CHAPTER

17

Genetic Manipulation of *Toxoplasma gondii*

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OUTLINE

Toxoplasma gondii, Second Edition
http://dx.doi.org/10.1016/B978-0-12-396481-6.00017-9

17.1 INTRODUCTION

The first genetic manipulations applied to *Toxoplasma* were performed by using chemical mutagenesis. These studies were pioneered in the 1970s by the Pfefferkorn laboratory (Pfefferkorn and Pfefferkorn, 1976; Pfefferkorn, 1988) who developed 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 analyse *Toxoplasma* population structure and evolution. See Chapters 3 and 16 for further discussion of these topics.

The reverse genetics approach, which introduces foreign DNA into parasites, was achieved using electroporation. Initially the transient

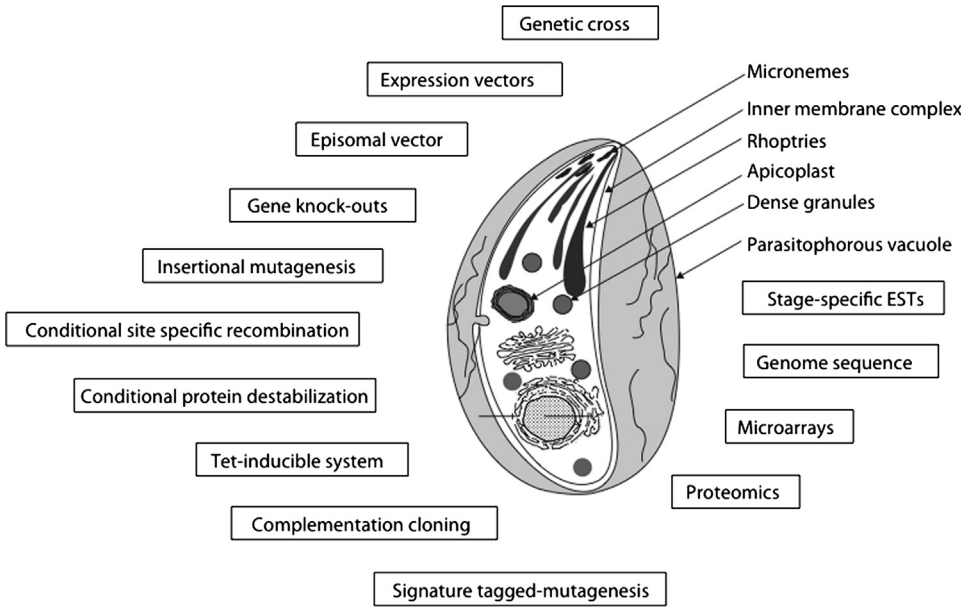


FIGURE 17.1 **Sources of information and manipulation strategies.** Schematic drawing of an intracellular parasite with the subcellular structures and organelles, and the list of the tools currently available for functional analysis. Figure modified from Soldati, D. and Meissner, M. '*Toxoplasma gondii* a model organism for the Apicomplexans' in 'Genomes and the Molecular Cell Biology of Malaria Parasites'. Horizon Press. 5, (2004), 135-167.

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 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 (http://www.Toxodb.org) and the availability of other apicomplexan genomes for comparison are delivering an unprecedented amount of exciting information. A genome comparison with the closely related parasite *Neosopora caninum* illustrates superbly how rather subtle genetic variation affects the biology of these parasites (Reid *et al.*, 2012). 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 17.1 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.

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.

17.2 THE MECHANICS OF MAKING TRANSGENIC PARASITES

17.2.1 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 multi-well 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 a different range of promoter strength and stage specificities, they can be chosen appropriately according to the purpose of the experiment.

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 has 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 *et al.*, 1995; Yang and Parmley, 1997). See Chapter 18 for a discussion of regulation of gene expression.

Importantly, a recent study has highlighted the considerable cell cycle dependency of gene expression (Behnke *et al.*, 2010) which stresses the importance of using the endogenous promoter to control the expression of the gene of interest (GOI).

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 an element is not included. In *Plasmodium* partial deletion of 3′ UTR regions have been exploited to modulate the level of expression of essential genes offering a way to analyse their function (Thathy and Menard, 2002).

At the start codon, a consensus sequence termed the 'Kozak sequence' is recognized by the ribosome as a favourable 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 amino acid following the initiation methionine confirmed the existence of an N-end rule in *T. gondii* (Matrajt *et al.*, 2002b). Amino acids such as Ala, Glu and Asp confer high level of expression of the transgene.

17.2.2 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 17.1.

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 seven days) before an effect of the drug is evident. At this point parasites 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 (Messina *et al.*, 1995; Soldati *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 and Boothroyd, 1995) and to disrupt genes by homologous recombinations (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 exploited as negative selectable markers. Loss of uracil phosphoribosyl transferase (*UPRT*)

TABLE 17.1 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 seven days before cloning	20 μM CM
DHFR−TS *T. gondii*	Wild type	Pyrimethamine; treatment during two days before cloning	1 μM PYR
Ble *Streptoalloteichus* or Tn5	Wild type	Phleomycin: two cycles of treatment during 5−10 hours on extracellular parasites	5 μg/ml PHLEO
HXGPRT *T. gondii*	RH hxgprt- ME49 hxgprt- PRU hxgprt-	Positive selection: Mycophenolic acid + xanthine: treatment during three days before cloning Negative selection: 6-Thioxanthine	25 μg/ml MPA 50 μg/ml XAN. 80 μg/ml 6−TX
UPRT *T. gondii*	RH uprt-	Negative selection: 5′-fluo-2′-deoxyuridine	5 μM FUDR
GFP/YFP *Aequorea victoria*	Wild type	FACS	
Essential genes *T. gondii*	TATi-1 conditional KO	Anhydrotetracycline	Max 1 μM 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	10 μM GCV
CD *E. coli*	Wild type	5-fluorocytosine	40 μM FLUC

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) cannot be converted into an inhibitor of GMP synthase (Donald *et al.*, 1996). 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, 1993). The *DHFR−TS* based selection is unique and shows an exceptional frequency of

chromosomal integration of up to 5% (Donald and Roos, 1993). 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.

Fluorescence activated cell sorting (FACS) is another way to select for stable transformation, when using a fluorescent tag as the marker (Sheiner *et al.*, 2011). To obtain clonal parasite lines stably expressing fluorescent protein, two rounds of fluorescence activated cell sorting and expansion of sorted parasites in culture (Gubbels *et al.*, 2003) is routinely used. Multiple fluorescent proteins can be used and sorted simultaneously; however, an instrument with multiple lasers might be required (see protocol section).

Furthermore, restriction enzyme-mediated integration (REMI) can be used to further enhance the frequency of transformation up to 400-fold (Black *et al.*, 1995) 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).

17.2.3 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 (Fig. 17.2). For example, positive/negative selection can be employed for 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.*, 2002a). Signature tagged mutagenesis has also been successfully applied to discover virulence genes in forward genetic screens, where growth of insertional mutants has been compared *in vitro* and *in vivo* (Frankel *et al.*, 2007).

Homologous recombination leading to gene replacement is instrumental to study gene function and can be accomplished in *T. gondii* using different strategies (Fig. 17.2). In wild type parasites the efficiency of homologous recombination is favoured if long contiguous stretches of homologous DNA are used to target the locus (Donald and Roos, 1994; Kim, 1993). Previously, the construction of vectors for the homologous removable of genes was a relatively cumbersome approach mainly due to the cloning of long flanking regions. The multisite Gateway® (Invitrogen) recombination technique is an improved strategy to avoid restriction enzyme mediated steps and was efficiently applied to *T. gondii* (Upadhya *et al.*, 2011).

Another restriction enzyme-free system is based on overlap extension polymerase chain reactions where only a few PCR steps are required for the construction of the knockout vector. The 5′ and 3′ flanking regions of the GOI are amplified and fused in a second PCR to either the first or second half of a selection cassette. These two constructs containing an incomplete cassette are then transfected into

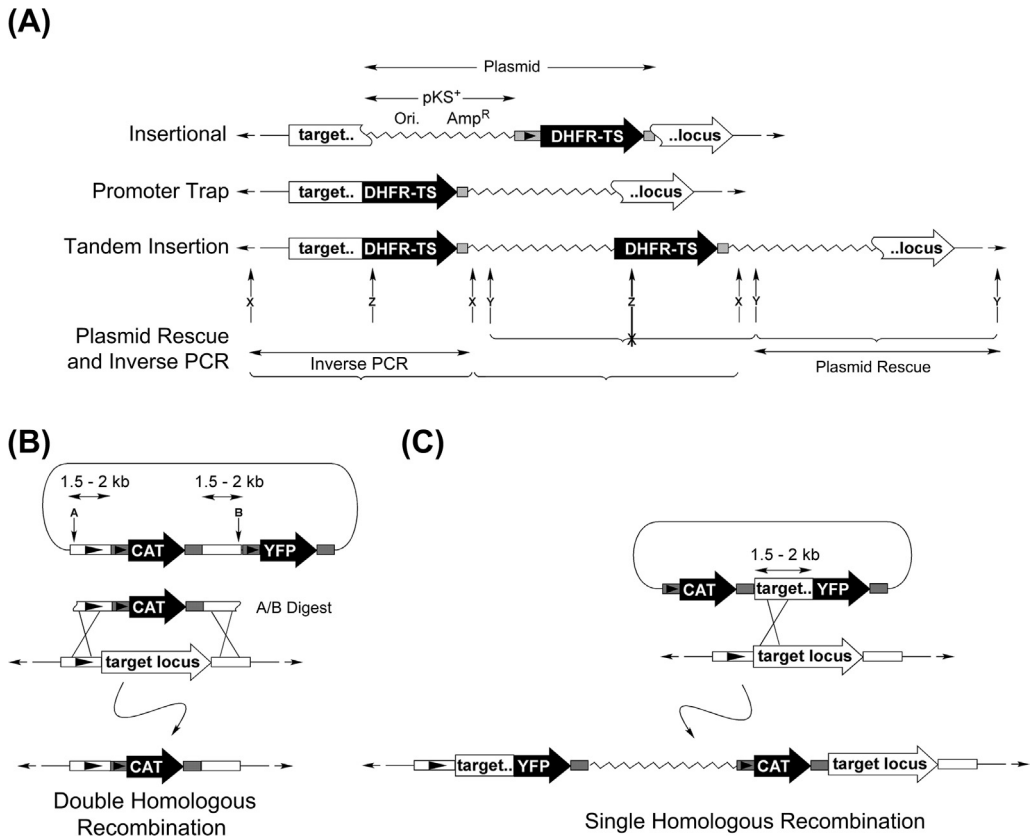


FIGURE 17.2 **Exploiting non-homologous insertion and homologous recombination to manipulate the *T. gondii* genome.**

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 Y) 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.*, 1999; 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) (Mazumdar *et al.*, 2006).

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, 2004.

the parasites. In this system, three single independent homologous recombinations are necessary to achieve gene knockout by replacement with a functional (reconstituted) selectable marker gene, i.e. in the 5′, in the 3′ and within the selection cassette (Upadhya *et al.*, 2011).

Additionally, the recent establishment of recombineering approaches using a cosmid library helped to address the need for long regions of homologous DNA (Brooks *et al.*, 2010; Francia *et al.*, 2012). To further enhance this approach a new large insert DNA library was constructed in a copy-control fosmid backbone. Recombineering can thus be performed at single copy, which enhances stability and fidelity, followed by DNA production at higher copy number. The library was arrayed and 100,000 will be end sequenced (Vinayak and Striepen, unpublished). Another way to increase homologous recombination frequencies is by a combination of positive/negative selections (Table 17.1) (Fox *et al.*, 1999, 2001; Mazumdar *et al.*, 2006; Radke and White, 1998).

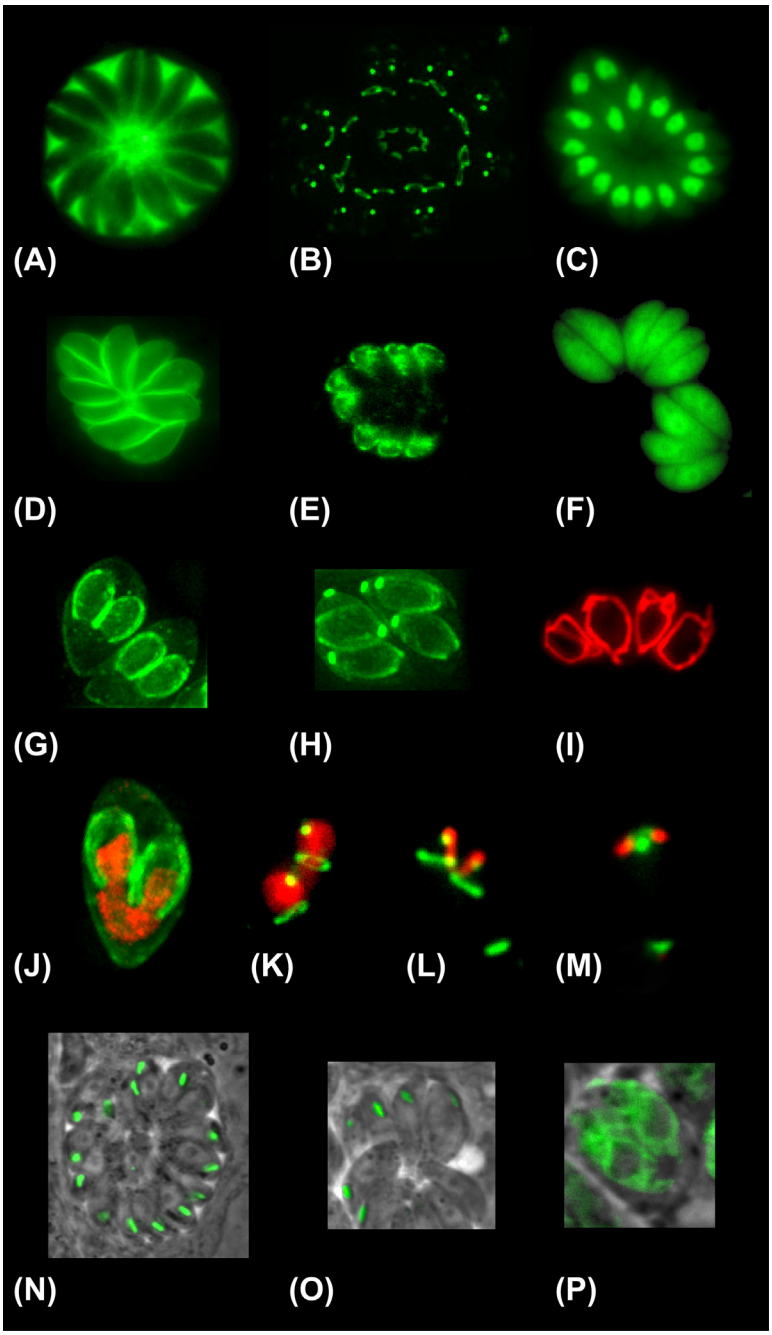
The efficiency of gene targeting was dramatically enhanced by the isolation of ΔKu80 strains. Ku80 is a component of the non-homologous end joining pathway of DNA repair present in *T. gondii* yet lacking in many other apicomplexans including the *Plasmodium* species. Consequently the usually high frequency of random integration events observed in wild type parasites is almost completely abolished in ΔKu80, resulting in a parasite strain that allows efficient gene replacement and endogenous tagging of genes (Huynh and Carruthers, 2009; Fox *et al.*, 2009).

17.3 USING TRANSGENIC PARASITES TO STUDY THE FUNCTION OF PARASITE GENES

17.3.1 Tagging Subcellular Compartments

Visualizing and following the morphology and behaviour of different subcellular compartments through the cells' life cycle is an essential tool for cell biological analysis. Proteins localizing to almost all organelles of *T. gondii* have been described and a large number of constructs allowing expression of organelle-specific fluorescent proteins is now available (see Fig. 17.3 for examples). 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 colours is available now for the simultaneous use of multiple markers. Cyan (CFP) and yellow fluorescent protein (YFP) are a suitable pair for double labelling 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 that are now widely used to track parasites in tissue culture and in infected animals (Gubbels *et al.*, 2003, 2004, 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 marker 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 newer '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* (van Dooren *et al.*, 2008).

Fluorescent labelling of organelles can be achieved by simple transient transfection of specific fluorescent proteins. This approach should be used with caution as the transient expression might result in unspecific targeting due to overexpression or inappropriate timing of expression. This is particularly common for proteins that are targeted for the secretory pathway. Alternatively, the respective marker can be stably expressed as a transgene via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random integration or via endogenous tagging, using a ΔKu80 strain that allows a high efficiency of endogenous tagging (Huynh and Carruthers, 2009; Fox *et al.*, 2009). Parasites expressing fluorescent proteins can also be analysed and sorted by flow cytometry. Additionally, fluorescent protein expression can be detected using a plate reader. This provides a convenient growth assay for drug screening and genetic selections (Gubbels *et al.*, 2003).

17.3.1.1 *Tagging of Parasite Proteins*

The cellular localization of a protein of interest is a first important step in order to characterize its function. 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 expressed as second copies from a heterologous promoter can be tagged by gene fusion using a generic epitope (for which antibodies are already available) or using fluorescent proteins. 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)).

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 and can lead to localization artefacts. For example, the overexpression of microneme proteins often results in accumulation in the early compartment of the secretory pathway or mistargeting to the parasitophorous vacuole (Soldati *et al.*, 2001). To

FIGURE 17.3 **Tagging subcellular compartments with fluorescent protein markers in *T. gondii*.** This figure provides examples of single and dual fluorescent protein labelling *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.*, 2004).
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).

overcome this issue, localization of a protein of interest can also be achieved via endogenous tagging in ΔKu80 strain, this provides the advantage of well-matched expression with respect to strength and timing as the native promoter element is used to drive transcription. Several C-terminal tagging constructs have been generated, taking advantages of ligation independent cloning (LIC) (Huynh and Carruthers, 2009). In combination with homologous recombination in a ΔKu80 strain the endogenous protein can be directly tagged (Huynh and Carruthers, 2009) and localized. Similar experiments can be performed using recombineered genomic cosmids and fosmids (Brooks *et al.*, 2010). However, C-terminal tagging can interfere with the function of some proteins preventing the isolation of the tagged strain.

17.3.2 Genetic Analysis of Essential Genes

In order to study the function of essential genes in a haploid organism, tools needed to be developed that allowed the engineering of conditional knockout, knockdown or trans-dominant mutants. Currently, several strategies operating on different levels, such as site-specific recombination, transcriptional control or control of protein stability, have been implemented in *T. gondii*. Each of these technologies has its advantages and disadvantages to be considered for gene-function analysis.

17.3.2.1 Site-Specific Recombination (SSR)

The yeast recombinases Cre and Flp recognize DNA sequences (the LOX and FRT sites, respectively) that are short enough for convenient cloning, but long enough to be specific and absent from even large genomes when not deliberately introduced. Both recombinases are highly efficient in excising DNA that lies in between the recognition sites and recombination requires only a minimal amount of recombinase expression. However, in order to generate conditional knockouts, temporal control of Cre is required. This can, in principle, be achieved via transient transfections with a Cre expression construct (Heaslip *et al.*, 2010); however, transfection efficiencies can vary and Cre overexpression is toxic (most likely due to non-specific recombination events (Xiao *et al.*, 2012)). A solution for this problem is provided by conditional Cre-systems, such as ligand controlled Cre-recombinases (Metzger *et al.*, 1995) or dimerisable Cre (DiCre) (Jullien *et al.*, 2003). While fusions of Cre to hormone binding domains have been shown to be constitutively active in *T. gondii* (Brecht *et al.*, 1999), the DiCre-system allows rapid, specific and efficient temporal control of Cre activity (Andenmatten *et al.*, 2013). Here, the Cre recombinase is split into two inactive fragments that are fused to the rapamycin binding proteins FRB and FKBP, respectively. Addition of the ligand rapamycin results in reconstitution of the functional enzyme, and excision of the GOI flanked by LoxP sites.

A clear advantage of the DiCre approach is that the GOI is under the control of its endogenous promoter, ensuring correct timing and levels of gene expression. In addition, future constructs can be easily modified to allow high-content cloning of knockout vectors, comparable to approaches applied in mice (Skarnes *et al.*, 2011). Challenges of the DiCre system include a difficulty to obtain clonal knockout population, since induction of DiCre results in recombination rate between 20% and 90% leading to a mixed population of KO and wild type parasites. Another disadvantage is the irreversibility of the recombination event.

17.3.2.2 Tetracycline Inducible Systems

One widely used approach to modulate expression 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 optimized 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 (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 isolation of conditional knockouts in *T. gondii*. Indeed, the necessity to keep the parasites in the 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.*, 2002). 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 seemed to also be active in *P. falciparum* (Meissner and Soldati, 2005). This system is suitable for the conditional disruption of *Toxoplasma* essential genes with no apparent reversion effect and operates on the parasites in the animal model. A line expressing one of the transactivators (TATi-1) was implemented to functionally analyse numerous genes including TgMyoA (Meissner *et al.*, 2002), TgAMA-1 (Mital *et al.*, 2005), TgMIC2 (Huynh and Carruthers, 2006), TgACP (Mazumdar *et al.*, 2006) and profilin (Plattner *et al.*, 2008), in several pioneering studies.

So far, the tet-inducible system has been relatively laborious, requiring two steps of selection (Fig. 17.4A). The first step is the construction of a stable line expressing an inducible copy of the gene of interest via random integration. The second step is the actual knockout of the target gene (see protocol section for details). More streamlined single step strategies have been established recently (Fig. 17.4B–E). In the first system the native promoter of the gene of interest is either replaced or distanced from the ATG by double homologous recombination with a selectable marker (DHFR) and the tet-inducible promoter (Fig. 17.4B). This can be performed in a Δ*Ku80* TATi-1 expressing line (Sheiner *et al.*, 2011) to favour the homologous recombination event. In the resulting mutant parasite, the GOI is directly controlled by the tet-inducible promoter in its genomic context, and addition of ATc induces knockdown (Sheiner *et al.*, 2011). Analysis of several essential genes was already achieved using this strategy (Francia *et al.*, 2012; Sampels *et al.*, 2012).

An alternative single step approach consists of using vectors that carry the coding sequence of TATi-1 under the control of a tubulin promoter (pTub8) downstream of the 5′ UTR of the GOI. The 3′ recombination results in the replacement of the endogenous promoter by the inducible tet-Operator containing promoter. Simultaneously, a tag can be placed at the N-terminus of the GOI (Fig. 17.4C). To establish C-terminal tagging the plasmid contains the same 5′ UTR, the targeted gene cDNA with the tag under the control of the tet-Operator and a 3′ UTR region for recombination (Fig. 17.4D). In all the strategies, including the two steps, the expression of TATi-1 is under the control of a constitutive promoter, which might not be suitable for genes with a very particular level or timing of expression. To solve this problem the pTub8 promoter in the 'all in one' strategy can be exchanged by the promoter of the GOI, to better mimic the timeline of GOI expression (Fig. 17.4E).

So far the frequency of double homologous recombination was found variable with the 'all in one' approach with frequent integrations only on one side and presumably in more than

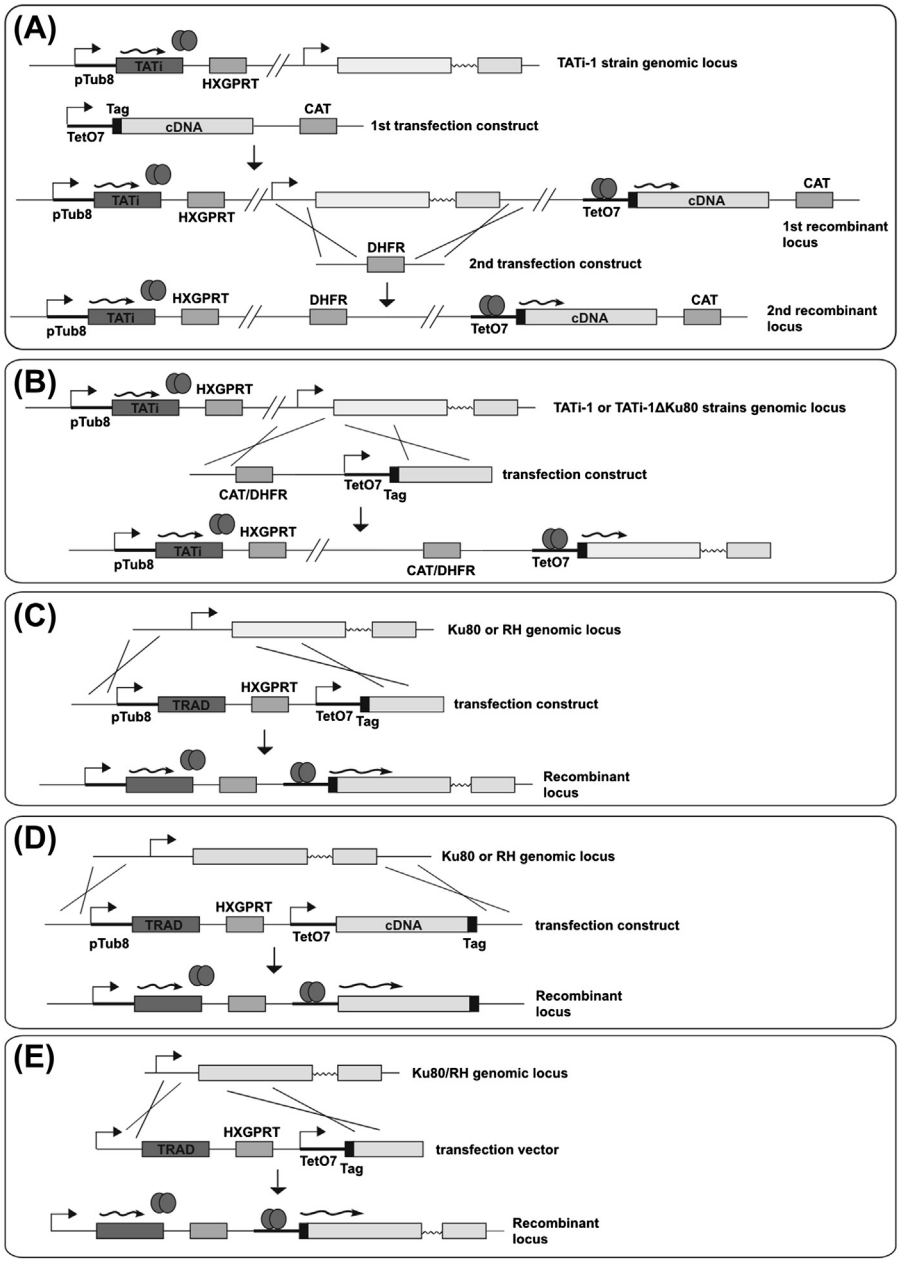

FIGURE 17.4 **Tetracycline inducible systems.**

A) Schematic representation of the 'two steps' process in the TATi-1 expressing strain. The first step consists with the random insertion of the GOI cDNA under the control of the TetO7 promoter. The second step is the knockout of the GOI.

B) The disruption of Ku80 in TATi-1 expressing strain favoured the recovery of double homologous recombination events where the promoter of the GOI is replaced by the TetO7 promoter.

C–D) Alternatively, an 'all in one vector' includes all the different elements for transactivation. This system allows N-term (B) or C-term (D) tagging but also can insert TATi-1 under the control of the GOI promoter.

E) Additionally, the TRADs can be explored to optimize the level of transactivation. GOI, gene of interest; TRAD, transactivator.

one copy. To adjust the expression, more than one copy of TATi-1 might be necessary and several could be integrated. In fact, the TATi-1 strain probably expressed several copies of this transactivator. In consequence, the 'all in one' strategies that result in the integration of one copy of TATi-1 might not be sufficient to drive the expression particularly if the promoter used is weak.

A series of new transactivators have recently been generated to optimize the tet-system for the malaria parasites (Pino *et al.*, 2013). The TATi-2 was previously adapted to *Plasmodium falciparum* and resulted in a successful and tight regulation of transgene expression for multi-copy episomal plasmids (Meissner *et al.*, 2005). In contrast, the level of expression as a single integrated copy dropped dramatically and hence hampered the generation of conditional knock-outs. A group of conserved proteins containing putative Apetala2 DNA-binding domains referred to as the Apicomplexan AP2 (ApiAP2) protein family have been used in a screen in *T. gondii* to identify new transcription activating domains. Four new transactivators (TRADs) have been created that are as or more potent than TATi-1. TRAD4 was exploited in *Plasmodium berghei* to generate knock-downs of essential genes for the intraerythrocytic development of the rodent malaria parasite (Pino *et al.*, 2013). These TRADs could be explored in the 'all in one' strategies to overcome the issue of insufficient transactivation.

The 'two steps' strategy in TATi-1 is a robust but laborious method to produce conditional knockout parasites (Fig. 17.4A). The first step results in different clones of various levels of expression (due to the number of random integrations, integration in highly transcribed loci, etc.) and allows therefore the selection of the most suitable expression. The 'all in one' strategies reduce this process in only one step but the transactivation only takes place in the GOI endogenous locus and in a single copy. The level and the proper timing of transactivation are critical for the establishment of conditional systems and therefore each method has to be considered for different genes.

17.3.2.3 *Regulation of Protein Stability*

A major limitation of the above conditional systems is their relatively slow response kinetics, as the protein of interest is still present after removal or down-regulation of the respective gene. In the case of stable proteins, it can take up to 96 hours before a phenotype becomes fully evident. Such long incubation times can complicate the interpretation of the observed phenotypes in particular when it comes to distinguishing primary from secondary effects. A more rapid method, based on conditional regulation of protein stability has been developed for mammalian cells (Banaszynski *et al.*, 2006) and successfully adapted to apicomplexans (Herm-Gotz *et al.*, 2007; Armstrong and Goldberg, 2007). This system is based on mutated forms of the FKBP12−rapamycin binding protein that result in its fast degradation. Fusion of this degradation domain (ddFKBP) to a protein of interest results in degradation of the entire protein. Addition of the inducer Shield-1 (a rapamycin analogue) results in rapid stabilization of the protein (Banaszynski *et al.*, 2006). Regulation can be achieved by fusing ddFKBP N- or C-terminally, with N-terminal fusion providing superior performance (Herm-Gotz *et al.*, 2007).

Proteins residing within organelles typically cannot be regulated using ddFKBP, since the protein needs to be accessible to the proteasome, which resides in the cytosol. This system in principle should be suitable to construct conditional mutants by direct endogenous tagging. While direct allelic replacement has been successful in some cases in *P. falciparum* (Dvorin *et al.* 2010; Farrell *et al.*, 2011; Russo *et al.*, 2009), attempts to use this strategy in *T. gondii* failed and resulted in the expression of ddFKBP-tagged proteins that remained stable in the absence of Shield-1.

The ddFKBP-system has been further optimized and new FKBP12 mutants have been

tested in the mammalian system (Egeler *et al.*, 2011). In addition, other destabilization domains have been developed, such as DHFR-based systems (DDD) (Iwamoto *et al.*, 2010) that work well in *P. falciparum* (Muralidharan *et al.*, 2011). It will be interesting to see whether these optimized versions can confer better regulation to endogenously tagged proteins in *T. gondii.*

Despite these obstacles the ddFKBP system is very well suited to generate overexpression or trans-dominant mutants (van Dooren *et al.*, 2009; Santos *et al.*, 2011; Daher *et al.*, 2010; Breinich *et al.*, 2009; Agop-Nersesian *et al.*, 2010). The rapid response kinetic of the ddFKBP-system is of particular advantage, when rapid processes are to be analysed, such as components of trafficking systems or signalling cascades.

17.3.3 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. 17.2). 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.*, 1996). Several non-essential genes have been identified using random insertion of a DHFR−TS or HXGPRT element (Sullivan *et al.*, 1999; Donald and Roos, 1995; Chiang *et al.*, 1999; 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 *et al.*, 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 favour 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 harbour 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 it is possible to generate a library of parasite mutants for essential genes by coupling random insertion to the tet-inducible system (Jammallo *et al.*, 2011).

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 been successfully 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 (Craver *et al.*, 2010; Frankel *et al.*, 2007; Payne *et al.*, 2011; Skariah *et al.*, 2012).

17.3.4 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 *et al.*, 2008) 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 workhorse 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 element that maintains stable episomes in *T. gondii* (Black and Boothroyd, 1998) allowing convenient rescue by heat lysis and transformation of bacteria. A library harbouring an episomal maintenance sequence on the backbone successfully complemented the HXGPRT locus in the knockout 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 built 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 Striepen, unpublished). Genes encoding large mRNAs and/or transcribed at low levels are typically underrepresented in cDNA libraries.

To overcome these problems, a large insert (40−50 kb) genomic cosmid library built 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, this library complemented numerous mutants with a ts cell division defect (Gubbels *et al.*, 2008). Note that the increased power of sequencing technology now also allows sequencing of the complete genome of mutants thus permitting to pinpoint the genetic basis of temperature sensitive defects even in mutants that fail to complement efficiently (Gubbels *et al.*, 2008).

17.4 PERSPECTIVES

T. gondii has proven itself as an excellent experimental model and reverse genetic approaches were key to building a detailed molecular picture of apicomplexan biology. The reverse genetic toolbox has seen constant extension and refinement. The potential and limits of the genetic approaches have been reviewed in light of the biological specificities that differ between *Toxoplasma* and rodent or human malaria parasites (Limenitakis and Soldati-Favre, 2011).

To this joins the tremendous resource in the form of the completed genome sequence, where one can identify a large number of 'candidate' genes of interest by computational screens. With knock-outs and conditional knock-outs becoming easier, one can also target a larger number of candidates. A suitable step forward in the genetic research of *Toxoplasma* biology might be a community effort to establish a phenome project.

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.

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

17.5.1 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 protocols below are based on HFF cells but can be used for HTERT cells as well (note the 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.

17.5.1.1 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 minutes. Inspect cells for rounding and detachment using an inverted microscope equipped with phase or interference contrast optics. If cells are still attached after 2 minutes, 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% newborn 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.

17.5.1.2 Maintenance of Tachyzoites

- Aspirate medium from a confluent HFF culture.
- Add 10 ml of infection medium (DMEM supplemented with 1% foetal calf serum (FCS, Invitrogen. For experiments which require tight control over the small molecule composition use dialysed foetal 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 used, 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.

17.5.1.3 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 labelled, 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% newborn calf serum into a 15 ml sterile centrifuge tube. Pellet cells in a table-top centrifuge at 900 g for 10 minutes at 4°C using a swing bucket rotor.
- Discard the supernatant and re-suspend 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 bookkeeping to keep 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 minutes, 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.

17.5.1.4 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 six 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, 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 three 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.

17.5.1.5 Passaging *Toxoplasma* Tachyzoites/Bradyzoite Cysts in Animals

- 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 three days later (for type I strains) and five 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 neutrophils) will also be seen in this lavage material.
- Passage through mice can be useful to remove microorganisms that have contaminated *T. gondii* tissue culture, provided that they cannot replicate in murine peritoneum. Anecdotal data indicate that periodic murine passage of a *T. gondii* strain passaged continuously in tissue culture helps to maintain the vigour and biologic characteristics of the strain.

17.5.2 Transfection and Stable Transformation Protocols

17.5.2.1 Transient Transfection

- Cytomix (120 mM KCl, 0.15 mM CaCl2, 10 mM K2HPO4/KH2PO4 pH 7.6, 25 mM HEPES pH 7.6, 2 mM EGTA, 5 mM MgCl2) 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 μM 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 minutes 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 minutes 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). Re-suspend DNA in 100 μl cytomix.
- Filter parasites from a freshly lysed T175 flask into a 50 ml polypropylene tube and count in a haemocytometer (dilute sample 1:10 in PBS for counting). Pellet parasites at 1500 g,

20 minutes, 4°C and re-suspend in complete cytomix to 3.3 10^7 parasites per ml (if required the parasite concentration can be increased up to eight 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 tub YFP−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.

17.5.2.2 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 depends on the vector used but 10−50 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, 1993), e.g. plasmid pDHFR*−TScABP (Sullivan *et al.*, 1999). After electroporation culture in the presence of 1 µM 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.*, 1996 for RH). Twenty-four hours after transfection add 25µg/ml mycophenolic acid (25 mg/ml stock in ethanol) and 50 µg/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 occurs (24-48 hours later). The lysed culture is forced three times through a 25-ga needle to ensure 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°C) and incubated at 37°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 microtitre plates containing HFF cells in the presence of 5 μg/ml of phleomycin.

17.5.2.3 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.*, 1995; Gubbels and Striepen, 2004).

17.5.2.4 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 haemocytometer and dilute to 250 parasites per ml.
- Add 100 μl (25 tachyzoites) to each well in the first and seventh vertical column.
- Using a multichannel pippetor perform a serial dilution from left to right transferring 100 μl at each step (mix each well by pipetting up and down three times). Discard medium after you reached column 6 and start over at row 7.
- Incubate for seven days without disturbing the culture.
- Inspect each row from left to right using an inverted microscope and identify wells that contain a single plaque and mark those wells. Expand clonal lines by passage into a T25 flask.

17.5.3 Measuring Parasite Survival and Growth

17.5.3.1 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.*, 1999). 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 1 μM pyrimethamine.
- Incubate for seven 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

flatbed 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 $\pi ab/4$ to calculate the area.

17.5.3.2 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 (Becton, Dickinson and Company) 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.

17.5.3.3 β-Galactosidase (LacZ) Assay

- This is an endpoint growth assay that can be used in multi-well 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 colour to desired intensity (if you wait too long all wells will turn red, use your negative and positive controls as a guide) and read absorbance at 570 nM. Plot end points as percent positivity as described above.

17.5.3.4 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 hours 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 hour.

- 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 hour 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.

17.5.4 Live-Cell and Indirect Immunofluorescence Microscopy

- Sterilize round 23 mm glass cover slips in 70% ethanol (or autoclave) and transfer to 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 minutes.
- Block in 2 ml 1% w/v BSA in PBS/0.25% Triton X100 for 30 minutes.
- React with primary antibody (diluted 1:100–1:5000 in PBS/BSA/0.25% Triton X100 depending on titre) 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 six-well dish (cell side up) and wash three times with 3 ml PBS (5 minutes each).
- React with secondary antibody diluted in BSA/PBS for 1 hour.
- Wash four times in 3 ml PBS (5 minutes each). To counter-stain DNA add 2 µl of a 2 mg/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 minutes 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).

17.5.5 Cytometry of Parasites and Infected Cells

Toxoplasma tachyzoites can be efficiently sorted using a fluorescence activated cell sorter (FACS) after labelling 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 *et al.*, 2004; Gubbels and Striepen, 2004). 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 multi-well plates. Using a MOFLO sorter we found three 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 re-suspend 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).

17.5.6 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 seven days and mark clones.
- Suspend parasites by pipetting up and down and transfer 100 μl of each well into a well of a six well plate. Replenish medium in the 96 well plates and keep in the incubator.
- Six well cultures will lyse within 3–4 days. Re-suspend lysed parasites by pipetting and harvest by centrifugation.
- Wash parasites with PBS and pellet again.
- Re-suspend 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 overnight).
- 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 minutes at −20°C.
- Spin for 10 minutes at full speed in a microcentrifuge, wash pellet with 70%

ethanol, spin again, briefly air dry and re-suspend 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.

17.5.7 Disruption of Essential Genes

17.5.7.1 Tetracycline Inducible Systems

As detailed above the first approach involves several 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.*, 2002) 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.*, 2002). If you omit the stop codon this should result in an N-terminal translational fusion to a c-myc epitope tag.
- Transfect into the TATi transactivator line (Meissner *et al.*, 2002), 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-myc 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 five days of treatment and then titre 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 analyse regulation of the ectopic copy in confirmed KO clones by IFA and Western blot. To facilitate double homologous recombination, vectors should preferentially be linearized by digestion at both ends of the construct to remove vector sequences. The choice of selectable marker depends on the background strain. A large variety of strains are readily available to the research community but the genetically modified strains significantly differ regarding the set resistance and sensibility to selectable marker genes.

17.5.7.2 Regulation of Protein Stability

The generation of transgenic parasites expressing ddFKBP-fusion proteins can be selected either in the presence (knock-down of an essential gene) or absence (expression of a dominant negative mutant) of Shield-1. Conditional expression is performed with 1 µM Shield-1.

17.5.8 Insertional Mutagenesis and Tag Rescue

- Electroporate tachyzoites by 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 20 μl restriction digests using several restriction enzymes that cut once in your plasmid (e.g. EcoRI, HindIII, XhoI, XbaI for pDHFR*−TScABP see Sullivan *et al*. (1999) 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 10 × 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 minutes at −20°C.
- Wash pellet with 1 ml 70% ethanol, air dry briefly, and re-suspend 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 hour at 37°C while shaking.
- Plate entire transformation onto an LB agar plate containing suitable antibiotic (in this case ampicillin).
- Tags can also be rescued by inverse PCR. See Sullivan *et al*. (1999) for primer design and a detailed protocol.

17.5.9 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 minutes.
- Add 50 l ENU to flask A and 50 μl sterile tissue culture grade DMSO to flask B.
- Treat for 4 hours at 37°C.
- Wash cultures three 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 1500 × g at 4°C for 20 minutes.
- Re-suspend 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.

17.5.10 Complementation Cloning using the *Toxoplasma* Genomic Libraries

- Prepare 50 large and 10 small LB−agar Petri dishes (10 μg/ml Kanamycin).
- To titre the ToxoSuperCos library prepare five 1.5 ml Eppendorf tubes with 135 μl LB (no antibiotics), one with 1 ml LB and one 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 at 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 tabletop 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 instructions, re-suspend DNA pellet in 150 µl TE per column and store cosmid DNA at 4°C in the dark.
- To complement *T. gondii* mutants perform five 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.
- 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.

17.5.11 Recombineering Cosmids of *Toxoplasma* Genomic Libraries

- Find a cosmid that covers your gene (using http://www.toxodb.org), and identify the corresponding bacterial clone number (using http://toxomap.wustl.edu/cosmid.html).
- Prepare the cosmid from an overnight 28–30°C culture, confirm by digest and electroporate 100–300 ng into *E. coli* strain EL250 (electroporate in 1 mm gap cuvette at 1.75 kV, 250Ω, and 25 µF).
- Induce the λ phage recombination machinery in a fresh 100 ml culture of EL250 containing cosmid (grown from 2 ml overnight culture at 28–30°C to OD = 0.4) by immediately transferring it to 43°C and shaking 20 minutes at 100 rpm, following 20 minutes cooling in ice-water.
- Use the cooled culture to make competent cells by three consecutive washes in ice cold sterile ddH2O (in 50, 20 and 3 ml, centrifugations at 4000 rpm, 10 minutes at 4°C). Re-suspend the competent pellet in 600 µl 10% sterile glycerol and aliquot 50 µl into ice-cold sterile microfuge tubes for storage at −80°C.
- PCR amplify a modification cassette (see Fig. 17.5 and Table 17.2 below on how to design your desired manipulation) from 0.1 to 50 ng of plasmid template.
- Use 100–300 ng gel purified targeting cassette to electroporate (same as above) to one 50 µl aliquot, rescue in SOC media at 28–30°C for one hour and plate on gentamycin + kanamycin to select for recombineering.

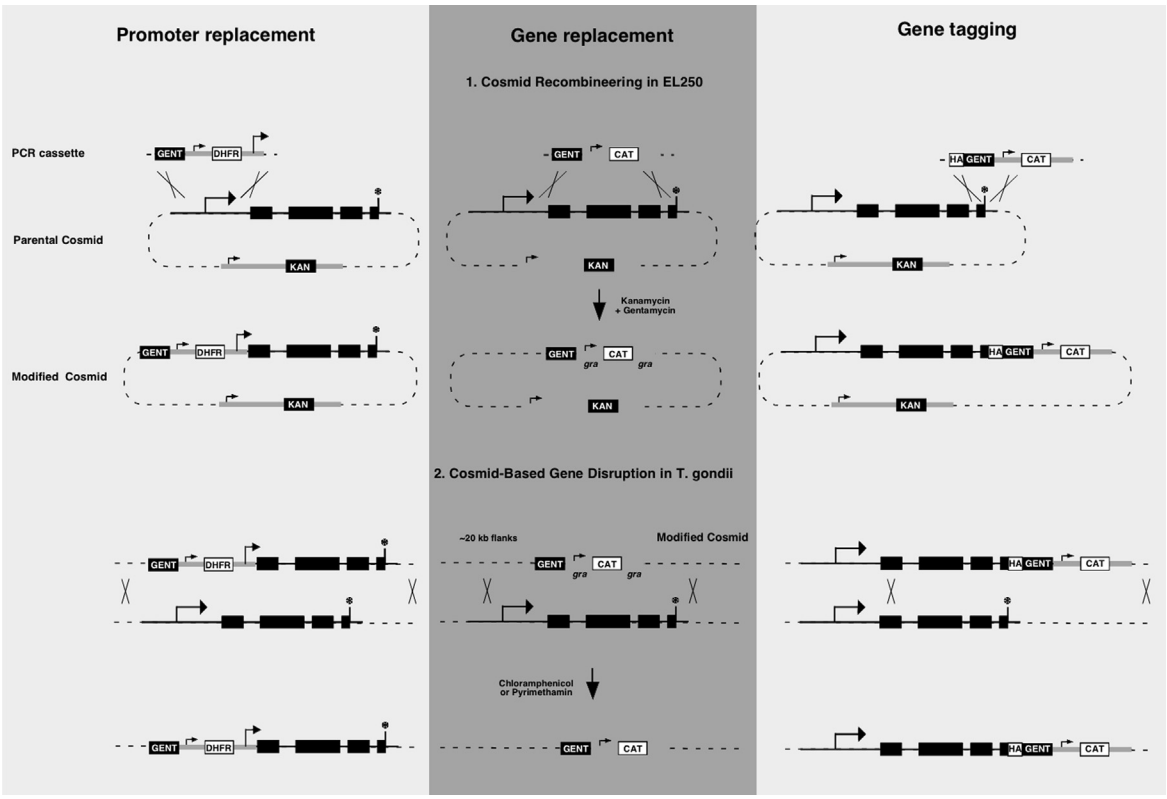

FIGURE 17.5 **Using cosmid recombineering to modify GOI.** Schematic representation of the three available strategies for cosmid modification: promoter replacement, gene replacement and C-terminal gene tagging. The PCR cassette and recombination even into the cosmid are depicted on the top of each panel as Step 1 with the resulting modified cosmid. Step 2 demonstrates the recombination into the genome using each modified cosmid (here shown as linear) and the resulting modified locus.

TABLE 17.2 Primers for PCR Amplification of Cosmid Modification Cassettes

Type of Modification	Toxoplasma Selectable Marker	F Primer	R Primer
Promoter replacement	DHFR	50 bp GOI at the 5′ of the promoter + GAATGGTAACCGACAAACGCGTTC	GCTTTCGTCTGTCTTCAACCAGATCT + 50 bp GOI just upstream of ATG
Gene replacement	BLE/CAT	50 bp GOI upstream of start codon + CCTCGACTACGGCTTCCATTGGCAAC	50 bp GOI downstream from stop codon + ATACGACTCACTATAGGGCGAATTGG
HA tagging	BLE/CAT	50 bp of GOI upstream of stop codon + AGGTACCCGTACGACGTCCCGGACTAC	50 bp GOI downstream from stop codon + ATACGACTCACTATAGGGCGAATTGG

17.5.12 Safety Concerns Working with *T. gondii*

Several aspects of the parasite's biology make working with *T. gondii* relatively safe. In immunocompetent persons the infection usually produces 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 tachyzoite 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 harbour 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 clean-up. Needle sticks are the most common source of laboratory infections. The safest approach 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 millimetres 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, sulphadiazine, 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.

Acknowledgements

Work in our laboratories is currently funded by grants from the National Institutes of Health (to Boris Striepen) and from the Wellcome Trust, Swiss National Foundation and Howard Hughes Medical Institutes (to Dominique Soldati-Favre). Boris Striepen is a Georgia Research Alliance Distinguished

Investigator. Dominique Soldati-Favre is supported by the Swiss National Foundation (FN3100A0-116722) and Damien Jacot by the Indo-Swiss Joint Research Programme. We thank Louis Weiss for providing protocols for propagation of parasites in mice. We thank all current and former members of our laboratories and many investigators in the field for their protocols and discussion.

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