Supporting information

A tracer-based method enables tracking of *Plasmodium falciparum* malaria parasites during human skin infection

Methods

Synthesis of Cy5-methyl-methyl ($Cy5M_2$)

Indol-Methyl

In brief, 2,3,3-trimethylindolenine (25 mmol) and methyl iodide (30 mmol) were stirred in toluene (40 ml) for 16h at 40 °C. The formed suspension was filtered and the precipitate was dried in vacuo yielding pink crystals (4 g) and used without further purification.

Cy5-Methyl-Methyl

Indol-Methyl (13.28 mmol), 3-anilinoacraldehyde (6.64 mmol) and sodium acetate (15.49 mmol) were stirred in ethanol absolute (150 ml) and refluxed for 8h under nitrogen atmosphere followed by stirring at room temperature for 6h. The mixture was concentrated and purified by silica gel chromatography using acetonitrile:methanol 3:1, followed by methanol, followed by methanol + 0.25%AcOH. After lyophilization a dark powder (148 mg) was obtained.

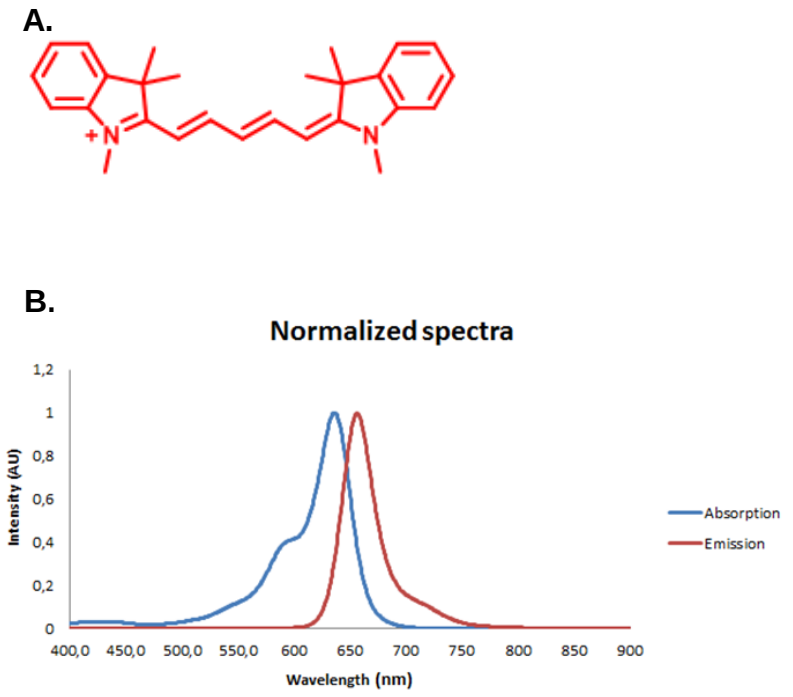

Figure S1. Properties of $Cy5M_2$

Molecular structure (**A**) and normalized absorption (blue) and emission spectra (red) of $Cy5M_2$ (**B**).

$Cy5M_2$ binding specificity for TSPO

Confocal microscopy

The binding specificity of $Cy5M_2$ was initially evaluated *in vitro* using two mammalian cell lines that have a higher mitochondrial density and thus TSPO expression levels than sporozoites: Human breast cancer cell line MDAMB213 X4 cells (X4-cells), kindly provided by Dr. G. Luker (University of Michigan,

Ann Arbor, USA), wherein CXCR4 expression was acquired after transfection with a GFP-tagged version of the human CXCR4-gene[1] and RT4-D6P2T rat Schwannoma cell line (RT4-cells), obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Both cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) enriched with 10% fetal bovine serum and 1% penicillin/streptomycin (all Life Technologies Inc. Carlsbad, CA, USA). Cells were kept under standard culture conditions. The cultured cells were trypsinized and seeded onto coverslips (ø35mm; MatTek Corporation) and incubated overnight in medium.

To validate the mitochondrial localization, $Cy5M_2$ was applied together with Mitotracker® green (Thermo Fisher Scientific, Waltham, MA, USA). RT-4 cells were incubated with 1 µM Mitotracker green® (for 1 hour at 37˚C) before the incubation with $Cy5M_2$ (5 min at RT with 3nM). To confirm TSPO binding specificity of $Cy5M_2$, blocking experiments were performed with the known TSPO inhibitor PK11195[2] (Sigma Aldrich). Both RT4-cells and X4-cells were incubated with 50 µM PK11195 (40 minutes at 37˚C), before the incubation with $Cy5M_2$ (5 min at room temperature (RT); 3nM). All samples were washed with PBS prior to confocal microscopy analysis (Leica TCS SP8X WLL microscope (Leica Microsystems, Wetzlar, Germany)). Cy5 was excited at 633 nm and the emission was collected between 650-700 nm, the laser power was kept constant during the comparative experiments.

$SMOOT_{human\ skin}$

MATLAB (The MathWorks Inc. Natick, MA, USA) software was created for in skin sporozoite analysis. Using this software we were able to extract the following features per sporozoite over time: movement pattern, angular dispersion, straightness index and velocity. Sporozoite tracks were characterized as motile or stationary based on their displacement. Subsequently, the movement patterns: *sharp turn, slight turn* and *linear* were segmented from motile tracks.

Straightness index (SI)

The SI is a measurement for the deviation of a track from a straight line and is used to quantify track tortuosity. It is defined as the ratio of distance between track end point (*C*) and track length (*L*), as calculated using formula (1). e.g. SI = 1 in a perfect linear path, SI = 0 when the path describes a circle.

$$SI = \frac{C_{track}}{L_{track}} = \frac{x(i) - x(0)}{\sum_{k=1}^{i}\big(x(k) - x(k-1)\big)}$$

(1)

Angular dispersion (AD)

The AD describes tortuosity by quantifying changes in direction by measuring deviation from the mean angle of movement. It is calculated according to the following formula:

$$AD = \frac{1}{I}\sqrt{C^2 + S^2}$$

(2)

Where *I* is the last step of the track and *C and S* are defined as:

$$C = \sum_{i=1}^{I} \cos\theta_i \qquad\qquad S = \sum_{i=1}^{I} \sin\theta_i$$

Where θ is the turn angle of the sporozoite track, defined by the angle difference between path directions in consecutive frames.

$$\boldsymbol{\theta_i = \delta_i - \delta_{i-1}}$$

e.g. AD = 1 indicates a consistent angle (either a straight line or a perfect circle) and smaller AD values represent the presence of random turns over the track course.

Velocity

Sporozoite velocity was measured using the displacement between frames. We defined step number in the track *i* to measure velocity *v* using formula (3), with *x as* the median pixel location of the sporozoite and *t* as the time duration in seconds.

$$v(i) = \frac{x_i - x_{i-1}}{t_i - t_{i-1}} = \frac{dx}{dt}$$

(3)

Results

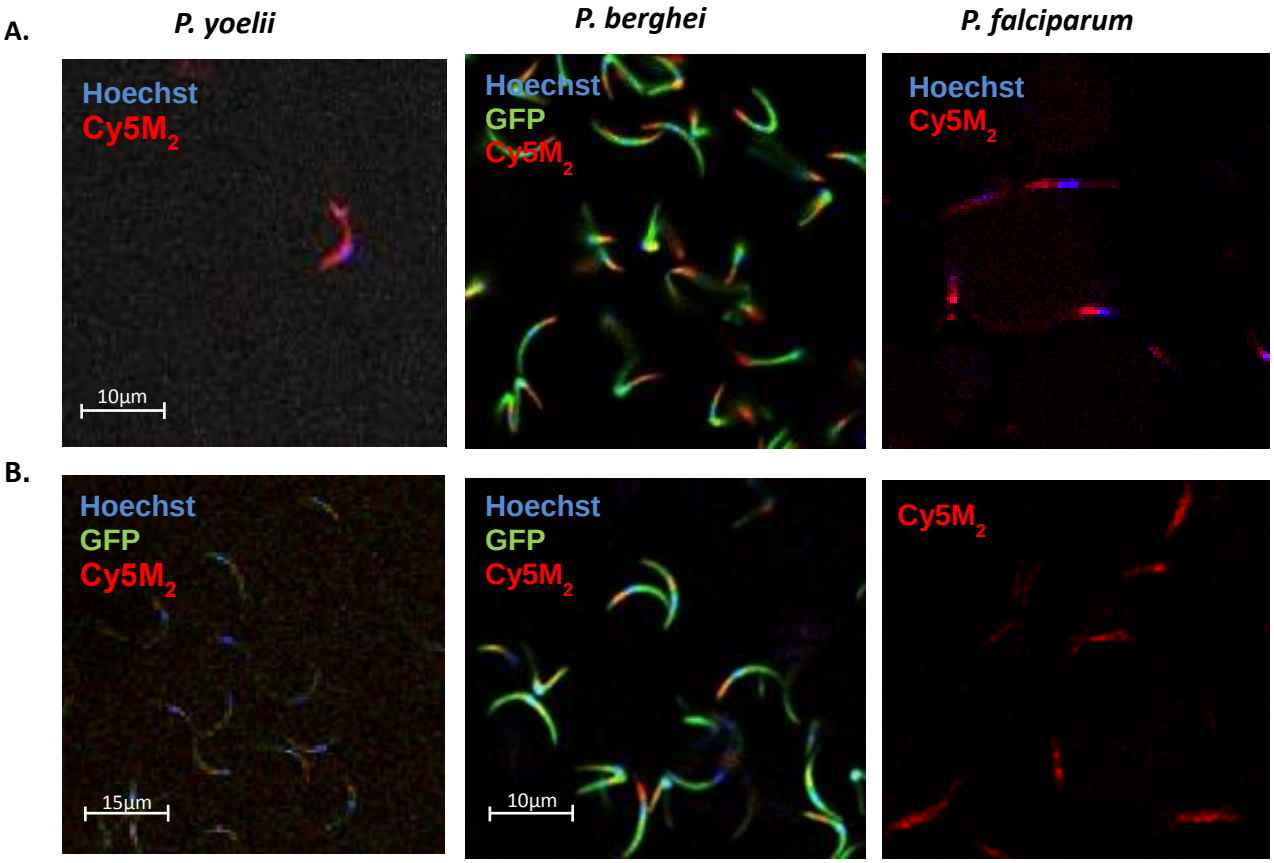

Figure S2. $Cy5M_2$ labels rodent and human malaria sporozoites
Labelling of different *Plasmodium* species with $Cy5M_2$, both *in vitro* (**A**, upper panels) as well as *in vivo* (**B**, lower panels) within the mosquito host. Overlay images of indicated colours. Brightfield in grey, Hoechst in blue, GFP in green and $Cy5M_2$ in red. Scale bar 10µM

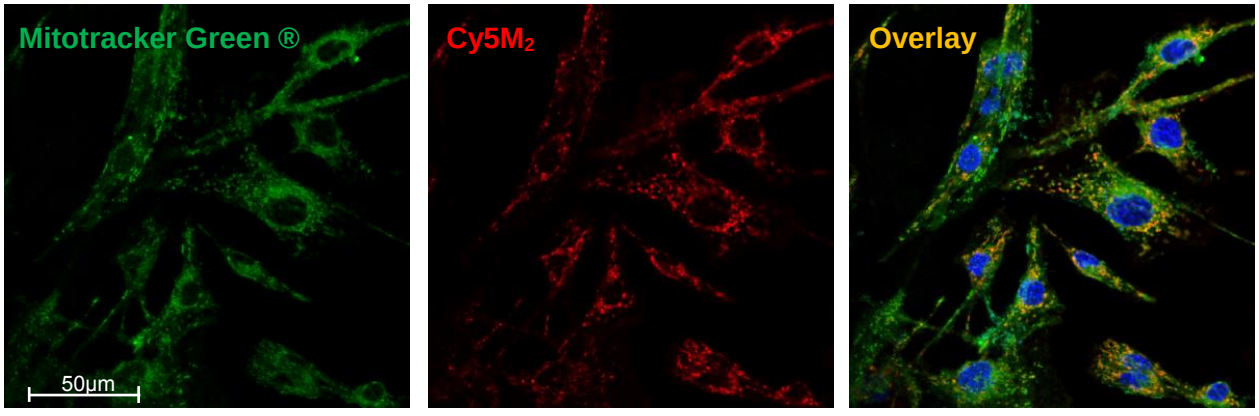

Figure S3. Co-localization of mitochondrial $Cy5M_2$ and MitoTracker® Green
RT4-cells were stained with Mitotracker® green (green) and $Cy5M_2$ (red). Nuclei are stained with Hoechst (blue). Scale bar 50µM.

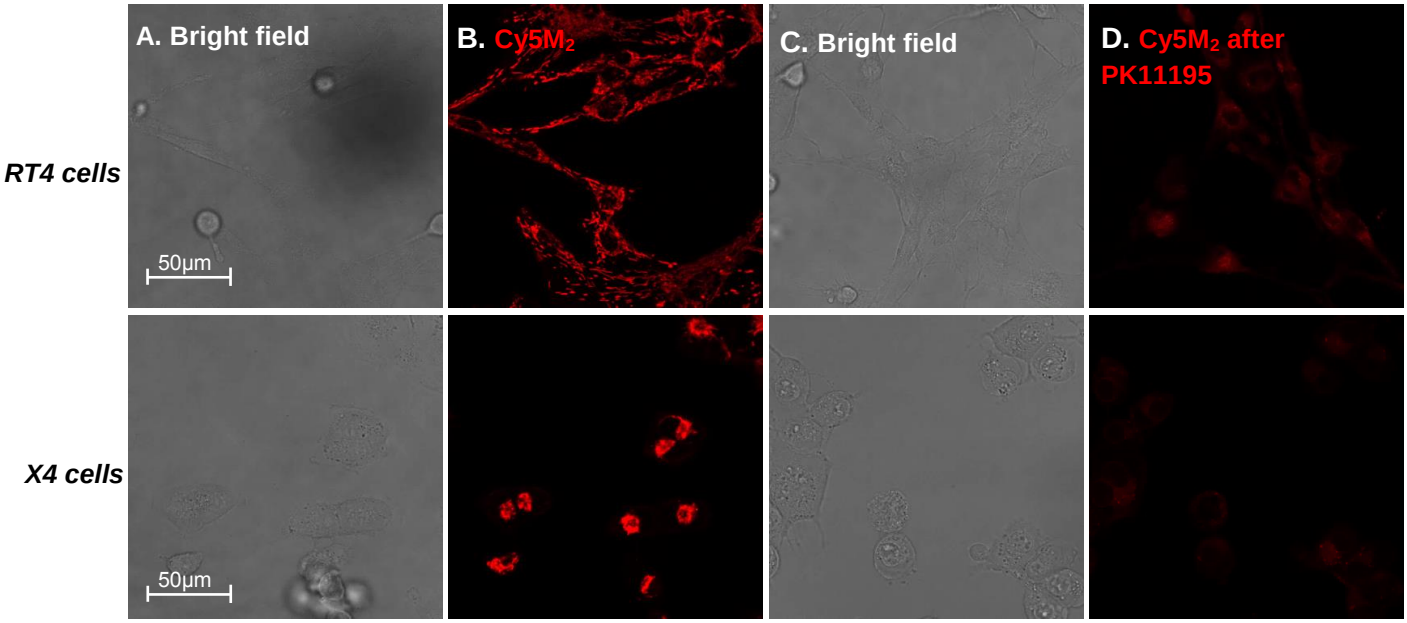

Figure S4. Competitive binding assay of $Cy5M_2$ with PK11195 in cell lines
RT4-cells (top) and X4-cells (bottom) are stained with $Cy5M_2$ (**A, B;** red). $Cy5M_2$ binding is blocked with PK11195 (**C,D**).

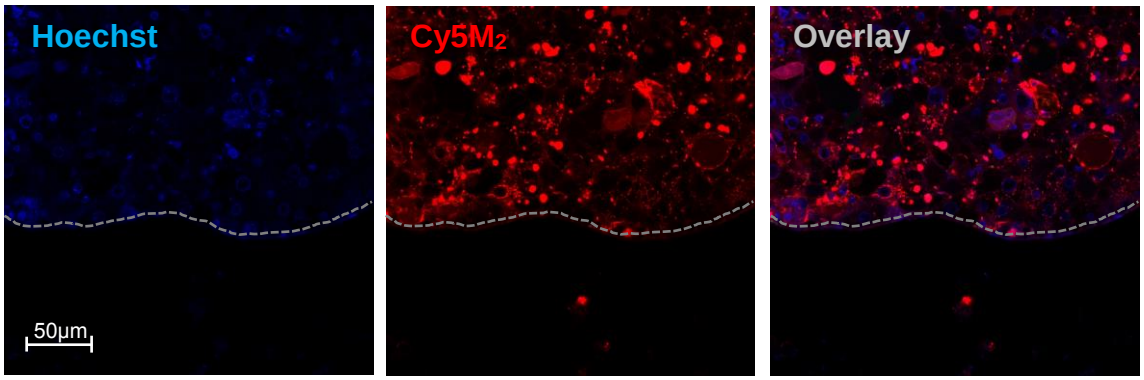

Figure S5. $Cy5M_2$ staining of the mosquito midgut
Midguts of *Pb* infected *Anopheles* mosquitoes fed on $Cy5M_2$. Mosquitoes were dissected 15 minutes after feeding. Midgut cells stained with $Cy5M_2$ (red), nuclei are stained with Hoechst (blue). Scale bar 50µM

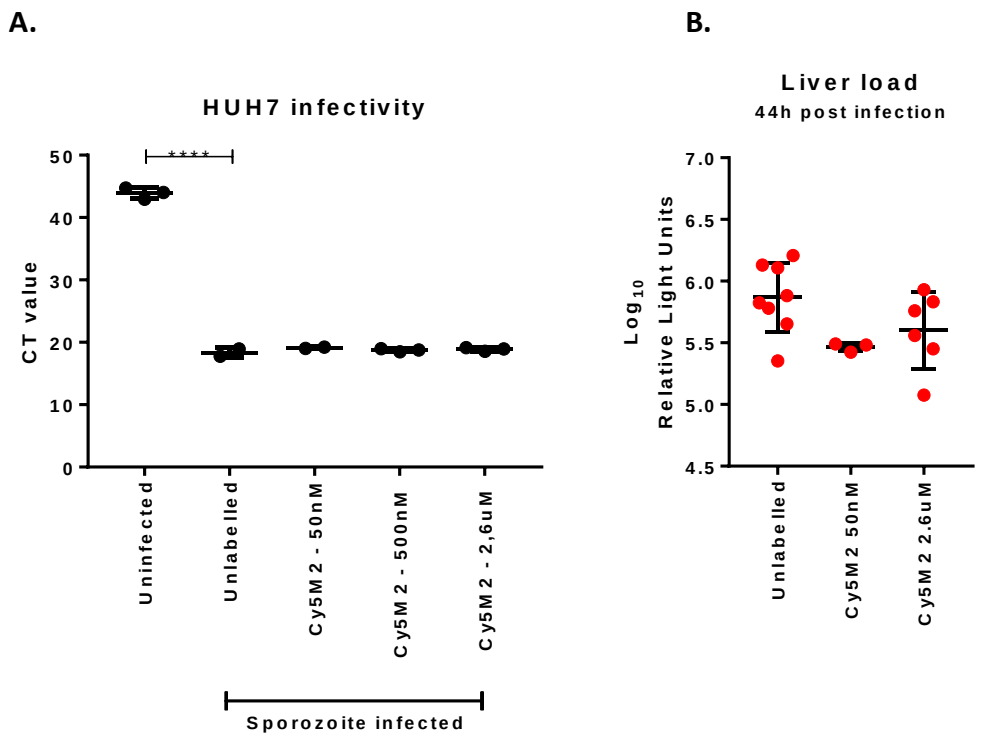

Figure S6. Sporozoite infectivity after $Cy5M_2$ labelling.
Sporozoite infectivity in HUH7 hepatoma cells (**A**) or in mice (**B**) was not affected by increased dosages of $Cy5M_2$. (**A**) PCR data showing similar levels of HUH7 cell infection with all dosages of $Cy5M_2$ labelled sporozoites compared to unlabelled controls. (**B**) Quantification of liver load measured by IVIS 44h post infection Cumulative results from 2 separate experiments (n=8 unlabelled; n=3 $TCy5M_2$ 50µM and n=8 $Cy5M_2$ 2,6µM). Statistical analysis: Student's T test. ****= p<0,0001

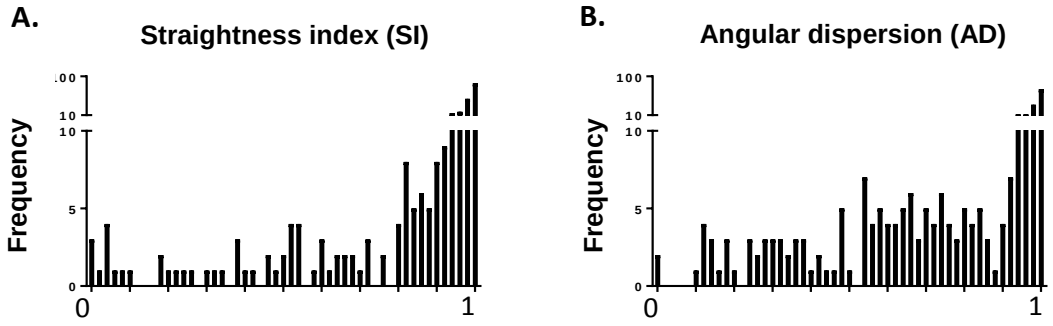

Figure S7. Tortuosity *Pf* tracks in human skin
The tortuosity of sporozoite tracks is quantified using the Straightness index (SI; **A**) and Angular dispersion (AD; **B**). The SI was non-parametrically distributed with the majority of tracks displaying high values (relatively straight tracks; median SI 0.97). Track AD values showed three peaks, indicating three subgroups of tracks. The majority of tracks had a high AD, indicating consistent directions of movement, as opposed to random variations.

References

1 Luker, K. E., Gupta, M., & Luker, G. D. Bioluminescent CXCL12 fusion protein for cellular studies of CXCR4 and CXCR7. BioTechniques. 2009; 47: 625-632

2 Hatty, C. R., & Banati, R. B. Protein-ligand and membrane-ligand interactions in pharmacology: the case of the translocator protein (TSPO). Pharmacological Research. 2015; 100, 58-63.