for multiple strains, see e.g. (Donald et al., 1996b) for RH). 24 hours after transfection add 25ug/ml mycophenolic acid (25 mg/ml stock in ethanol) and 50ug/ml xanthine (50 mg/ml stock in 0.1 N KOH). MPA/xanthine should kill parasites within 2-3 days.

BLE: For phleomycin selection electroporate parasites with an expression vector encoding the resistance marker BLE (Messina et al., 1995) transfer to HFF cells until complete lysis of the host culture occurred (12 to 24 hours later). The lysed culture is forced three times through a 25-ga needle to assure that all the parasites are extracellular (see safety section for concerns about needle passing before using this protocol). The suspension of parasites is adjusted to 5 mg/ml of phleomycin (stock solution: 20 mg/ml in water and stored at -20^{o}C) and incubated at 37^{o}C for 10 hours. Parasites are transferred for recovery to HFF cultures in media containing 5 μg/ml of phleomycin. After a new cycle of lysis the extracellular parasites are treated again in presence of drug for 10 hours

and cloned thereafter by limiting dilution in 96-well microtiter plates containing HFF cells in the presence of 5 μg/ml of phleomycin.

5.2.3 Restriction enzyme mediated integration (REMI)

Transformation efficiency can be enhanced by adding 50-100 u of BamHI, NotI, or SacII to the cuvette immediately prior to electroporation (these three enzymes have worked in the past; choose one that does not cut an essential part of your plasmid[s]). Note that REMI often results in multi-copy integration of plasmid(s) (Black et al., 1995a, Gubbels et al., 2004b).

5.2.4 Cloning of transgenic lines by limiting dilution in 96 well plates

- Seed tissue culture treated 96 well plates with HFF cells and grow to confluency. Remove medium and add 100 μl DMEM 1% FCS to each well.
- Harvest freshly lysed parasites by filtration and centrifugation as described above.
- Count using a hematocytometer and dilute to 250 parasites per ml.
- Add 100 μl (25 tachyzoites) to each well in the first and 7th vertical colum.
- Using a multichannel pippetor perform a serial dilution from left to right transferring 100 μl at each step (mix each well by pippetting up and down three times. Discard medium after you reached column 6 and start over at row 7.
- Incubate for 7 days without disturbing the culture.
- Inspect each row from left to right using an inverted microscope and identify wells which contain a single plaque and mark those wells. Expand clonal lines by passage into a T25 flask.

5.3 Measuring parasite survival and growth

5.3.1 Plaque assay

- Plaque assays are a reliable way to measure the number of viable and infectious parasites in a sample and are well suited to measure stable transfection efficiency. The following protocol will measure stable transformation using a DHFR-TS resistance plasmid.
- Electroporate tachyzoites as described above using 50 μg of pDHFR*-TScABP (Sullivan et al., 1999b). After electroporation, dilute 50 μl of the content of the cuvette into 950 μl cytomix or medium.
- Infect T25 HFF cultures in drug-free medium with 3 μl and 6 μl diluted parasite suspension and two cultures with 6 μl and 60 μl to be cultured in the presence of 1μM pyrimethamine.
- Incubate for 7 days without disturbing the flasks (optimal time may depend on strain used, 2-3 mm plaques are best for scoring, a few extra flasks can be added in a larger experiment to be 'developed' individually to test when the right plaque size is achieved). The period of selection takes longer with type II and III strains.
- To stain the monolayer aspirate the medium, rinse with PBS, fix for 5 minutes with ethanol, and stain for 5 minutes with a crystal violet solution (dissolve 12.5 g crystal violet in 125 ml ethanol and mix with 500 ml 1% ammonium oxalate in water).
- Remove crystal violet solution and rinse with PBS.
- Air dry and count the number of plaques.
- This assay can also be used to quantify parasite growth by measuring plaque area. To

do this scan stained flasks with a standard flat bed scanner at 600 dpi and use image analysis for measurements. The area of plaques can be reasonably approximated using an ellipse. Measure the longest and shortest diameters of each plaque and use πab/4 to calculate the area.

5.3.2 Fluorescence assay

- This assay will produce dynamic growth curves over the time of the experiment (usually a week).
- Seed tissue culture treated black 384 or 96 well plates with special optical bottom (Beckton Dickinson) with HFF cells. For larger scale assays an automatic liquid dispenser (e.g. Genetix Q-Fill) will increase throughput and reproducibility.
- Once plates are confluent replace medium with DMEM (without phenol red, Hyclone) 1% FCS and antibiotics as described above.
- Infect each well with 2000 (384 well) or 5000 (96 well) tachyzoites (e.g. the YFP-YFP strain (Gubbels et al., 2003)). Plan to have quadruple wells for each experimental condition (e.g. drug concentration) and include negative (no parasites) and positive controls on each plate. Fill all wells with medium but do not use the outermost wells as they evaporate faster which affects parasite growth.
- Measure fluorescence daily for each well for 5-8 days using a sensitive plate reader (BMG Fluostar, bottom excitation and emission 510/12 and 540/12 nm respectively).
- Plot the results (average of four wells and standard deviation) as percent positive in relation to the untreated positive control in each plate.

5.3.3 β-Galactosidase (LacZ) assay

- This is an endpoint growth assay that can be used in multiwell formats (McFadden et al., 1997), a yellow substrate will be turned into a red product.
- Seed HFF cells into standard tissue culture treated 384 well plates as described above.
- Change medium of confluent cultures to DMEM 1% FCS without phenol red (50 µl/well) and infect with 2000 β-galactosidase expressing tachyzoites (wash parasites in PBS before infection to eliminate phenol red).
- At the desired read-out day (usually 5 days after infection, optimal staining has to be established empirically for each strain and condition) add 4.5 µl chlorophenol red-b-galactopyranoside (CPRG, Boehringer Mannheim, 4.5 mM stock in medium without penol red)
- Develop color to desired intensity (if you wait too long all wells will turn red, use your negative and postive controls as guide) and read absorbance at 570 nm. Plot end points as percent positivity as described above.

5.3.4 Uracil incorporation assay

- In contrast to mammalian cells *T. gondii* can directly salvage uracil through UPRT. This can be exploited to measure parasite growth as a function of [^{3}H]-uracil incorporation into parasite TCA precipitable nucleic acids (Pfefferkorn and Guyre, 1984, Roos et al., 1994). The advantage of this assay is that it can be used in all strains and does not require a transgene. Recently a 96 well real-time format has been developed for this assay which is described in detail in (Nare et al., 2002).
- Infect 24 well cultures with parasites and incubate under test conditions (e.g. in

presence of a drug)

- Add 5 μCi of [5,6-^{3}H]-uracil (30-60 Ci/mmol) to each well and incubate for 2 h at 37°C
- Chill cultures and add an equal volume of ice-cold 0.6 N trichloroacetic acid to the medium of each well and incubate on ice for at least 1 h.
- Remove TCA and rinse plates under running water overnight (make sure to use a sink designated for radioactivity work).
- Dry plates, add 500 μl of 0.1 N NaOH to each well, incubate for 1 h and measure radioactivity in half of the sample by liquid scintillation counting. Depending on the scintillation cocktail used neutralization of the base can help to avoid background.

5.4 Live-cell and indirect immunofluorescence microscopy

- Sterilize round 23 mm glass cover slips in 70% ethanol (or autoclave) and transfer to a six well plates. Seed cover slips with host cells and culture to confluency. Infect wells with tachyzoites 24-36 hours before microscopic examination.
- To observe parasites expressing fluorescent protein transgenes remove coverslip from dish with sterile forceps, wipe off medium from the bottom side and gently invert onto a microscope glass slide. If longer observation is required (e.g. for time lapse microscopy) use spacer circles (e.g. Secure Seal, Invitrogen) to generate a small reservoir of medium. Alternatively use dishes that have a cover slip bottom (e.g. ΔT3 dishes, Bioptechs).
- To use antibodies to stain cells remove medium, and fix cells in 2 ml of 3% paraformaldehyde in PBS for 10-20 min.

- Remove fixative and permeabilize cells in 2 ml 0.25% Triton X100 (in PBS) for 10 min.
- Block in 2 ml 1% w/v BSA in PBS/0.25% Triton X100 for 30 min.
- React with primary antibody (diluted 1:100 – 1:5000 in PBS/BSA/0.25% Triton X100 depending on titer) for 1 hour. This can be done with minimal reagent by inverting the coverslip onto 100 µl drops on parafilm in a moist chamber.
- Place back into 6 well dish (cell side up) and wash 3 times with 3 ml PBS (5 min each).
- React with secondary antibody diluted in BSA/PBS for 1 hour.
- Wash 4 times in 3 ml PBS (5 min each). To counter-stain DNA add 2 µl of a 2mg/ml DAPI stock solution to the first wash.
- Apply a drop of mounting medium to a microscope slide.
- Briefly wash coverslip in dH_2O (to prevent crystal formation after drying) and invert into mounting medium (cells down).
- Some epitopes are sensitive to aldehyde fixation. In that case use 2 ml of methanol for 20 min as fixative (Methanol will also permeabilize the cells, and no Triton treatment is required). This protocol also works better to stain proteins secreted into the parasitophorous vacuole (these are often washed out by Triton permeabilization). A more elaborate protocol for secreted protein which preserves subcellular structures better than methanol can be found in (Lecordier et al., 1999).

5.5 Cytometry of parasites and infected cells

Toxoplasma tachyzoites can be efficiently sorted using a fluorescence activated cell sorter (FACS) after labeling with specific antibodies to the surface of the parasite (Kim

and Boothroyd, 1995, Radke et al., 2004) or based on the expression of autofluorescent protein (Striepen et al., 1998, Gubbels and Striepen, 2004, Gubbels et al., 2004a). Parasites expressing fluorescent proteins can also be sorted within their host cells (Gubbels and Striepen, 2004, Gubbels et al., 2005).

- For sorting autofluorescent parasites harvest a freshly lysed culture and filter parasite through a 3 μm polycarbonate filter. Count parasites and take up in sterile PBS at 10^7/ml.
- Use a high-speed sorter equipped with a 488 nm argon laser and the following filter and mirrors (GFP or YFP: DM: 555 SP, F: 530/40 BP; DRFP or Tomato DM: 555 SP, F: 570/40 BP**).** Note that for sorting the flow stream is broken into droplets, which carries the potential to produce aerosolized parasites. Extra safety can be provided by an evacuated and HEPA filtered enclosure of the sorting chamber. Discuss biosafety aspects with the FACS facility director and operator.
- For enrichment sort into tubes preloaded with 0.5 ml of PBS or medium and transfer to a confluent T25 HFF culture. For cloning sort directly into seeded multiwell plates. Using a MOFLO sorter we found 3 events per well to result in the maximum number of single clones per plate.
- To sort infected cells, inoculate parasites into a confluent HFF culture 1-24 hours prior to sorting.
- Aspirate medium and wash twice with sterile PBS.
- Trypsinize cells as described above and recover in 10 ml DMEM 1% FCS.
- Filter through a 75 μm cell strainer (Becton Dickinson), spin down and resuspend in 0.5 ml PBS and sort as described above.

- Detail on antibody staining for FACS of tachyzoites is provided in (Radke et al., 2004).

5.6 Disruption of non-essential genes

T. gondii is haploid and non-essential genes can be disrupted by homologous recombination using single or double cross-over. As discussed in detail the main challenge is to overcome the background of non-homologous plasmid insertion. Above we have described and cited several approaches, here we describe a CAT/YFP positive/negative selection for homologous recombination by double cross-over in detail.

- Construct a targeting plasmid that flanks a sagCATsag selectable marker cassette with 1.5-3 kb homologous sequence from the target gene (typically the 5' and 3' genomic sequences flanking the actual coding sequence). Introduce a YFP expression cassette 3' adjacent to the 3' homologous flanking region. Be sure that your targeting plasmid contains a unique restriction site that will allow you to linearize the construct without cutting into markers or flanking regions (e.g. in the multi-cloning site of the plasmid backbone).
- Test for YFP expression in a transient transfection experiment (~30% of the vacuole should show cytoplasmic fluorescence)
- Transfect with 10, 25 and 50 μg of linearized plasmid and select for stable transformation in the presence of 20 μM chloramphenicol.
- Subject the drug resistant population (typically after 3-4 passages) to FACS (use the non-transfected parent strain and a YFP expressing strain as positive and negative controls). Gate events to be sorted to 'viable' tachyzoites by forward and side scatter and clone non-fluorescent parasites by sorting into confluent 96 well plate cultures.

- Leave plates undisturbed and check for single plaques after 7 days and mark clones.
- Suspend parasites by pipetting up and down and transfer 100 µl of each well into a well of a 6 well plate. Replenish medium in the 96 well plate and keep in the incubator.
- 6 well cultures will lyse within 3-4 days. Resuspend lysed parasites by pipetting and harvest by centrifugation.
- Wash parasites with PBS and pellet again
- Resuspend parasites in 500 µl TE, add 1 µl RNAse (10mg/ml), 10 µl 10% SDS and 20 µl Proteinase K (10 mg/ml)
- Incubate at 55°C for at least 1 hour (can go over night)
- Extract twice with 500 µl phenol: chloroform: isoamylalcohol (25:24:1, molecular biology grade), and once with chloroform, always keep the water phase.
- Add 1/10 volume of 3 M NaOAc and 2.5 volumes of ethanol and precipitate DNA for 20 min at –20°C
- Spin for 10 min at full speed in a microcentrifuge, wash pellet with 70% ethanol, spin again, briefly airdry and resuspend DNA in 50 µl TE.
- Use 5 µl as template in a PCR reaction with primers that will produce different size products for the native and the KO locus (make sure that your primers do not pick up the ectopic mini-gene copy).
- Confirm putative allelic replacements by Southern blot using appropriate probes

5.7 Disruption of essential genes

As detailed above this approach has three steps: 1. Introduce an ectopic tet-regulatable copy of the target gene, 2. Target the native locus by homologous recombination, and 3.

knock-down of the expression of the ectopic copy using ATc treatment. The choice of selectable markers may differ from experiment to experiment (the tet-transactivator line (Meissner et al., 2002a) is resistant to mycophenolic acid), this example will use CAT, YFP and DHFR-TS.

- Construct a plasmid for ectopic expression of the target gene e.g. by replacement of the ACP coding sequence in plasmid ptet07sag4-ACPmyc/DHFR-TS (Mazumdar et al., submitted, (Meissner et al., 2002a). If you omit the stop codon this should result in a N-terminal translational fusion to a c-myc epitope tag.
- Transfect into the TAti transactivator line (Meissner et al., 2002a), select stable transformants in the presence of 1 μM pyrimethamine and clone by limiting dilution.
- Test clones for transgene expression by IFA and Western blot using an anti-cmyc antibody (mAb 9E10, Roche).
- Choose clones that express the transgene at a similar level as the native gene. Depending on the size of the target gene addition of the tag may result in a noticeable mobility shift on SDS PAGE. In this case an antibody against the target protein can be used to compare both proteins side by side.
- It is critical to identify a tightly regulated clone before proceeding to the KO experiment. Careful characterization of clones will pay off with a clean interpretable phenotype. Test for regulation by culturing parasites in the presence or absence of 1 μg/ml of ATc (0.2 mg/ml stock in ethanol) followed by IFA and Western blot. Note that stable proteins might have to be diluted out by growth. Do your first screen after 5 days of treatment and then titer the minimal treatment time for complete suppression using your tightest clone.

- Target the native locus as described above (using CAT/YFP positive negative selection), establish allelic replacement, and analyze regulation of the ectopic copy in confirmed KO clones by IFA and Western blot.

5.8 Insertional mutagenesis and tag rescue

- Electroporate tachyzoites as using 50 µg of linearized (e.g. restricted with NotI) plasmid pDHFR*-TScABP. Select for stable transformants in 1 µM pyrimethamine and apply the desired phenotypic screen. Clone mutants by limiting dilution, expand into T25 cultures and isolate genomic DNA as described above.
- Set up parallel 20µl restriction digests using several restriction enzymes which cut once in your plasmid (e.g. EcoRI, HindIII, XhoI, XbaI for pDHFR*-TScABP see (Sullivan et al., 1999b) for maps and a detailed discussion of enzyme choice). Use 2 µg genomic DNA for each digest and incubate overnight at 37°C.
- Purify DNA from digest using a Qiagen spin column following the manufacturer's protocol and elute in 30 µl elution buffer.
- Mix 5 µl eluate with 2 µl 10x NEB ligase buffer, 13 µl H_2O and 1 µl T4 DNA ligase and incubate overnight at 16°C.
- Add 1 µl glycogen, 2 µl 3 M NaAc, pH 5.2 and 50 µl ethanol and precipitate DNA for 30 min at -20°C.
- Wash pellet with 1 ml 70% ethanol, airdry briefly, and resuspend pellet in 10 µl H_2O
- Electroporate 1 µl into 25 µl library efficient electrocompetent bacteria (we found DH12S to result in best recovery).

- Transfer into sterile microcentrifuge tube, add 200 µl LB medium and incubate for 1 hr at 37°C while shaking.
- Plate entire transformation onto an LB agar plate containing suitable antibiotic (in this case ampicilin).
- Tags can also be rescue by inverse PCR. See (Sullivan et al., 1999b) for primer design and a detailed protocol.

5.9 Chemical mutagenesis

- ENU is highly toxic and carcinogenic. Use utmost care with all materials that have come into contact with this chemical. Label tubes and flasks to warn members of your laboratory, and dispose contaminated solutions appropriately.
- The mutagenic potency can vary from batch to batch and has to be titrated by plaque assay. Prepare a stock solution (100 mg/ml in DMSO) and store multiple aliquots at -20°C. Perform triplicate plaque assays using 0, 25, 50 and 75 µl of mutagen. Optimal mutagenesis results in 70% parasite killing compared to untreated controls (the protocol below assumes 50 µl as the optimal dose)
- Infect two confluent T25 HFF cultures with 1.2 ml of a freshly lysed culture 24 hours prior to the experiment.
- Replace medium with 10 ml DMEM 0.1% FBS medium.
- Incubate at 37°C for 30 min.
- Add 50 l ENU to flask A and 50µl sterile tissue culture grade DMSO to flask B.
- Treat for 4 hours at 37°C.

- Wash cultures 3 times with 10 ml cold sterile PBS and discard into a dedicated waste container.
- Add 10 ml PBS, scrape cells with a cell scraper, liberate parasite by two passages through a 25-ga needle (see safety section), and filter through a 3 μm polycarbonate filter.
- Transfer to 50 ml tube, add 40 ml PBS, and spin at 1500x g at 4°C for 20 minutes.
- Resuspend in 5 ml PBS and count parasites. Proceed to cloning by limiting dilution. It is advisable to control the mutagenesis efficiency of each experiment by plaque assay.

5.10 Complementation cloning using the ToxoSuperCos library

- Prepare 50 large and 10 small LB-agar Petri dishes (10 μg/ml Kanamycin)
- To titer the ToxoSuperCos library prepare five 1.5 ml Eppendorf tubes with 135 μl LB (no antibiotics), 1 with 1 ml LB and 1 empty tube
- Remove library from the -80°C freezer and keep on ice (work quickly to avoid thawing and immediately refreeze library)
- Scrape a small amount of library (~20 μl) into the empty tube
- Add 1 μl of thawed scraped bacteria to 1 ml LB ($1:10^3$ dilution)
- Keep the remainder of the thawed library at 4°C (stable for 1-2 days)
- Prepare a dilution series (10^4-10^8), plate 100 μl of each dilution on pre-warmed small LB-Kan plates, grow overnight at 37°C and count colonies to calculate the number of colony forming units (cfu) per ml.
- To amplify the library DNA pre-warm large LB-Kan plates at 37°C, Prepare 10 ml of LB containing 50,000 cfu/ml and plate 200 μl per plate.

- Grow overnight 37°C (incubate longer if colonies are too small).
- To harvest, add 2 ml of LB to the plate and scrape colonies using a cell scrape, transfer into a 250 ml centrifugation bottle (on ice) and wash with 1 ml of LB. Repeat for each plate and pool.
- Pellet bacteria in a table top centrifuge, remove liquid and weigh the pellets (bacteria can be stored at -20°C at this step).
- Purify cosmids using a commercial kit e.g. Qiagen large construct kit according to the manufacturer's instructrions, resuspend DNA pellet in 150 µl TE per column and store cosmid DNA at 4°C in the dark.

- To complement *T. gondii* mutants perform 5 independent transfections as described above (8 10^7 parasites and 25 μg cosmid DNA per cuvette). Include at least one mock transfection to control for reversion.
- Transfer independently into T175 HFF cultures, incubate overnight at permissive conditions then apply selective pressure. The ToxoSuperCos backbone contains a DHFR-TS cassette. In addition to the selective pressure you can also apply pyrimethamine selection. This can be helpful to ensure integration of the entire cosmid, facilitating rescue of a tag later on.
- For ts mutants plaques can be identified 10-14 days after transfection
- Clone by limiting dilution, prepare genomic DNA and rescue a sequence tag exploiting the Kan marker on the ToxoSuperCos backbone as describe for insertional mutagenesis (use BglII, HindIII and XhoI).
- BLAST rescued sequences against ToxoDB. You should obtain hits to the same genomic region from independent complementations. Check if your candidate region is represented among the sequenced and arrayed cosmids displayed on ToxoDB, acquire these cosmids and test for complementation.

5.11 Safety concerns working with *T. gondii*

Several aspects of the parasite's biology make work with *T. gondii* relatively safe. In immunocompetent persons the infection produces usually no or only modest symptoms. Depending on the region of the world 20-70% of the population is already infected and resistant to reinfection. Lastly, the tachyzoites stage, which is most widely used in experimental work is not highly infective by aerosol or ingestion. However, *T. gondii* is a

human pathogen with the ability to cause severe disease and should be handled with appropriate care (severe lab accidents have occurred in the past).

We summarize a few ground rules in the following (this section does not represent a comprehensive laboratory safety manual).

- Laboratory workers who belong to a specific risk group (active or potential severe immunosuppression, pregnancy) should not work with live parasites
- Safety procedures should be frequently reviewed with all members of the laboratory.
- Handle parasites in designated biosafety cabinets. Label all work areas, flasks, tubes and waste containers that might harbor infectious material accordingly.
- Wear a lab coat, gloves and goggles. Goggles are especially important for workers who do not wear glasses. An eye splash could potentially deliver a high inoculum of parasites.
- The main route of infection with tachyzoites is direct inoculation by injury or through eye splash. Be extremely careful in all situations that involve sharps. Note that coverslips, microscope slides as well as plastic or glass tubes can break and produce sharp edges. Should you break something, sterilize using 70% ethanol before you attempt cleanup. Needle sticks are the most common source of laboratory infections. The safest approach is to minimize such situations is to avoid them. Consider if the use of sharps is really essential to your experiment. If you really have to needle pass infected cells to liberate parasites leave the plastic sheath on the needle and cut off its tip using sturdy scissors several mm before the tip of the actual needle. This can help to protect you from accidental sticks and provides extra safety at no additional cost or effort.

- Be especially careful working with strains that encode resistance to drugs commonly used for treatment of humans including pyrimethamine, sulfadiazine, clindamycin and azithromycin.
- Sterilize all materials that were in contact with live parasites (autoclave all plastic tissue culture material, bleach all liquids accumulating in e.g. vacuum bottles and frequently sterilize surfaces by spraying and wiping down with 70% ethanol).
- Have a plan for a potential accident. While the goal is to prevent accidents, they might happen nonetheless. Establish local as well as national contacts to infectious disease specialists who could provide advice for diagnosis and treatment. (Reference laboratories include the Palo Alto Research Foundation (http://www.pamf.org/serology) and the Laboratory of Parasitology and FAO/WHO International Centre for Research and Reference on Toxoplasmosis, Statens Seruminstitut, 2300 Copenhagen S, Denmark).
- Ensure good communication about lab safety and **always** disclose any contamination, accident or inoculation. Inform the head of your laboratory about any accident, even if you feel this was a minor incident.

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FIGURES & TABLES:

Table 1

Selection strategies, gene markers and conditions

Figure 1

Sources of information and manipulation strategies.

Schematic drawing of an intracellular parasite with the subcellular structures and organelles and the list of the tools currently available for functional analysis. Figure modified from Soldati, D. and Meissner, M. "*Toxoplasma gondii* a model organism for the Apicomplexans" in "Genomes and the Molecular Cell Biology of Malaria Parasites". Horizon Press. 5, (2004), 135-167.

Figure 2. Exploiting non-homologous insertion and homologous recombination to manipulate the *T. gondii* genome. (**A**) Schematic representation of insertional genomic tagging using a DHFR-TS plasmid (based on (Roos et al., 1997). Plasmid DNA is indicated on top, genomic insertions below. For insertional mutagenesis expression of the DHFR-TS pyrimethamine resistance gene is driven by its own promoter, the insertion therefore is not necessarily within the open reading frame but might also act through inactivating a regulatory region (e.g. promoter). In case of promoter trapping DHFR-TS does not carry its own promoter, and expression of the resistance gene depends on insertion close to an active promoter, or as an in frame fusion into an expressed gene. Tandem insertions can complicate the identification of the tagged locus by plasmid rescue (using restriction enzyme X) and/or inverse PCR (using restriction enzyme X or

Y). However, simultaneously applying restriction enzyme Z, cuts the tandem into two fragments incompatible with plasmid rescue or inverse PCR (Sullivan et al., 1999b, Roos et al., 1997).

(**B**) Schematic representation of gene knock-out through double homologous recombination. The homologous regions destined for homologous recombination are represented by white boxes. Restriction enzymes A and B are used to generate fully homologous ends. In this case YFP is used as a negative selectable marker to enrich for homologous recombination (YFP is lost and parasites are FACS negative).

(**C**) Schematic representation of allelic replacement through single homologous recombination. In this strategy a circular plasmid inserts and tags the locus with a YFP fusion (which can be omitted, or replaced by a shortened ORF to create a functional knock-out). The gene-locus 3' of the plasmid backbone is functionally inactivated by the lack of a promoter. This figure is taken from "The Biology of *Toxoplasma gondii* " Manipulating the *Toxoplasma* genome**.** Gubbels, M-J., Mazumdar, J., van Dooren, G., and Striepen, B. Horizon Press, In press.

Figure 3. Tagging subcellular compartments with fluorescent protein markers in *T. gondii*. This figure provides examples of single and dual fluorescent protein labeling T. gondii, all images were obtained by live cell microscopy. **(A)** Dense granules and parasitophorous vacuole, P30-GFP (Striepen et al., 1998); (**B**) centrocones (outermost dots) and posterior IMC rings of mother (innermost) daughter cells (lines), MORN1-YFP (Gubbels et al., 2006); (**C**) nuclei, PCNA-GFP (Radke et al., 2001); (**D**) plasma

membrane, P30-GFP-GPI (Striepen, unpublished); (**E**) micronemes, MIC3-GFP (Striepen et al., 2001); (**F**) cytoplasm, YFP-YFP (Gubbels et al., 2003); (G) inner membrane complex, IMC3-YFP (Gubbels et al., 2004b); (**H**) microtubules, YFP-TUB (Hu et al., 2002), (**I**) mitochondria, HSP60-RFP (G. van Dooren, unpublished); (**J**) dividing tachyzoites IMC3-YFP and H2b-mRFP (Hu et al., 2004), (**K**) nuclear division and cytokinesis, H2b-mRFP and MORN1-YFP (Gubbels et al., 2006); (**L**) apicoplast division, FNR-RFP and MORN1-YFP (Striepen et al., 2000), (**M**) Golgi division, GRASP-RFP and MORN1-IMC; (**N**) apicoplast, ACP-GFP (Waller et al., 1998); (**O**) rhoptries, ROP1-GFP (Striepen et al., 1998); (**P**) endoplasmatic reticulum, P30-GFP-HDEL (Hager et al., 1999)

Table 1

Selection strategies, gene markers and conditions

Selectable marker genes	Recipient strain	Drug or selection procedure	Concentration range
CAT *E. coli*	Wild type	Chloramphenicol Drugs treatment during 7 days before cloning	20µM CM
DHFR-TS *T. gondii*	Wild type	Pyrimethamine; treatment during 2 days before cloning	1µM PYR
Ble *Streptoalloteichus* or Tn5	Wild type	Phleomycin: 2 cycles of treatment during 5 to 10 hours on extracellular parasites	5µg̃/ml PHELO
HXGPRT *T. gondii*	RHhxgprt- ME49hxgprt- PRUhxgprt-	Positive selection: Mycophenolic acid + xanthine: treatment during 3 days before cloning Negative selection: 6Thioxanthine	25µg/ml MPA 50µg/ml XAN. 80 µg/ml 6-TX
UPRT *T. gondii*	RH uprt-	Negative selection: 5'-fluo-2'-deoxyuridine	5µM FUDR
GFP/YFP *Aequorea victoria*	Wild type	FACS	
Essential genes *T. gondii*	TATi-1 conditional KO	Anhydrotetracycline	Max 1µM ATc
Cre recombinase *Enterobacteria phage P1*	Transgenes flanked by loxP sites (recycling of markers)	Transient transfection with Cre expressing plasmid. Cloning immediately after electroporation	No selection
TK *Herpes simplex*	Wild type	Ganciclovir 24 hours treatment	10µM GCV
CD *E. coli*	Wild type	5-fluorocytosine	40µM FLUC

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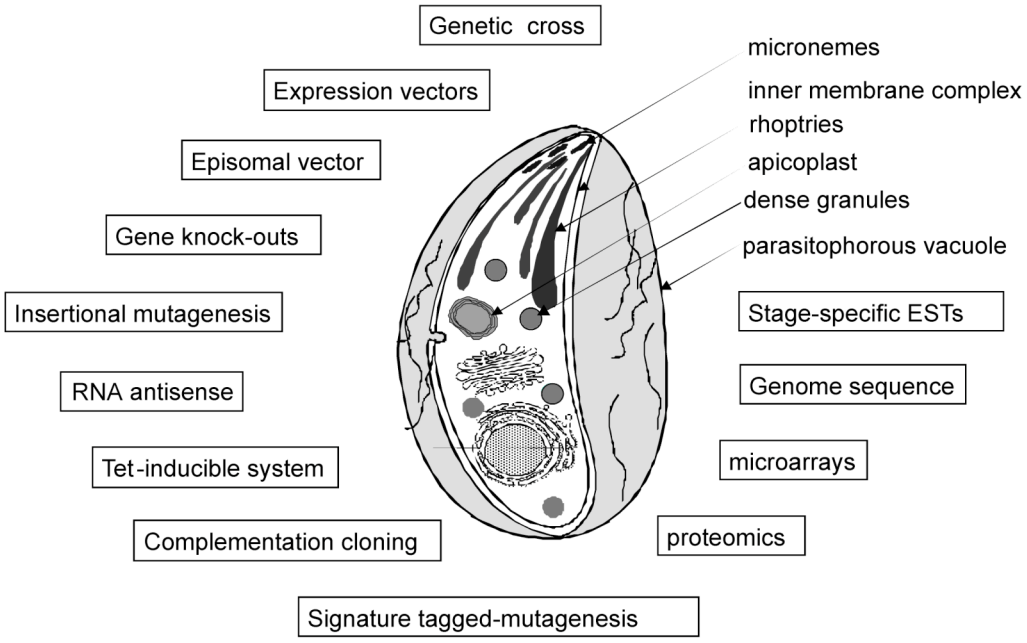
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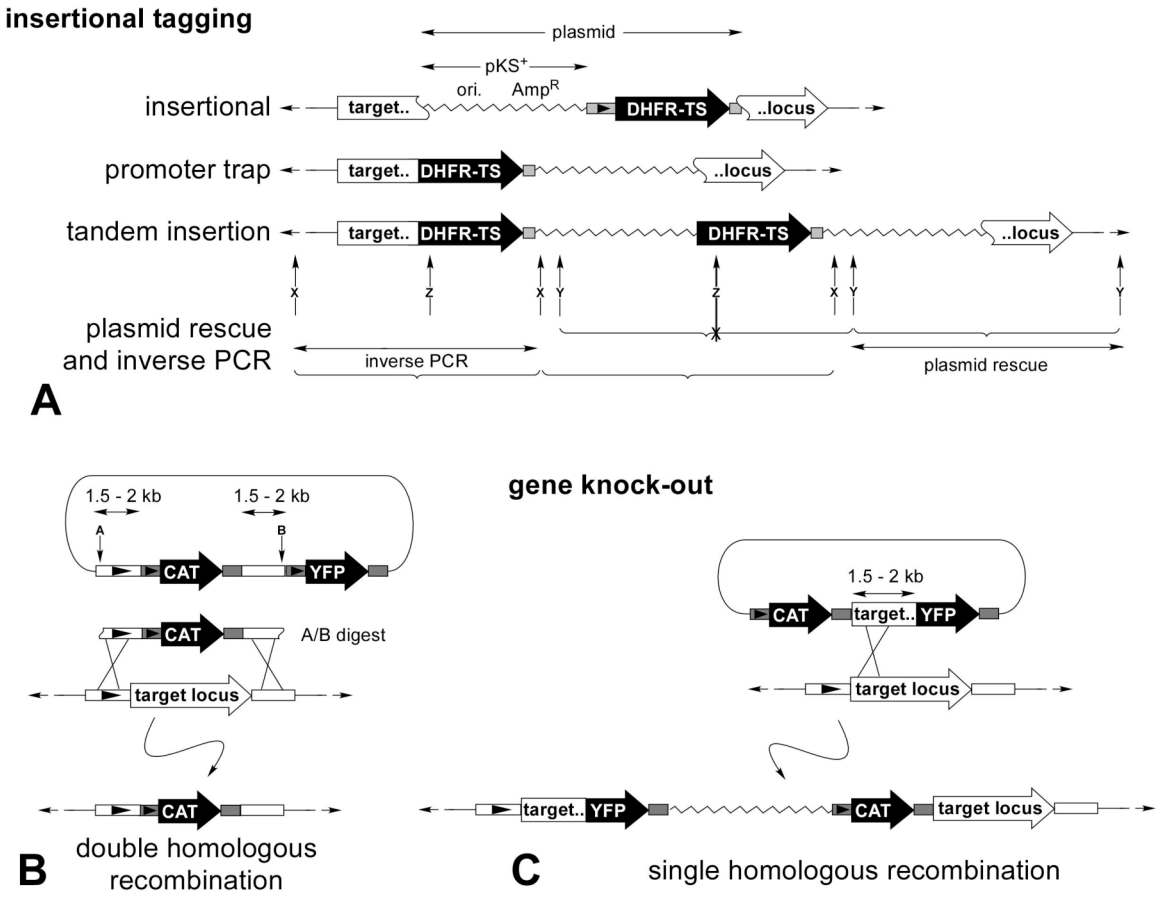
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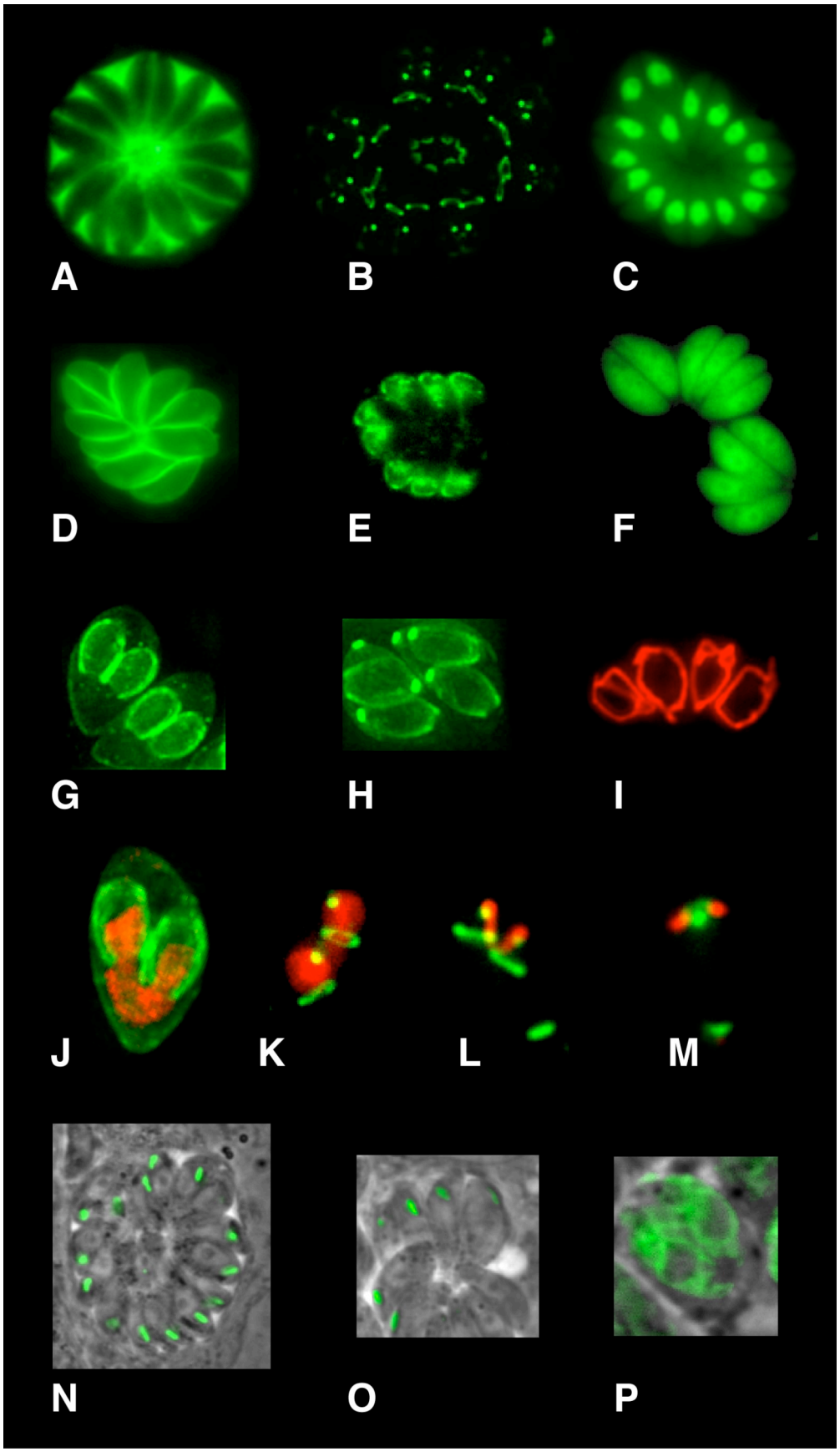
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