**Supplementary Figures**

![Optical Setup Diagram](image)

**Figure S1. Illustration of the optical setup for detection system.** Fluorescence microscope 2 is same with fluorescence microscope 1 and not represented in the figure. Also shown is a microscopic image of the linear focused beam on the chip near the inlet channels.
Figure S2. Optimization of pulse delay, voltage, and width for actuator activation.

(a) Schematic representation of detection setup for measurement of sorting efficiency. Sorting efficiency was optimized at a linear velocity 2.89 cm/s. (b) Sorting efficiency at different delay times to trigger the sorter. The voltage applied to the sorter is 15 V and the pulse width is 10 ms. (c) Sorting efficiency with different voltages applied to the sorter. The delay time is 0 and the pulse width is 10 ms. (d) Sorting efficiency at different pulse widths. The voltage applied to the sorter is 15 V and the delay time is 0.
Figure S3. Ca$^{2+}$ association and dissociation kinetics of Y-GECO1f in intact *E. coli* cells. (a) Fluorescence changes versus time for Ca$^{2+}$-association. (b) Fluorescence change versus time for Ca$^{2+}$-dissociation.
Supplementary Tables

**Table S1. Distribution of the cell-to-cell time difference at different throughput at the flow rate of 5.25 μL/min**

<table>
<thead>
<tr>
<th>Throughput (cells/s)</th>
<th>Percentage of cells at different cell-to-cell time difference</th>
<th>Total number of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 0.05 s</td>
<td>&lt; 0.1 s</td>
</tr>
<tr>
<td>3</td>
<td>6%</td>
<td>15%</td>
</tr>
<tr>
<td>5</td>
<td>13%</td>
<td>31%</td>
</tr>
<tr>
<td>10</td>
<td>27%</td>
<td>51%</td>
</tr>
<tr>
<td>20</td>
<td>59%</td>
<td>86%</td>
</tr>
</tbody>
</table>
Table S2. Mutations in Y-GECO variants.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-GECO1m</td>
<td>-</td>
</tr>
<tr>
<td>Y-GECO1f</td>
<td>ΔG13(^1)</td>
</tr>
<tr>
<td>Y-GECO2f</td>
<td>ΔG13, M1T, T30S, S128P</td>
</tr>
</tbody>
</table>

\(^1\) ΔG13 represents that G13 is deleted.
Table S3. Properties of Y-GECO variants.

<table>
<thead>
<tr>
<th>Protein variant</th>
<th>λ&lt;sub&gt;abs&lt;/sub&gt; (nm) with ε&lt;sup&gt;1&lt;/sup&gt; (mM&lt;sup&gt;−1&lt;/sup&gt;cm&lt;sup&gt;−1&lt;/sup&gt;) in parenthesis</th>
<th>λ&lt;sub&gt;ex&lt;/sub&gt;, λ&lt;sub&gt;em&lt;/sub&gt; (nm) in parenthesis</th>
<th>ε * ϕ&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Ca&lt;sup&gt;2+&lt;/sup&gt;-dependent change in brightness&lt;sup&gt;3&lt;/sup&gt; with λ&lt;sub&gt;ex&lt;/sub&gt; (nm) in parenthesis</th>
<th>pK&lt;sub&gt;a&lt;/sub&gt;&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-GECO1f</td>
<td>412 (17)</td>
<td>0.05 (412, 538)</td>
<td>0.9</td>
<td>6× (412)</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>522 (26)</td>
<td>0.75 (522, 538)</td>
<td>19.5</td>
<td>4× (522)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>412 (23)</td>
<td>0.16 (412, 538)</td>
<td>3.7</td>
<td>6× (522)</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>522 (6)</td>
<td>0.58 (522, 538)</td>
<td>3.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y-GECO2f</td>
<td>412 (20)</td>
<td>0.06 (412, 538)</td>
<td>1.2</td>
<td>2× (412)</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>522 (31)</td>
<td>0.77 (522, 538)</td>
<td>23.9</td>
<td>4× (522)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>412 (30)</td>
<td>0.09 (412, 538)</td>
<td>2.7</td>
<td>24× (522)</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>522 (2)</td>
<td>0.51 (522, 538)</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Quantum yield ϕ for emission (m) at the 538 nm peak measured at the two excitation (x) peaks.

<sup>2</sup> The product of ε and ϕ is proportional to the overall fluorescent brightness in units of mM<sup>−1</sup>cm<sup>−1</sup>.

<sup>3</sup> Up and down arrows indicate fluorescence increases and decreases, respectively.

<sup>4</sup> The pK<sub>a</sub> is defined as the pH at which the ratio fluorescence response (excitation at 526 nm/ excitation at 416 nm) is 50% of maximum.
Table S4. *In vitro* $k_{\text{off}}$ and $K_d'$ of Y-GECO variants

<table>
<thead>
<tr>
<th>Protein variant</th>
<th>$k_{\text{off}}$ (s$^{-1}$)</th>
<th>$K_d'$ (nM) with Hill coefficient in parenthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-GECO1f</td>
<td>11.65</td>
<td>2500 (2.7)</td>
</tr>
<tr>
<td>Y-GECO2f</td>
<td>5.96</td>
<td>2200 (1.4)</td>
</tr>
</tbody>
</table>
### Table S5. Oligonucleotides used in this work

<table>
<thead>
<tr>
<th>Name</th>
<th>5’ to 3’ sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FW_XbaI_6His</td>
<td>GCGATGTCTAGAGTTTCATCATCATCATCATCATGGTATGGCTAGC</td>
</tr>
<tr>
<td>RV_stop_HindIII</td>
<td>GCGATGAAGCTTCTACTTCGTGTCATCATTTGTACAAAATCTCTTCGTAGTTT</td>
</tr>
<tr>
<td>FW_BamHI_Kozak_6His</td>
<td>AAACAGGAGGAATTAGCTTTGGAGATCCACCATGGGTTCATCATCATCATGTGGTCATCATGGTATGGC</td>
</tr>
<tr>
<td>RV_CaM_stop_EcoRI</td>
<td>CGCGAATTTCTACTTCGTGTCATCATTTTGTAC</td>
</tr>
</tbody>
</table>