Sub-micrometer ultra-short pulse laser surgery in *Theileria*-infected cells

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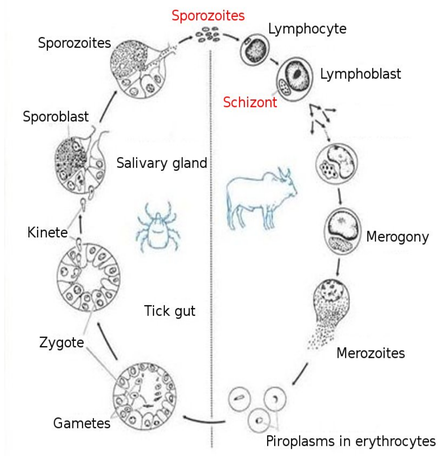
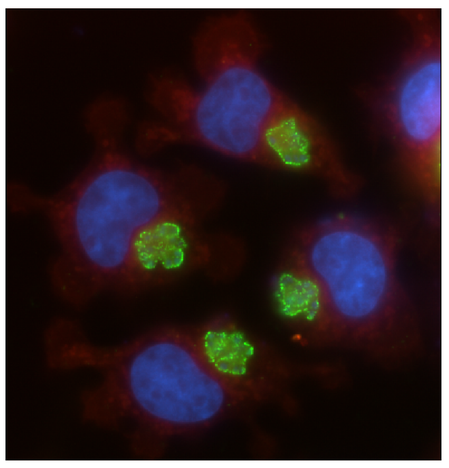
Introduction

The protozoan parasite *Theileria* infects lymphocyte cells after it is injected by a tick into a bovine host; it takes up residence in the cytoplasm of infected cells. The interaction is still poorly understood, and the ability to transfect *Theileria* would enable new studies to shed light on how *Theileria* infects and transforms its host cell, which acquires many of the properties of tumor cells.





The goal of our research is to achieve intracellular transfection of the schizont stage of the parasite's life cycle. Transfection has been achieved for *Theileria* sporozoites, but in order to study *Theileria* in the sporozoite stage, elaborate facilities to house both infected calves and the ticks that transmit the parasite are required[1].
Femtosecond laser ablation has been used to transfect single mammalian cells, and we seek to extend this technique to the transfection of the intracellular parasite.

Fluorescence image of fixed, stained Theileria infected macrophages. Blue: host cell nucleus, red: host cell membrane (nile red), green: *Theileria* parasite.

Ultrashort pulse laser ablation

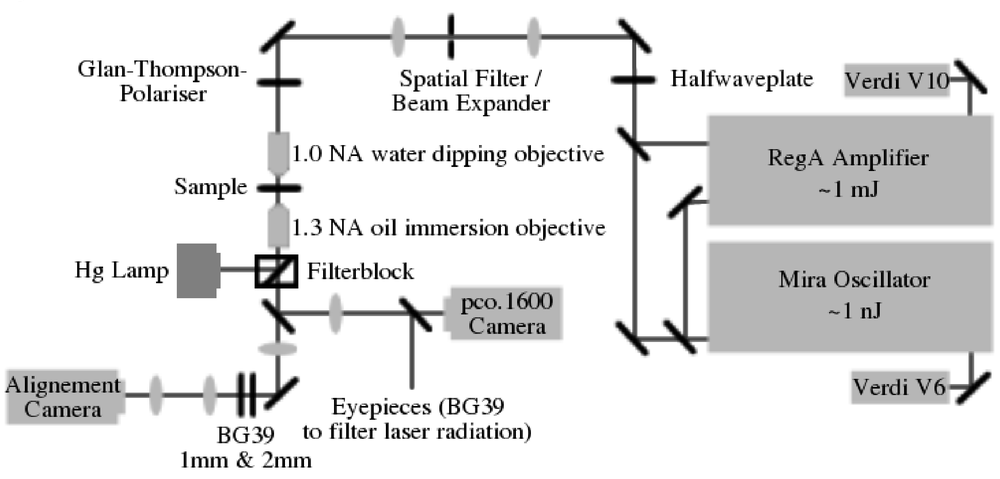
Advantages:
- nonlinear interaction occurs only in the focal volume where intensity is high enough
- damage is tightly confined to the focal volume
- no significant heating and associated thermal damage

Two possible ablation modes[2]:

high repetition rate	low repetition rate
Ti:Sapphire-Oscillator	Ti:Sapphire-Amplifier
≳ 1 MHz	< 1MHz
of order 1 nJ/pulse	of order 100 nJ/pulse
of order 10 GW/cm²	of order 10 TW/cm²
multiple shots	single shot
photochemical effects due to free e^-, no cavitation bubble	mechanical damage due to cavitation bubbles

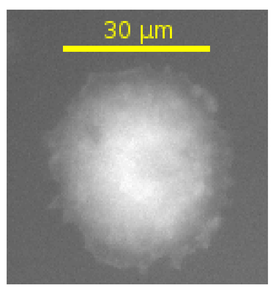
Setup

Both a Mira oscillator (λ=800 nm, f=76 Mhz, τ=180 fs) and a RegA amplifier (λ=800 nm, f=single shot to 250 kHz, τ=300 fs) were used for the experiments.
The laser was focused onto the sample through a 1.0 NA water-dipping objective. Fluorescence was collected using a 1.3 NA oil immersion objective from below. A spatial filter / beam expander was used to obtain a tightly focusable Gaussian beam.



Transfection of host cells

Macrophages were kept in culture medium to which plasmids coding for green fluorescent protein were added. Holes in the cell membrane were made using ultrashort pulse laser ablation (4.8 TW/cm², single shot). This resulted in transfection of the cells and subsequent expression of green fluorescent protein.



macrophage one day after laser treatment; cell expressed GFP

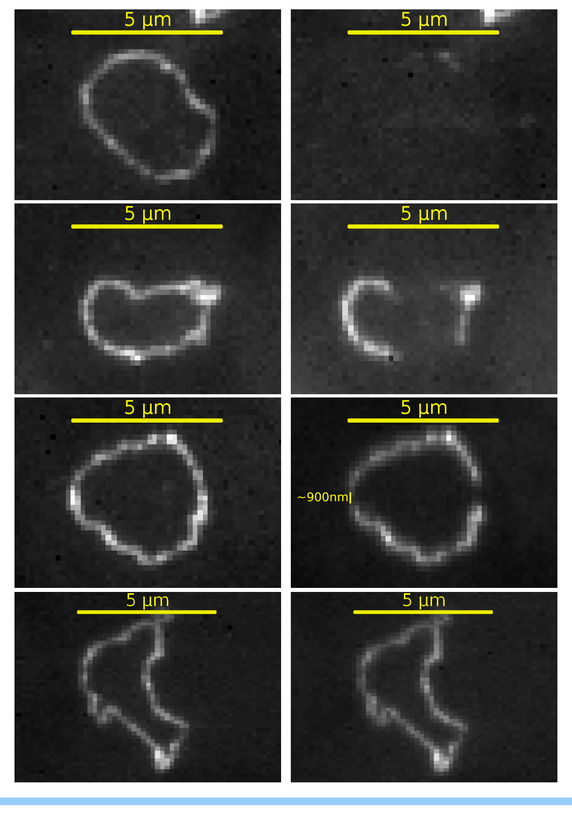
Transfection of *Theileria* parasite

Challenges:
- perforation of a membrane inside a living cell without compromising cell viability (requires finding a location on parasite membrane where collateral damage to cell is minimized)
- overcoming of restricted movement of DNA within the cell due to cytoskeleton[3]
- visualization of parasite inside the cell (fluorescence microscopy)
- achievement of transfection despite short lifetime of foreign (plasmid) DNA in cytoplasm[4]

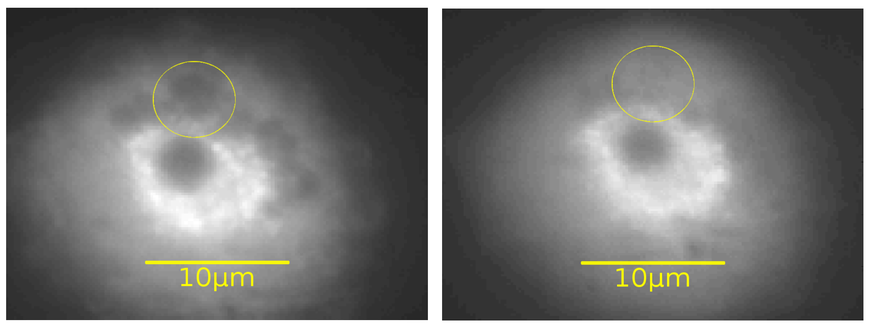
Stained sections

The effect of single shots with the amplified laser was measured. The laser focus was moved over the membranes of fixed sections of *Theileria* parasites, with a shot every 0.5 µm. (Spot size ~300nm)

The irradiance must be kept near the ablation threshold (~24 TW/cm²) in order to obtain precise ablation in the membrane and to avoid damaging the parasite (and the host cell).



Perforation of the *Theileria* membrane

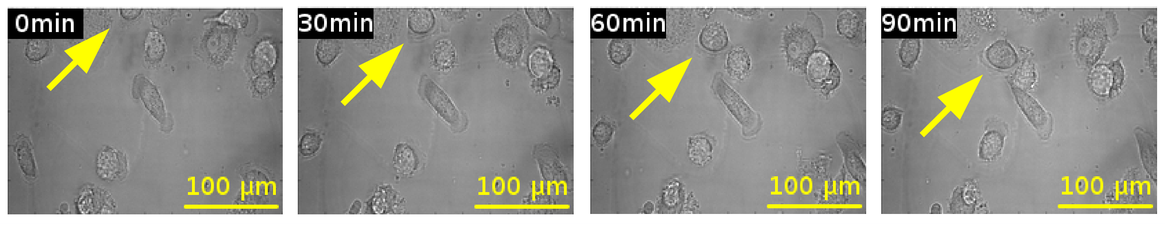


left image: *Theileria* parasite inside a macrophage, before laser treatment
right image: same cell after laser treatment; parasite was filled with GFP

Perforation of the membrane of a *Theileria* parasite (using 46 GW/cm² oscillator pulses for 2 s) leads to filling of the parasite with green fluorescent protein without compromising cell viability. Membrane perforation could be achieved using 1 ms (100 GW/cm²) to 5 s (20 GW/cm²) exposure times. Currently we are working on transporting DNA plasmids into the parasite.

Moving cells

The macrophages move with a speed of up to 50 µm/hr at room temperature in the culture dishes, making it difficult to track treated cells overnight. (Expression of green fluorescent protein in transfected parasites may require 24 hrs or longer.)



Outlook

Work to transfect the *Theileria* parasite in its schizont stage is currently in progress. The movement of DNA plasmids longer than 250 kbp is probably hindered by the cytoskeleton and this is likely the reason why our transfection experiments have not yet been successful. CytochalasinD can be used to depolymerize the actin cytoskeleton, allowing the DNA to move more freely. Additionally, the lifetime of foreign DNA in cytoplasm is limited[4], requiring experiments to be carried out shortly after injection of plasmids into the cytoplasm.

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