**Cyanine Phototruncation Enables Cell Labeling with Spatiotemporal Control**


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**ABSTRACT:** Photoconvertible tracking strategies examine the dynamic migration of various cell populations. Here we develop phototruncation-assisted cell tracking (PACT) and apply it to evaluate the migration of immune cells into tumor-draining lymphatics. This approach is enabled by a recently discovered cyanine photoconversion reaction that leads to the two-carbon truncation and consequent blue-shift of these commonly used probes. By examining substituent effects on the heptamethine cyanine chromophore, we find that introduction of a single methoxy group increases the yield of the phototruncation reaction in neutral buffer by almost 8-fold. The resulting cell-tracking probes are applied in a series of in vitro and in vivo experiments, including quantitative, time-dependent measurements of the migration of immune cells from tumors to tumor-draining lymph nodes. Unlike previously reported cellular photoconversion approaches, this method does not require genetic engineering. Overall, PACT provides a straightforward approach to labeling cell populations with precise spatiotemporal control.

Cell migration is a critical component of host immunity against foreign organisms and cancer. Tumor-draining lymph nodes (TDLNs) lie immediately downstream of tumors and play an important role in cancer immunology and immunotherapy. Immune cell migration between tumors and TDLNs determines tumor immune status. However, it is difficult to quantitatively assess cell dynamics between tumors and TDLNs. Photoconvertible cell-tracking strategies allow cell types of interest to be examined with precise control. The advantage of these strategies over standard direct labeling (i.e., with fluorescent proteins or with antibody labeling) is that they enable the spatio-temporal selection of the cell population to be tracked through site-specific photoconversion. Green-to-red photo-convertible proteins such as Kaede, EosFP, and Dendra2 have been explored for these applications. However, these photosensitive proteins require genetic engineering, are not activated by tissue penetrant near-infrared (NIR) light, and can be rejected by immune-competent mice. Consequently, methods to facilitate in vivo cellular photoconversions remain a significant need.

The rich photochemistry of broadly used cyanine probes has enabled applications ranging from super-resolution microscopy to in vivo drug delivery. We recently found that the hypsochromic photoconversion (i.e. “photoblueing”) of cyanines involves a previously uncharacterized phototruncation reaction (Figure 1a). This chemistry, mediated by singlet oxygen, involves the formal excision of the ethene diradical from the polymethine chain resulting in the two-carbon truncated homologue. We demonstrated that cyanine phototruncation could be applied for single-molecule localization microscopy (SMLM) applications. However, the modest yield of the cyanine photoconversion reaction in neutral buffer limited the scope of this photoconversion chemistry.

**Figure 1.** a) Cyanine phototruncation reaction. b) Depiction of the application of (i) spatially controlled PACT to (ii) cellular migration from tumor to TDLN.
Here we report the development of phototruncation-assisted cell tracking (PACT) (Figure 1). Enabled by efforts that significantly improve the yield of cyanine phototruncation, we apply this chemistry to cell-tracking applications and employ it to examine immune cell migration from the tumor to the TDLN. Building on our prior observation that additives, pH and buffers dramatically impact phototruncation yield, we hypothesized that polar functional group on the cyanine chromophore might impact reaction conversion in neutral aqueous conditions.\textsuperscript{36} We find that 3'-OMe-substitution on the polymethine chain leads to a dramatic increase (~8×) under physiological conditions. We then demonstrate that PACT can be implemented in vivo through intratumoral injection of a 3'-OMe-variant of the cell-tracking DiR dye to track immune cell migration into TDLNs. These studies provide a quantitative means for the temporal characterization of the tumor-derived immune-cell population in the TDLN.

**Table 1: Phototruncation screen of substituted heptamethine cyanines**

<table>
<thead>
<tr>
<th>Cmp.</th>
<th>R\textsubscript{1}</th>
<th>R\textsubscript{2}</th>
<th>R\textsubscript{3}</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>HITCl</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>1.3 ± 0.12</td>
</tr>
<tr>
<td>1</td>
<td>I</td>
<td>H</td>
<td>H</td>
<td>0.3 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>CN</td>
<td>H</td>
<td>H</td>
<td>0.7 ± 0.03</td>
</tr>
<tr>
<td>3</td>
<td>MeO</td>
<td>H</td>
<td>H</td>
<td>10.2 ± 0.92</td>
</tr>
<tr>
<td>4</td>
<td>COOMe</td>
<td>H</td>
<td>H</td>
<td>4.4 ± 0.21</td>
</tr>
<tr>
<td>5</td>
<td>H</td>
<td>COOH</td>
<td>H</td>
<td>4.1 ± 0.02</td>
</tr>
<tr>
<td>6</td>
<td>H</td>
<td>COMe</td>
<td>H</td>
<td>4.5 ± 0.04</td>
</tr>
<tr>
<td>7</td>
<td>H</td>
<td>COOMe</td>
<td>H</td>
<td>2.4 ± 0.11</td>
</tr>
<tr>
<td>8</td>
<td>Br</td>
<td>H</td>
<td>Me</td>
<td>1.8 ± 0.02</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>0.5 ± 0.02</td>
</tr>
<tr>
<td>10</td>
<td>Ph</td>
<td>H</td>
<td>H</td>
<td>0.4 ± 0.01</td>
</tr>
<tr>
<td>11</td>
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<td>COOMe</td>
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<tr>
<td>12</td>
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<td>CN</td>
<td>H</td>
<td>0.2 ± 0.10</td>
</tr>
<tr>
<td>13</td>
<td>OMe</td>
<td>CN</td>
<td>H</td>
<td>0.2 ± 0.01</td>
</tr>
<tr>
<td>14</td>
<td>H</td>
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<tr>
<td>16</td>
<td>Me</td>
<td>H</td>
<td>H</td>
<td>0.8 ± 0.03</td>
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<td>17</td>
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<td>EtO</td>
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<tr>
<td>18</td>
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<td>0.4 ± 0.03</td>
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<tr>
<td>19</td>
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<td></td>
<td>0.9 ± 0.15</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td>1.2 ± 0.09</td>
</tr>
</tbody>
</table>

A 50 μM solution of cyanines in the PBS (10 mM; pH = 7.4) buffer was prepared from a 5 mM DMSO stock solution. Irradiations were conducted at 22 °C using either 690, 730, or 780 nm LED (Marubeni America Co.) set to 0.5 W/cm\textsuperscript{2} for 1 h. The sample was then analyzed by UV-vis absorbance, and the yield was determined assuming that the peak at ~650 nm is the unsubstituted pentamethine product (See Figure S1).

![Figure 2](image-url)

**Figure 2.** a) Absorbance curves of 3 (R\textsubscript{1} = OMe, R\textsubscript{2} = H) before and after irradiation with 730 nm LED (500 mW/cm\textsuperscript{2} for 1 h) (50 μM, pH 7.4 PBS). b) Samples of 3 and HITCl (50 μM, pH 7.4 PBS) were irradiated with a 730 nm LED (50 mW/cm\textsuperscript{2}) for up to 1 h and monitored by UV-vis absorbance designated time intervals. c) Cellular photoconversion data. MC38 cells were incubated with PBS containing 3'-OMe-DiR (20 μM) or DiR (20 μM) for 30 min at 37 °C. The cells were then washed with PBS containing 1% FBS twice and then exposed to NIR light at 10 and 100 J/cm\textsuperscript{2} (780 nm, 150 mW/cm\textsuperscript{2}). Photoconversion was analyzed by flow cytometry using Cy7 and Cy5 filter sets with identical gating (Figure S3).

We first set out to improve the yield of phototruncation in neutral buffer. These studies involved examining a series of 3', 4', and 5'-substituted cyanines, which were prepared using a recently described method and whose spectral properties are well documented.\textsuperscript{37–38} Following LED irradiation of 50 μM solution (PBS, pH = 7.4, 37 °C), the yield of the pentamethine product was assessed by UV-vis spectroscopy (Table 1, Figure S1). While the yield of the conversion of the parent probe was modest (1.3% ± 0.12%), we identified several substituents that significantly improved the yield (Table 1). Most dramatically, 3'-OMe substitution led to a roughly 8-fold improvement in the phototruncation yield (10.2 ± 0.92%). LC-MS analysis of the phototruncated product found the major product was the parent pentamethine cyanine lacking polymethine substitution. In our prior report, we proposed a computationally supported mechanism involving an exothermic 4-membered-ring-driven elimination of oxidized ethene.\textsuperscript{36} This involved the asynchronous attack of O\textsubscript{2} at C1' on the polymethine. However, the formation of the non-substituted pentamethine suggests that O\textsubscript{2} attacks at C3 on the polymethine, presumably due to the increased electrophilicity imposed by 3'-OMe substitution. Irradiation experiments monitoring the disappearance of the parent heptamethine reveals the 3'-OMe derivative is photoconverted more efficiently the unsubstituted dye (Figure 2b). This observation is
likely due to the effect of increased electron-density in the chromophore on $^{1}{\text{O}}_2$ reactivity, as observed in prior results from our lab and others.\textsuperscript{34}

The C18-substituted heptamethine cyanine cell membrane probe, DiR, is broadly used to label and track individual cell populations for up to 48 h without effect on their homing or proliferation.\textsuperscript{13, 39-40} Prior to our report of the underlying phototruncation chemistry, two studies observed a hypsochromic photoconversion of cells labeled with the DiR dye.\textsuperscript{28, 41} While establishing the feasibility of this approach, the modest conversion of the DiR affected detection efficiency. Based on the
outcome of our optimization efforts, we synthesized 3'-OMe-DiR (See Supplementary Information) and evaluated its potential for PACT. We first established hepta-to-pentamethine cyanine phototransition in a series of in vitro studies. The effect of irradiation on 3'-OMe-DiR or DiR labeled EL4 and MC38 cells was first assessed by fluorescence microscopy (Figure S2). Dramatically higher time-dependent fluorescence in the Cy5 channel was observed in cells stained with 3'-OMe-DiR. In line with our initial phototransition experiment, fluorescent signal remained in the Cy7 channel for the DiR-labeled samples under these conditions (50 J/cm²). We then evaluated the fraction of phototruncated cells by flow cytometry using the commonly found allophycocyanin (APC) and APC-Cy7 filter set for pentamethine cyanines, respectively. We observed significant increases in the fraction of cells in the Cy5 channel following 780 nm irradiation with both probes, however, the conversion was much more efficient for 3'-OMe-DiR (Figure 2c and Figure S4). We also established that light exposure had a negligible effect on the cell viability (Figure S5) up to 100 J/cm². These experiments demonstrate the potential for cellular phototruncation, and that flow cytometry provides straightforward means to assess photoconversion efficiency.

To investigate the potential for spatiotemporally controlled PACT, the probes (10 nmol) were injected into the tumor in the right lower limb of the C57BL/6 mice bearing MC38 xenograft tumors. After 1 h, the tumor was selectively irradiated with an external laser source (780 nm, 150 mW/cm², 100 J/cm², 65 sec). Subsequent in vivo fluorescence imaging showed significant photoconversion in the case of 3'-OMe-DiR dye (Figure 3a, Figure S6), which was also confirmed in the fluorescence microscopy of frozen tumor slices (Figure 3b). Flow cytometry analysis of various immune cells indicated that phototruncation could be implemented to quantify photoconversion in specific cell types, with readily detectable conversion being detected with 3'-OMe-DiR (Figure 3c-d, Figure S3). These studies demonstrate that 3'-OMe substitution provides critical improvements in photoconversion yield relative to the parent DiR probe and establishes the potential for in vivo PACT.

PACT was then applied to investigate cell migration from MC38 and LL/2-luc tumors (inoculated in the right dorsum) to TDLNs. To track the migration of the intratumoral immune cells, the TDLN (at approx. 1.5 cm) was extracted 1 and 24 h after NIR light exposure (as described above), and a single-cell suspension was obtained. Subsequent flow cytometry analysis identified labeled, photoconverted tumor cells and immune cells including macrophages and dendritic cells 24 h, but not 1 h, post-injection (Figure 3e-f, Figure S7-8). Critically, without photoconversion, the initial transfer of the cell labeling dye throughout the tumor and TDLN make it impossible to clearly define the migrated cell population (up to 0.06% of non-photoconverted cells at 1 h, Figure S7-8). These efforts establish a means to quantify the relative abundance of tumor-derived macrophages and dendritic cells in the TDLN.

In conclusion, we demonstrate that PACT can facilitate irradiation-dependent cell tracking experiments without genetic modification. These studies were enabled by efforts to examine the role of chromophore substitution, which identified a 3'-OMe substituent that improved the yield of photoconversion. Motivated by the key role of TDLNs in tumor immune status, we apply PACT to track the migration of innate immune cells from tumors to the TDLNs. This approach represents an easily implemented method for investigating in vivo cell migration from a specific location of interest. These PACT experiments rely on commonly used laser/filter sets found on numerous instruments and may be coupled with fluorescence-activated single-cell sorting (FACS) for further analysis of PACT-labeled cells. Going forward, the development of antibody-targeted variants of the 3'-OMe-substituted probes would extend this approach to address cellular migration in an antigen-specific manner. Further efforts are currently underway to define and optimize the underlying chemistry and apply this approach to characterize immune cell and other dynamic migration processes.

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge on the ACS Publications website.

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ABBREVIATIONS

PACT: Phototruncation-assisted cell tracking, TDLN: tumor draining lymph node, FACS: fluorescence-activated single-cell sorting, HITC1: 1',1',3,3,3',3'-Hexamethylindotricarbocyanine iodide, PBS: phosphate-buffered saline

REFERENCES


