Experimental Parasitology 180 (2017) 19–26

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Full length article

Toxoplasma gondii plaque assays revisited: Improvements for ultrastructural and quantitative evaluation of lytic parasite growth

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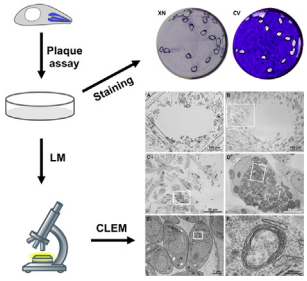
HIGHLIGHTS

- Protocol for correlative light and electron microscopy (CLEM) of single *T. gondii* plaques provided.
- Comparison of 'positive' and 'negative' plaque staining methods.
- Description of ImageJ toolset for plaque size measurement.
- Chemically defined serum-free medium supports superior parasite growth during plaque assays.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 11 October 2016
Received in revised form
3 December 2016
Accepted 18 December 2016
Available online 21 December 2016

Keywords:
Plaque assay
Apicomplexa
Replication
Histochemical staining
Correlative light and electron microscopy

ABSTRACT

Lytic growth of intracellular *Toxoplasma gondii* tachyzoite stages over a period of days results in plaques within mononolayers of host cells. Plaque assays are in frequent use to isolate single clones and to investigate invasion, replication and egress over a longer time frame. To allow correlating plaque morphology and/or size with ultrastructural examination of individual parasites we introduce a simple protocol for correlative light and electron microscopy (CLEM) of entire plaques. We also illustrate the advantages of visualizing only the boundaries of plaques by staining for infected cells ('positive staining') rather than the traditional staining of the intact cell monolayer, thus outlining the area of lysed cells ('negative staining'). Tachyzoites expressing β-galactosidase of *Escherichia coli* are an easy to visualize histochemical marker for this purpose. Quantitative measurements of plaque area with our compiled user-friendly ImageJ macros are compared to commercial software for ease and shown to be more accurate for some applications. Finally, a chemically defined medium is shown to be superior to the fetal bovine serum-containing medium for plaque assays, resulting in larger plaques. The reported additions and changes of the plaque assay procedure offer improved ways to analyze subtle differences in invasion, pathogen growth and egress. Our chemically defined medium will improve standardization of e.g. drug screening assays.

Abbreviations: βGal, β-galactosidase; CI, confidence interval; CLEM, correlative light and electron microscopy; CV, Crystal Violet; dDMEM, chemically defined growth medium; DMEM, Dulbecco's Modified Eagle's Medium; EM, electron microscopy; FBS, fetal bovine serum; lacZ, β-galactosidase gene from *E. coli*; LM, light microscopy; NTB, Nitrotetrazolium blue chloride; TEM, transmission electron microscopy; X-Gal, 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside; XN, X-Gal/ Nitrotetrazolium blue chloride staining solution.

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http://dx.doi.org/10.1016/j.exppara.2016.12.015
0014-4894/

1. Introduction

Plaque assays are based on the ability of an intracellular replicating agent to lyse its host cell within a monolayer and subsequently infect neighboring cells so that over time (days) a macroscopically visible hole in the cell monolayer becomes apparent. Established in animal virology to quantitate virus titer or isolate individual particles decades ago (Dulbecco and Vogt, 1954) it was introduced later for similar purposes in research of the intracellular apicomplexan parasite *Toxoplasma gondii* (Chaparas and Schlesinger, 1959; Foley and Remington, 1969; Pfefferkorn and Pfefferkorn, 1976). Only recently was a plaque assay also described for the blood stages of the related *Plasmodium falciparum* (Thomas et al., 2016).

This assay is still one of the major methods to obtain clones of new parasite isolates or to study the growth behavior of genetically or chemically generated mutants compared to wild-type tachyzoites (Roos et al., 1994). Its major advantage over other in vitro methods that report parasite growth, like cell staining and counting, ^{3}H-uracil incorporation into parasite DNA (Pfefferkorn and Pfefferkorn, 1977) or enzymatic assays reflecting parasite numbers (e.g. GFP, β-galactosidase, luciferase, (Dube et al., 2009)) is its ease of setup and low hands-on time. In addition, it also monitors the repeated processes of the lytic cycle (i.e. invasion – replication – egress). This is done over the course of 5–7 days, which also allows detection of subtle defects in any of those processes that would not be visible in shorter growth assays.

Direct observation of such plaques at the ultrastructural level by electron microscopy (EM) would be advantageous. However, with conventional protocols direct EM observations are difficult to obtain since in traditional protocols monolayers are scraped off the culture vessel's surface and subsequently sedimented by centrifugation prior to processing for EM (Dubremetz and Ferguson, 2009; McGovern and Wilson, 2013). This results in the loss of the overall plaque morphology and subsequently, due to the inherent low number of parasites per plaque assay, also in difficulties to find infected cells to examine.

Traditionally, plaques have been visualized by staining of the remaining cells with unspecific dyes like crystal violet (CV), resulting in a clear zone within the otherwise intact monolayer due to cell lysis when viewed against a white background (here called 'negative staining'). However, a disadvantage of this simple method is that the actual proliferative zone of tachyzoites within the infected cells at the rim of the plaque is not stained, which might result in loss of some useful information. Alternatively, staining for the parasites within those cells will create a visible outline of the plaque in an otherwise clear (non-stained) monolayer ('positive staining'). The advantage is that mechanical monolayer destruction during handling or otherwise caused cell death can be clearly distinguished from parasite-induced cell lysis.

Here we describe experiments that aim to address both issues. We introduce a simple protocol for correlative light and electron microscopy (CLEM) of entire plaques that allows correlating plaque morphology and/or size with ultrastructural examination of individual parasites. Moreover, we have used *T. gondii* tachyzoites expressing lacZ of *Escherichia coli* (Seeber and Boothroyd, 1996) for positive staining and demonstrate the distinctive advantage of using this marker in several situations. During the course of these experiments we also found that a chemically defined medium is superior to medium containing fetal bovine serum (FBS) for plaque assays, resulting in larger plaque areas. For quantitative measurements of such plaques user-friendly ImageJ macros were written and compared to commercial software for ease and accuracy.

2. Material and methods

2.1. Parasite strains and cell culture

T. gondii strain RHβ1 expressing the *E. coli* lacZ gene under the tachyzoite-specific SAG1 promoter has been described previously (Seeber and Boothroyd, 1996), as has been strain RHΔKU80 (Fox et al., 2009). They were maintained in DMEM plus 10% FBS (here called FM) and stable glutamine (Biochrom, Germany) in human fibroblasts immortalized with hTERT (BJ5ta; ATCC CRL-4001).

2.2. Plaque assays

For plaque assays, confluent monolayers in 12-well plates (TPP, Trasadingen, Switzerland) were infected in FM with various numbers (25–75) of tachyzoites/well that had been freshly released from infected cells by passing through a 21G needle and microscopically counted. After 2 h wells were washed once with PBS which was then replaced with either FM or defined DMEM (dDMEM). The latter consists of DMEM where FBS was replaced with a chemically defined substitute consisting of 0.5 mg/ml fatty acid-free BSA (Sigma-Aldrich), 1× Lipid Mixture I (Sigma-Aldrich), 5 μg/ml holo-transferrin (Sigma-Aldrich), 10 μg/ml bovine insulin (Sigma-Aldrich), 1 μM lipoic acid, and the following trace elements: 0.5 nM $MnCl_2$ x4$H_2$0; 0.5 nM $(NH_4)_6Mo_7O_{24}$ × 4H_2O; 0.25 nM $NiSO_4$ x6H_2O; 250 nM Na_2SiO_3 x9H_2O; 0.25 nM $SnCl_2$; 2.5 nM Na_3VO_4; 1 nM $CuSO_4$ x5H_2O; 125 nM $ZnCl_2$; 10 nM Na_2SeO_3 (all final concentrations) (Crawford et al., 2006). The complete medium should be used within a few weeks.

Plates were then incubated at 36.8 °C/5%CO_2 for 5–7 days within a dedicated incubator to avoid its frequent opening during routine cell culture and thereby shaking of plates, which can lead to secondary plaques (i.e. freshly released tachyzoites do not invade the neighboring host cells but are transported further away, giving rise to further smaller plaques).

2.3. Plaque staining procedures and documentation

Histochemical βGal activity staining was performed as described (Seeber and Boothroyd, 1996), with the following adaptations. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature, then washed with PBS for 10 min before it was exchanged for the activity staining solution consisting of PBS containing 1.3 mM $MgCl_2$, 0.075% Nitrotetrazolium blue chloride (NTB), 0.02% 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) and 0.02% Triton X100 (Gossrau, 1978), called XN herein. The resulting black-blue precipitate provides better contrast for imaging than the blue X-Gal-ferricyanide precipitate. The reaction was terminated by removing the staining solution, washing in tap water, followed by 5 mM EDTA in 1 mM NaOH for 15 min.

Crystal violet (CV) staining solution consisted of 0.2% dye (Fluka) in 2% ethanol. Wells were filled with 500 μl CV solution, incubated for 30–60 min at room temperature before wells were then washed several times with tap water.

Plates were emptied, air-dried and photographed in RGB mode using a consumer-type digital camera (Canon Powershot G10) at the highest possible resolution. Plates were illuminated by an LED white light box (Lightpad A930; Artograph, USA). Higher magnification images were acquired with a Zeiss Observer Z1 microscope equipped with a Zeiss AxioCam ERc 5s camera.

Images were analyzed using the following softwares: Photoshop CS4 (Mac) and CS6 (Windows) - Adobe, USA; Affinity Photo 1.4 (Mac) - Serif Europe Ltd., UK; ImageJ 1.49u (https://imagej.nih.gov/ij/); Fiji 1.51d (http://fiji.sc/). Statistical analyses and data representation were performed with GraphPad Prism 5.04.