

## Laser Capture Microdissection of Hepatic Stages of the Human Parasite *Plasmodium falciparum* for Molecular Analysis

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### Summary

Despite the sequencing of parasite genomes and development of DNA microarray technology, gene profiling of parasites remains a difficult task. For example, transcriptome analysis cannot currently be applied to the hepatic stages of the malaria parasite *Plasmodium falciparum* due to difficulties in obtaining sufficient amounts of parasite material that lies among the large excess of host cell RNA. Here, we describe the isolation of *P. falciparum*-infected human hepatocytes by a laser capture microdissection approach. Reverse transcriptase polymerase chain reaction amplification of several *P. falciparum* transcripts demonstrates the high quality of the RNA recovered after microdissection. This approach should enable analysis of *P. falciparum* transcriptome during its hepatic development, a major step toward the identification of new therapeutic and vaccine targets.

**Key Words:** Gene expression; parasite; laser capture microdissection; *Plasmodium falciparum*.

### 1. Introduction

During the past few years, many parasite genomes have been completely sequenced and others are in progress. This sequence information, in combination with new large-scale genome analysis technologies, provides important tools for molecular studies such as gene expression profiling. However, in the field of parasitology, in the context of a complex background of host nucleic acids, such approaches remain difficult to perform because of the difficulty in isolating the parasite from its host to obtain parasite material pure enough to be

analyzed. This problem is strengthened when studying an intracellular parasite. For example, *Plasmodium falciparum*, the malaria parasite that kills more than one million children every year, invades and develops inside the hepatocytes before setting cycles of erythrocytic development associated with the clinical symptoms. Hepatic stages are important targets for vaccine-induced protective immunity and prophylactic treatment (1). However, little is known about the development of the parasites inside the liver. Indeed, molecular analysis, such as a DNA microarray approach, is hampered by the difficulty of isolating parasites from the host cells and by their low density either inside the liver or in hepatocyte cultures. Resulting preparations contain a very low proportion of nuclear material originated from the parasite. Therefore, it is necessary to enrich preparations for infected hepatocytes before molecular analysis.

Laser capture microdissection (LCM) technology appears to be one of the most appropriate approaches to isolate infected cells while saving nuclear material, notably RNA (2,3). In parasitology research, few papers have used the LCM approach. One publication does concern the isolation of mouse brain vessels sections to detect gene expression induced by *P. berghei* infection (4); another one demonstrates the feasibility of LCM on tissue sections from *P. yoelii* infected mouse liver for gene expression studies (5). In contrast to rodent malaria, which can be investigated *in vitro* and *in vivo*, most of the studies concerning the human plasmodium, *P. falciparum*, hepatic stages need to be performed in cultures. We have recently applied LCM technology to cell cultures in order to isolate *P. falciparum*-infected hepatocytes, the aim being to obtain RNA preparations enriched in parasite material (6).

## 2. Materials

1. HEPES buffer, pH 7.6: 8 g/L NaCl, 0.2 g/L KCl, 0.1 g/L Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, 2.38 g/L HEPES.
2. Collagenase D (Roche, Meylan, France).
3. Percoll.
4. Collagen I (Beckton-Dickinson, Franklin Lakes, NJ).
5. Lab Tek glass slides (Nalge Nunc International, Cergy Pontoise, France).
6. Williams medium E (Life Technology, Cergy Pontoise, France).
7. Fetal calf serum (Life Technology).
8. L-glutamine (Life Technology).
9. Sodium pyruvate (Bio-Whittaker, Miami, FL).
10. Insulin (Sigma, Steinheim, Germany).
11. 5-Fluorocytosin (Roche).
12. Penicillin (Life Technology).
13. Streptomycin (Life Technology).
14. Dexamethasone.

15. Methanol.
16. Mouse antibodies directed against the *P. falciparum* heat-shock protein 70 (gift from D. Mattei, Institut Pasteur, France).
17. FITC-conjugated goat anti-mouse immunoglobulin (Sigma).
18. RNase inhibitor (Promega, Madison, WI, USA).
19. Ethanol.
20. Xylene.
21. PixCell II LCM system equipped with a fluorescent microscope (Arcturus Engineering, Mountain View, CA).
22. Cap (CapSure™ TF-100).
23. Sterile 0.5 mL microcentrifuge tubes.
24. Micro RNA isolation kit (Stratagene, La Jolla, CA).
25. Sensiscript Reverse Transcriptase kit (Qiagen).
26. Fast-start Taq polymerase (Roche).
27. dNTPs.
28. Oligonucleotide primers.
29. Agarose and DNA electroporation equipment.

### 3. Methods

The following methods describe (1) hepatocyte infection, (2) parasite labeling and microdissection, and (3) RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR).

#### 3.1. Hepatocyte Infection

Descriptions of (1) the isolation and culture of human hepatocytes and (2) the infection with *P. falciparum* sporozoites are given below.

##### 3.1.1. Human Hepatocyte Culture

1. Primary cultures of human hepatocytes are isolated from liver segments using the two-step enzymatic perfusion technique (7). The hepatic fragments are successively perfused with HEPES buffer to eliminate all blood and with 0.05% collagenase D in HEPES buffer with 0.75 mg/mL CaCl<sub>2</sub> to dissociate the hepatocytes.
2. Viable cells are isolated by centrifugation at 800g on a 36% Percoll phase. Cells are seeded at a density of  $1.4 \times 10^5$  per cm<sup>2</sup> on a 16-chamber Lab-Tek glass slide (see **Note 1**) coated with collagen I (2 h with 100  $\mu$ L/well of a 50  $\mu$ g/mL solution in 0.02 N acetic acid) and incubated at 37°C in 4% CO<sub>2</sub> atmosphere.
3. Hepatocytes are cultivated in Williams medium E with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mg/L insulin, 2.5  $\mu$ g/mL 5-fluorocytosin, 200 U/mL penicillin, and 200  $\mu$ g/mL streptomycin. After adherence of the cells, culture medium is replaced by fresh medium supplemented with 10<sup>-7</sup> M dexamethasone.

### 3.1.2. *P. falciparum* Sporozoite Isolation and Hepatocyte Infection

1. *Anopheles stephensi* adult females were infected with the NF 54 strain of *P. falciparum*, using a membrane-based feeder system (8).
2. After 14–21 d, mosquitoes are killed and their salivary glands are aseptically dissected and disrupted by trituration in a glass tissue grinder. Sporozoites are counted in a KovaSlide® chamber and diluted in culture medium.
3. Hepatocytes are inoculated with  $2 \times 10^5$  sporozoites per well and incubated at 37°C in 4% CO<sub>2</sub> atmosphere for 3 h and washed three times in complete medium. Finally, fresh complete medium is added and renewed every day (9).

### 3.2. Parasite Labeling and Microdissection

Infected hepatocytes are (1) labeled with an antibody directed against the heat-shock protein 70 (HSP-70) (10), and (2) microdissected by laser capture.

#### 3.2.1. Parasite Labeling

1. At appropriate times after inoculation, rinse the cultures three times in 1X PBS. Fix for 5 min in methanol and wash again three times in 1X PBS.
2. Incubate for 30 min at 37°C with a monoclonal antibody, directed against *P. falciparum* HSP-70, diluted 1/500 in PBS and containing 400 U/mL of RNase inhibitor (see Note 2).
3. Wash three times in PBS.
4. Incubate in a FITC-conjugated goat anti-mouse immunoglobulin, diluted 1/100 in PBS with RNase inhibitor, for 20 min at 37°C.
5. Wash three times in PBS.

#### 3.2.2. Laser Capture Microdissection

To perform an efficient microdissection, it is essential to have the driest sample possible. Cultures are dehydrated through a freshly prepared series of increasing concentrations of ethanol.

1. Incubate first with 70% ethanol for 30 s, then incubate two times in 95% ethanol for 1 min each and two times for 1 min in 100% ethanol. Clear slides in xylene by incubating two times 10 min, and finally air-dry in the hood.
2. Without delay, proceed to microdissection using the PixCell II LCM system. Set up the microdissector to 90 mW of laser power and 15 µm diameter laser beam (see Note 3).
3. Put down the cap on the slide that has been inserted in the microdissector. Infected hepatocytes are distinguished from uninfected ones by the fluorescence of the parasites. Ensuring the attachment of the parasite to the membrane of the cap will require several laser pulses (see Note 3).
4. When all fluorescent cells have been captured, lift up the cap and control for the absence of the targeted cells. Place a sterile slide on the microscope and put the cap back down on this slide to verify the presence of the fluorescent hepatocytes on the cap.

5. Lift up the cap again and place into a 0.5-mL Eppendorf microcentrifuge tube for subsequent RNA extraction.

### 3.3. RNA Extraction and RT-PCR

In order to verify the integrity of the RNA after microdissection, we extract the RNA and perform a RT-PCR using primers designed from sequences of genes known to be expressed during the liver stage: *lsa-1* (11), *lsa-3* (12), and *hsp-70* (10). The RNA extraction should be performed directly in the 0.5-mL Eppendorf tube immediately after the microdissection to avoid any RNA degradation.

#### 3.3.1. RNA Extraction

The Micro RNA isolation kit used is designed for a small number of cells (small amount of RNA). The protocol is conducted according to manufacturer's recommendations with column centrifugation. A DNase treatment (DNase I at 37°C for 15 min) is included during the extraction. RNA is eluted with 30  $\mu$ L of preheated (65°C) elution buffer and stored at -80°C. Filter tips are used for the extraction and all Eppendorf tubes are autoclaved prior to use.

#### 3.3.2. Reverse Transcription

1. To make the first-strand cDNA, use 12  $\mu$ L of total RNA and mix with 1  $\mu$ M oligo dT, 0.5 mM each dNTP, 10 U RNase inhibitor, 1  $\mu$ L of Sensiscript reverse transcriptase in 1X buffer in 20  $\mu$ L final volume. Perform a negative control reaction with the same volume of RNA using the same reaction mixture lacking the reverse transcriptase enzyme.
2. Incubate the reaction at 37°C for 1 h.
3. Inactivate the enzyme by incubating at 93°C for 5 min.

#### 3.3.3. PCR Amplification

1. Take 2.5  $\mu$ L of first-strand cDNA and mix with 0.4  $\mu$ M of each primer, 1 unit of Fast Start Taq polymerase, 200  $\mu$ M each of dNTP in a 2 mM MgCl<sub>2</sub> 1X PCR buffer for a total volume of 25  $\mu$ L.
2. Amplify using the following PCR conditions: a first denaturation (and activation of the Taq polymerase) step of 4 min at 95°C preceding 40 cycles of amplification composed of a denaturation step at 95°C for 45 s, an annealing step at 45°C for 45 s, and an extension step at 60°C for 1 min. The PCR amplification is terminated by a final extension step at 60°C for 10 min.
3. If one round of amplification is not enough to detect the PCR products, amplify 1  $\mu$ L of each PCR product using 0.625 U Taq polymerase in the same mixture and the same amplification conditions as described above, except with an increase in the annealing temperature to 50°C.
4. Stain the resulting products with ethidium bromide and run on a 1% agarose gel in 1X TAE buffer.

#### 4. Notes

1. The diameter of the wells of the 16-chamber Lab-Tek glass slides used is the same as the diameter of the cap, so all the selected cells from one well are microdissected without moving the cap, thus avoiding any contaminations.
2. Hepatocytes are particularly RNase-rich. So it is very important to be careful throughout the experiment to avoid these RNases (similarly for parasite RNase). All steps preceding the microdissection itself should be performed under sterile conditions with sterile reagents. The remaining critical step is the parasite labeling, which is performed at 37°C. In order to avoid RNA degradation during the incubation at 37°C, add RNase inhibitor in a concentration of 400 U/mL to the primary and secondary antibodies. The labeling time can be reduced to 20 min for the first antibody, and to 15 min for the second one. Shorter labeling times have been tested, as described in Fend et al. (**13**), but parasites were not bright enough to be detected during the microdissection. This labeling step should be avoided when transgenic fluorescent sporozoites are used (**14**).
3. The main difficulty in performing microdissection with hepatocyte cultures is due to their tight attachment to the slide. It is therefore more difficult to capture the cells compared to microdissection of a tissue section, and a high laser power is required for the microdissection. Even so, the bottom membrane of the targeted cell is still attached to the slide and, consequently, the intracellular parasite is not easily captured and sometimes still remains on the slide after the cap is lifted. So, several laser shots (between 3 and 6) are necessary to capture only one parasite. This can explain why several noninfected hepatocytes may be captured along with the infected one. In this type of experiment, targeting an intracellular parasite in hepatocyte cultures, obtaining pure material is nearly impossible. Nevertheless, if the objective is to perform gene expression analysis, even microarray analysis, absolutely pure material is not required and experiments can be successful if the proportion of parasite RNA among the hepatocyte RNA is sufficient.
4. The use of GFP-*P. falciparum* infected human hepatocytes could be helpful to solve most of the encountered problems.

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