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Generation of transgenic *Leishmania donovani* expressing the Red Fluorescent Protein mRFP1: a tool for *in vivo* imaging of the parasite-host interaction

Maria Kotini [a&], Amalia Papadaki [a], Despina Smirlis [a], Ketty Soteriadou [a], Giorgos Diallinas [&], Haralabia Boleti [ab]

[a]Molecular Parasitology Laboratory and [b]Light microscopy Unit Hellenic Pasteur Institute, Vas. Sofias 127, Athens, 11521-Greece
[&]Biology Dep. of Kapodistrian University of Athens, Botany Department

Introduction

The parasites of the genus ***Leishmania*** are protozoan organisms of the *Trypanosomatidae* family. When transmitted to the mammal host by the bite of the insect host (sandflies of the genus *Phlebotomus* or *Lutzomyia*) cause a wide spectrum of diseases called Leishmaniases, the most serious of which is visceral Leishmaniasis (kala azar) which if left untreated is fatal. The *Leishmania* parasites exist in two forms, the promastigote which lives in the invertebrate host and the amastigote that propagates in the macrophages of the vertebrate host (***Fig.1***).

The **Monomeric Red Fluorescent Protein** (mRFP1) is derived from the tetrameric DsRed by random and directed mutagenesis. The mRFP1 consists of 226 aa and has the typical beta-barrel structure of the fluorescent proteins. The chromophore is protected in the interior of the molecule by the beta - sheets of the barell (***Fig. 2***). The mRFP1 is excited at 587 nm and emits at 607 nm. Like other red fluorescent proteins that are excited between 550-650 nm is ideal for *in vivo* imaging in deep tissues of experimental animals.

Aim of this project was to generate transgenic parasites *Leishmania donovani (L.donovani)* expressing mRFP1 to use in experiments of *in vivo imaging* of infection at the cellular and the whole animal level (mouse model of Leishmanisis infection) by fluorescence microscopy.

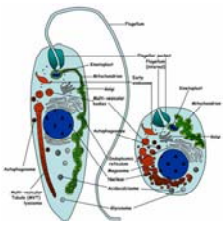


Fig. 1. *Promastigote (on the left) and amastigote (on the right) forms of Leishmania.*

Fig. 2. *mRFP1 structure. Blue color indicates the chromophore in the core of the molecule*

Methodology-Results

1 Constuction of the plasmid pLexsy-sat-mRFP1

The mRFP1 gene was amplified by PCR from the pRSET-b-mRFP1 plasmid (***Fig. 3***) using as primers the

5' oligo : 5'-GAAGATCTATGGCCTCCTCCGAGGACG-3' and

3' oligo : 5'-GGCCTCGAGTCAAGCTTCGAATTCTTAGGC-3' Sunsequently the mRFP1 PCR product was inserted in the vector pLexsy-sat (***Fig. 5***) which is spesific for expression in protozoan parasites of the Trypanosomatidae family. Insertion was realised after digestion of the PCR product (***Fig. 4***) and the vector with the Bgl II & Xho I restriction enzymes and ligation of the digested DNA fragments (***Fig. 6***).

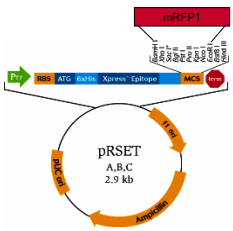


Fig. 3 *pRSET-b plasmid with the mRFP1 gene inserted in the BamHI & EcoRI sites*

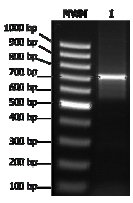


Fig. 4 *PCR product (1)*

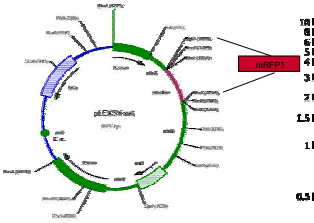


Fig. 5 *pLexsy-sat plasmid with mRFP1 insert*

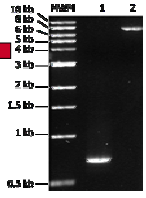


Fig.6 *Insert mRFP1 (1); Vector pLexsy-sat (2)*

2. Generation of L.donovani - mRFP1 parasites

Transgenic parasites *L.donovani - mRFP1* were generated by electroporation with the pLexsy-sat-mRFP1 plasmid and incubation in selection medium consisting of RPMI + 10% FBS + Hepes (100μg/ml) antibiotic nurseothricin (100μg/ml) at 26ºC. Positive clones were selected 3 weeks later and mRFP1 expression was verified by observation under fluorescence microscope, by Western Blot Analysis (***Fig. 7***) and by FACS (***Fig. 10***).

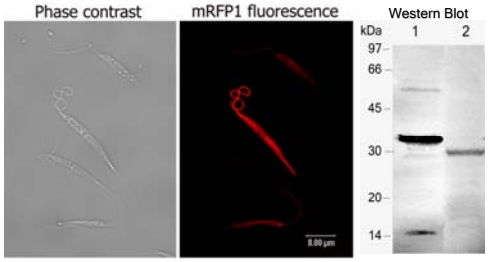


Fig. 7 & Fig 8 *Transgenic parasites L.donovani-mRFP1. Pictures from Confocal microscope (left). Western Blot analysis (on the right).[1: bacterial extracts;2: Leishmanial extracts].*

3 Characterization of the transgenic L.donovani parasites

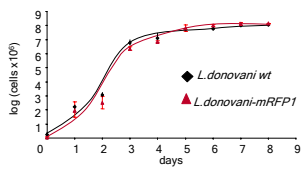
A. *Detection of mRFP1 in L.donovani-mRFP1 by Western Blot*

A specific anti-mRFP1 polyclonal Ab was raised in a rabbit after immunization with 6His-mRFP1 protein produced in bacteria. The affinity purified antibody was used to detect mRFP1 in whole cell extracts from *L.donovani-mRFP1.* In ***Fig. 8*** extracts from bacteria producing 6-His-mRP1 and from *L.donovani-mRFP1* were analysed side by side in a 12%(w/v) SDS-PAGE. The nitrocellulose filter was probed with 0.5 μg/ml affinity purified anti-mRFP1 antibody.

B. *Comparison of growth characteristics between transgenic parasite L.donovani-mRFP1 and the original strain LG13 L.donovani*

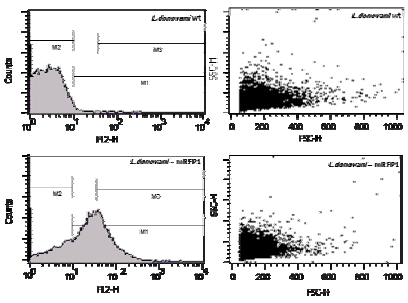
Parasite cultures of both the transgenic and the wild type strains were seeded at $1x10^6$ parasites/ml. They were enumerated by heamocytometry every 24 hrs for a period of 8 days (***Fig. 9***). The *L.donovani-mRFP1* parasites have similar growth characteristics as the wt strain LG13.

Fig. 9 *Growth curve*



C. *Analysis of transgenic L.donovani-mRFP1 parasites by FACS*

Parasites at the logarithmic phase of growth were analysed by FACS for morphological characteristics (FSC and SSC) and for Red Fluorescence (FL2) (***Fig.10***). The transgenic parasite strain is a mixed population of cells expressing varying amounts of the fluorescent protein but with similar morphological characteristics as the wt parasites.



L.Donovani wt	Events (cells)	% total	Mean fluorescence
total	20000	100.00	2,84
M1	152	0.76	27,74
M3	27	0.14	93,35
***L.donovani*-mRFP1**			
total	20000	100.000	36.02
M1	14999	75.00	46.46
M3	6759	33.79	75.98

Fig. 10. *Analysis of L.donovani-mRFP1 by FACS*

4 Phagocytosis of L.donovani-mRFP1 by Raw264.7 mouse macrophages

Raw264.7 macrophages expressing transiently actin-GFP were incubated for 30 min with *L.donovani-mRFP1* and were subsequently fixed by 4% w/v PFA and analysed by confocal microscopy (Leica TCS-SP).

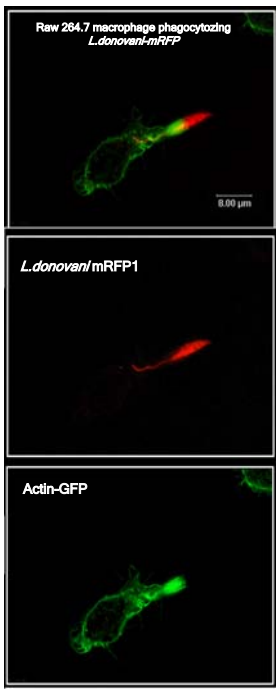


Fig. 11 *Confocal microscopy images of Raw 264.7 macrophase phagocytozing an L.donovani-mRP1 parasite. Top panel :merged image of the red and green fluorescence*

5 Survival of L.donovani-mRFP1 in Raw264.7 mouse macrophages

Raw264.7 cells were infected by overnight incubation with *L.donovani-mRFP1* parasites at a parasite:macrophage ratio 20:1. The next day the parasites were removed, fresh medium was added and the infected macrophages were further incubated up to 72 hrs. They were subsequently fixed by 4% w/v PFA, stained for the FCγ receptor with mouse IgG and analysed by confocal microscopy (Leica TCS-SP). Leishmanias were found intact (arrows) in several macrophages.

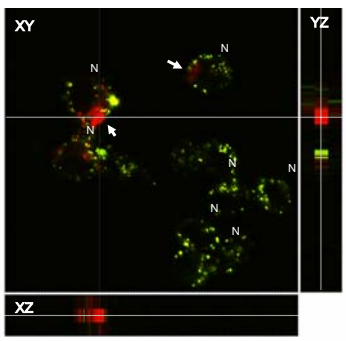


Fig. 12 *Confocal microscopy images. **Red:** L. donovani mRFP1. **Green:** Fcγ-receptor stained with mouse IgG and anti-mouse Alexa488. The arrows indicate the mRFP1-parasites inside macrophages. YZ and XZ sections of a 3 D image reconstructed out of 6 optical sections (1 μm thick) are presented on the right and at the bottom of the image. **N**: indicates the nuclei of the macrophages.*

Conclusions-Future perspectives

We have generated a transgenic *L.donovani-mRFP1* strain that expresses mRFP1 at high levels, has similar growth and morphological characteristics with the wt strain LG13 and is capable of surviving in tissue culture macrophages 72 hrs post infection. *The L.donovani-mRFP1 parasites* constitute a valuable cellular tool for *in vivo* imaging of parasite-host interaction at the cellular and tissue/animal level using Fluorescent Microscopy.