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Short communication

The fluorescent protein iLOV outperforms eGFP as a reporter gene in the microaerophilic protozoan *Trichomonas vaginalis*

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ABSTRACT

Trichomonas vaginalis is a flagellated protozoan causing a notorious urogenital infection in humans. Due to its anaerobic metabolism, an alternative fluorescent protein that can be readily expressed in oxygen-deprived conditions is ideal. This study assessed the performance of iLOV, which does not require oxygen to function, as compared to the conventional enhanced green fluorescent protein (eGFP) in *T. vaginalis*. The results indicated that iLOV outperforms eGFP in both transient and stable expression, being detectable earlier and producing higher fluorescent intensity than eGFP in *T. vaginalis*. This finding facilitates forthcoming genetic studies that will advance the knowledge on this human parasitic infection.

Trichomonas vaginalis is a flagellated protozoan causing the most common non-viral sexually transmitted disease worldwide [1]. The infection is restricted to the urogenital tracts of both sexes, habitats where oxygen level is extremely low [2]. *T. vaginalis* contains peculiar organelles known as hydrogenosomes, where a substrate-level phosphorylation is catalysed by oxygen-sensitive enzymes [3]. Although an antioxidant defence system does exist accordingly [4], exposure to oxygen can still affect the metabolism of this protist, becoming detrimental at high concentrations [5]. Therefore, *T. vaginalis* has been cultured in laboratories under microaerophilic or anaerobic conditions.

The green fluorescent protein (GFP) and its derivatives have vastly facilitated the study of protein dynamics in living cells [6]. In particular, enhanced GFP (eGFP) was once used to indicate the successful transgenic expression in *T. vaginalis* achieved by a viral RNA-based vector [7], which represents the only fluorescent protein (FP) applied to this protist to date. Nonetheless, eGFP (25 kDa) and its derivatives are generally thought unsuitable for anaerobic protozoa due to their intrinsic limitations, i.e. poor pH and thermal stability and, most importantly, the oxygen dependence for maturation of their chromophore [8,9]. Therefore, an alternative reporter gene that can be stably expressed and readily detectable under an anaerobic cellular milieu is desirable for *T. vaginalis*.

Comparatively, another group of smaller proteins (~10–19 kDa) with the characteristic light, oxygen or voltage (LOV) sensing domain are promising FP candidates in *T. vaginalis*. The LOV domain contains a flavin mononucleotide chromophore which is present ubiquitously in *T. vaginalis* [10] and does not undergo oxygen-dependent maturation [8]. Among the LOV-based FPs, some have been tested in anaerobic microorganisms under oxygen-deficient conditions and showed better performance over conventional GFP [11–14]. In this study, we chose iLOV protein, a photo-reversible fluorescent reporter derived from a photoreceptor in *Arabidopsis thaliana* [15,16] as our FP candidate. We compared the maturation rate and fluorescent intensity of iLOV and eGFP in *T. vaginalis*, at transient and stable expression stages respectively, under microaerophilic conditions.

The iLOV coding sequence (CDS) in this study (Fig. S1A) was chemically synthesized following the codon usage preference of *T. vaginalis* [17] and inserted into MasterNeo plasmid (pMN) [18] for expression. In the resulting plasmid pMN-α-SCS-iLOV, the iLOV gene is under regulation of the 5′ and 3′ untranslated region (UTR) of α-succinyl CoA synthetase (α-SCS) gene (TVAG_047890) and the iLOV coding domain (333 bp) is in frame with a double influenza hemagglutinin tag at its 3′ end (i.e. C-terminus of the protein) (Fig. S1B). Likewise, other three plasmids were engineered. These combined the iLOV or eGFP (720 bp) coding domains with the promoter of either α-SCS or ferredoxin (Fd) gene (TVAG_003900), 329 and 250 bp upstream of the transcription start site respectively (Fig. S1B). Both genes are abundantly transcribed in *T. vaginalis*, according to transcriptomic data (trichdb.org), indicating robust promoter activity. In addition to these elements, pMN carries a neomycin drug-resistance gene under the control of a *T. vaginalis* β-tubulin promoter [18] (Fig. S1B).

A standard electroporation protocol [18] was used to introduce 50 μg of each of these four plasmids into the G3 strain of *T. vaginalis*. In addition, one control group of *T. vaginalis* cells was transfected with the

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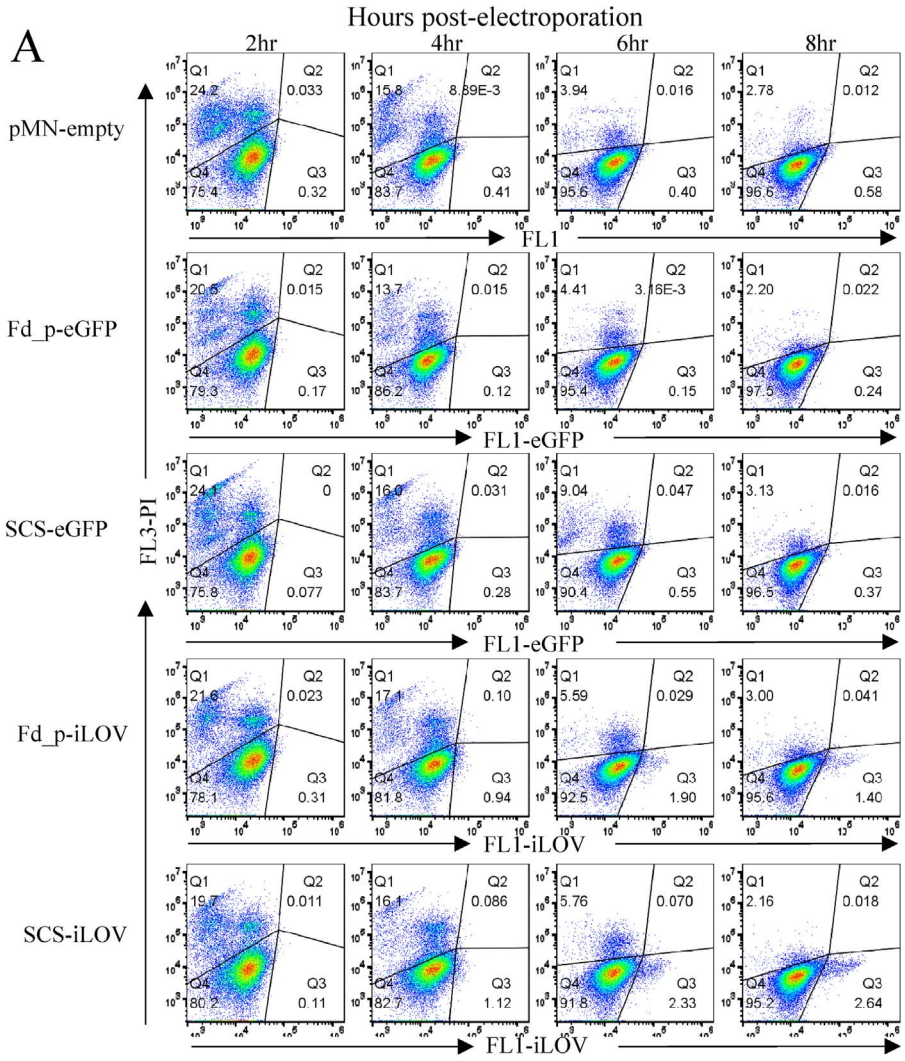

Fig. 1. Transient expression of iLOV and eGFP in *T. vaginalis* evaluated by flow cytometry. *T. vaginalis* was cultured in complete Diamonds media, *i.e.* supplemented with 10% heat-inactivated horse serum, penicillin (1000 units/ml) and streptomycin (0.1 mg/ml) [18]. Cultures were passaged daily at a 50–100 X dilution and grown overnight at 37 °C. For these experiments, cells were grown to a maximum number of 1–2 × 10^6 cells/ml where virtually all cells were alive and swimming. Cells were transfected with each of the five plasmids: α-SCS-iLOV, α-SCS-eGFP, Fd_p-iLOV, Fd_p-eGFP and pMN-empty, as depicted in Fig. S1, following a standard electroporation protocol [18]. Overnight cultures were harvested by centrifugation (3000 g for 20 min at 4 °C) and washed with ice-cold complete media. Cell pellet was re-suspended in ice-cold complete media at a concentration of 8.33 × 10^8 cells/ml. Aliquots of 300 µl cell suspension were transferred into pre-chilled 0.4 cm electrocuvettes, mixed with 50 µg of each plasmid and electroporated at 350 V with 975 µF capacitance using a Bio-Rad Gene Pulser II. Electroporated cells were immediately transferred to 50 ml centrifuge tubes, filled up with pre-warmed complete media and incubated at 37 °C. At the indicated time points (2 h, 4 h, 6 h and 8 h), cells were sampled from the supernatant, pelleted by centrifugation, resuspended in Phosphate-buffered saline (PBS) and taken for flow cytometry analysis. Green fluorescent intensity of each transient transfectant was detected by FL1, and dead cells excluded by FL3 after being stained by 0.2% propidium iodine (PI), using an Accuri C6 Flow Cytometer, BD Biosciences. A protocol was set up to count a total of approximately 30,000 live cells per sample. (A) Live eGFP- and iLOV-positive cells were detected in the quadrant Q3 (bottom right). Data is the representative of three experimental repeats. (B) Variations of the percent of live cells expressing green fluorescence across the period of transient expression. Error bars represent the standard deviation of the percentage values obtained by the three independent transfection assays. *p < 0.001 in a repeated measured ANOVA test (N = 3), rejecting the null hypothesis that there is no change in the percentage of cells expressing fluorescence during transient expression period. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

empty pMN plasmid without the integration of any FP gene (Fig. S1B). Cells were sampled 2, 4, 6 and 8 h post-transfection for analysis by fluorescence-activated cell sorting (FACS), in order to evaluate the transient expression of iLOV and eGFP (Fig. 1). After the last time-point (8 h), G418 was added to the cultures of transfected cells. Drug selection was achieved by passaging transfected cells daily in the presence of this drug for 2 weeks, after which stable expression of the FPs was re-examined by FACS and fluorescence microscopy (Fig. 2). Under excitation with blue light (450 nm), both iLOV and eGFP exhibit a broad range of emission wavelength with the maximum value at 495 nm [15] and 507 nm [19], respectively. Therefore, the optical filter used here (533/30 nm) suits the detection of fluorescent signals emitted by either