SUPPLEMENTAL MATERIAL

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Supplemental methods

gBlock molecular biology

Constructs were made by inserting custom oligonucleotides from IDT into either pcDNA3.1 or ppHluorin-N1 (ppHl-N1) into restriction sites as indicated. Consensus human coding sequences were obtained from listed accession numbers.

gBlock sequence to clone human tPA into ppHl-N1 (accession no. NM_000930.4)

Design notes: (1) Adds tPA CDS between HindIII and BamHI sites in ppHl-N1; (2) underline = silent mutation from consensus tPA CDS; three silent mutations were made to tPA CDS to satisfy oligonucleotide synthesis folding conditions; and (3) red GG maintains frame between tPA and pHluorin.

```
5′ Fill Hind3 Kozak Seq 5′ tPA >>>

------- ------ ---------
CGAGCTC-AAGCTT-GCCGCCACC-ATGGATGCAATGAAGAGAGGGCTCTGCTGTGTGCTGCTGCTGTGTGGAGCAGTCT
TCGTTTCGCCCAGCCAGGAAATCCATGCCCGATTCAGAAGAGGAGCCAGATCTTACCAAGTGATCTGCAGAGATGAAAAA
ACGCAGATGATATACCAGCAACATCAGTCATGGCTGCGCCCTGTGCTCAGAAGCAACCGGGTGGAATATTGCTGGTGCAA
CAGTGGCAGGGCACAGTGCCACTCAGTGCCTGTCAAAAGTTGCAGCGAGCCAAGGTGTTTCAACGGGGGCACCTGCCAGC
AGGCCCTGTACTTCTCAGATTTCGTGTGCCAGTGCCCCGAAGGATTTGCTGGGAAGTGCTGTGAAATAGATACCAGGGCC
ACGTGCTACGAGGACCAGGGCATCAGCTACAGGGGCACGTGGAGCACAGCGGAGAGTGGCGCCGAGTGCACCAACTGGAA
CAGCAGCGCGTTGGCCCAGAAGCCCTACAGCGGGCGGAGGCCAGACGCCATCAGGCTGGGTCTGGGGAACCACAACTACT
GCAGAAACCCAGATCGAGACTCAAAGCCCTGGTGCTACGTCTTTAAGGCGGGGAAGTACAGCTCAGAGTTCTGCAGCACC
CCTGCCTGCTCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACGCACAGCCTCACCGAGTC
GGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGATAGGCAAGGTTTACACAGCACAGAACCCCAGTGCCCAGGCAC
TGGGCCTGGGCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCTTGGTGCCATGTGCTGAAGAACCGCAGG
CTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAA
AGGAGGGCTCTTCGCCGACATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTCGCCCGGAGAGC
GGTTCCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCACTGCTTCCAGGAGAGGTTTCCGCCC
CACCACCTGACGGTGATCTTGGGCAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATA
CATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGATTCGTCCCGCT
GTGCCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGACTGGACGGAGTGTGAG
CTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTCTATTCGGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCC
ATCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACTCGGAGCG
GCGGGCCCCAGGCAAACTTGCACGACGCCTGCCAGGGCGATTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATG
ACTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACCAAGGTTACCAACTA
CCTAGACTGGATTCGTGACAACATGCGACCG-GGG-GAT-CCA-CCG-GTC
                                - --- --
                                  BamHI
```

gBlock sequence to add a stop codon after tPA in ppHl-N1

Design notes: (1) tPA was moved from ppHl-N1 to pCDNA3.1 with HindIII and BamHI; (2) this construct adds a stop codon (red) to 3′ end of tPA CDS; and (3) cut with endogenous BsrGI site (bold) and NotI (green).

```
5′ tPA (position 1496)

TTGCACGACGCCTGCCAGGGCGATTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAGCTGGGGCCTGGGCT
GTGGACAGAAGGATGTCCCGGGTGTGTACACCAAGGTTACCAACTACCTAGACTGGATTCGTGACAACATGCGACCG-TAG-GGATCCACTAGTCC
                        ------                                                  ---
                        BsrGI                                                    *

AGTGTGGTGGAATTCTGCAGATATCCAGCACAGTG-GCGGCCGC-TCGAGTCTA
                                    --------         3′
                                      NotI
```

gBlock sequence to clone human S513A tPA into ppHl-N1 or pCDNA3 (accession no. NM_000930.4)

Design notes: (1) GCT (red) = S478A/S513A mutation; (2) adds S513A tPA CDS between HindIII and BamHI sites in ppHl-N1; (3) adds S513A tPA CDS (with stop codon) between HindIII and NotI sites in pCDNA3; (4) underline =

silent mutation from consensus tPA CDS; (5) three silent mutations were made to tPA CDS to satisfy oligonucleotide synthesis folding conditions; (6) red GG maintains frame between tPA and mCherry; and (7) Kozak sequence 3′ of tPA is maintained.

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5′ Fill Hind3 Kozak Seq 5′ tPA>>>

------- ------ ---------
CGAGCTC-AAGCTT-GCCGCCACC-
ATGGATGCAATGAAGAGAGGGCTCTGCTGTGTGCTGCTGCTGTGTGGAGCAGTCTTCGTTTCGCCCAGCCAGGAAATCCA
TGCCCGATTCAGAAGAGGAGCCAGATCTTACCAAGTGATCTGCAGAGATGAAAAAACGCAGATGATATACCAGCAACATC
AGTCATGGCTGCCGCCCTGTGCTCAGAAGCAACCGGGTGGAATATTGCTGGTGCAACAGTGGCAGGGCACAGTGCCACTCA
GTGCCTGTCAAAAGTTGCAGCGAGCCAAGGTGTTTCAACGGGGGCACCTGCCAGCAGGCCCTGTACTTCTCAGATTTCGT
GTGCCAGTGCCCCGAAGGATTTGCTGGGAAGTGCTGTGAAATAGATACCAGGGCCACGTGCTACGAGGACCAGGGCATCA
GCTACAGGGGCACGTGGAGCACAGCGGAGAGTGGCGCCGAGTGCACCAACTGGAACAGCAGCGCGTTGGCCCAGAAGCCC
TACAGCGGGCGGAGGCCAGACGCCATCAGGCTGGGTCTGGGGAACCACAACTACTGCAGAAACCCAGATCGAGACTCAAA
GCCCTGGTGCTACGTCTTTAAGGCGGGGAAGTACAGCTCAGAGTTCTGCAGCACCCCTGCCTGCTCTGAGGGAAACAGTG
ACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAAT
TCCATGATCCTGATAGGCAAGGTTTACACAGCACAGAACCCCAGTGCCCAGGCACTGGGCCTGGGCAAACATAATTACTG
CCGGAATCCTGATGGGGATGCCAAGCCTTGGTGCCATGTGCTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGC
CCTCCTGCTCCACCTGCGGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCC
TCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTCGCCCGGAGAGCGGTTCCTGTGCGGGGGCATACTCAT
CAGCTCCTGCTGGATTCTCTCTGCCGCCCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCA
GAACATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTGTCCATAAGGAATTCGATGAT
GACACTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGATTCGTCCCGCTGTGCCCAGGAGAGCAGCGTGGTCCG
CACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAGG
CCTTGTCTCCTTTCTATTCGGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCATCCAGCCGCTGCACATCACAACAT
TTACTTAACAGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACTTGCACGA
CGCCTGCCAGGGCGATGCTGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGGCATCATCAGCTGGG
GCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACCAAGGTTACCAACTACCTAGACTGGATTCGTGACAACATG
CGACCG-GGG-GAT-CCG-TAG-GCGGCCGC-TTAATTC
 R  P   G   D   P   *  --------
         BamHI           NotI
```

gBlock sequence to clone human PAI-1 into pCDNA3 (accession number: NM_000602.4)
Design notes: (1) Adds PAI CDS between HindIII and BamHI sites in pCDNA3.

```
US Fill HindIII Kozak Seq 5-PAI>>>

------- ------ --------
CGAGCTC-AAGCTT-GCCGCCACC-
ATGCAGATGTCTCCAGCCCTCACCTGCCTAGTCCTGGGCCTGGCCCTTGTCTTTGGTGAAGGGTCTGCTGTGCACCATCCCCCATCCTACGTGGCCC
ACCTGGCCTCAGACTTCGGGGTGAGGGTGTTTCAGCAGGTGGCGCAGGCCTCCAAGGACCGCAACGTGGTTTTCTCACCCTATGGGGTGGCCTCGGT
GTTGGCCATGCTCCAGCTGACAACAGGAGGAGAAACCCAGCAGCAGATTCAAGCAGCTATGGGATTCAAGATTGATGACAAGGGCATGGCCCCCGCC
CTCCGGCATCTGTACAAGGAGCTCATGGGGCCATGGAACAAGGATGAGATCAGCACCACAGACGCGATCTTCGTCCAGCGGGATCTGAAGCTGGTCC
AGGGCTTCATGCCCCACTTCTTCAGGCTGTTCCGGAGCACGGTCAAGCAAGTGGACTTTTCAGAGGTGGAGAGAGCCAGATTCATCATCAATGACTG
GGTGAAGACACACACAAAAGGTATGATCAGCAACTTGCTTGGGAAAGGAGCCGTGGACCAGCTGACACGGCTGGTGCTGGTGAATGCCCTCTACTTC
AACGGCCAGTGGAAGACTCCCTTCCCCGACTCCAGCACCCACCGCCGCCTCTTCCACAAATCAGACGGCAGCACTGTCTCTGTGCCCATGATGGCTC
AGACCAACAAGTTCAACTATACTGAGTTCACCACGCCCGATGGCCATTACTACGACATCCTGGAACTGCCCTACCACGGGGACACCCTCAGCATGTT
CATTGCTGCCCCTTATGAAAAAGAGGTGCCTCTCTCTGCCCTCACCAACATTCTGAGTGCCCAGCTCATCAGCCACTGGAAAGGCAACATGACCAGG
CTGCCCCGCCTCCTGGTTCTGCCCAAGTTCTCCCTGGAGACTGAAGTCGACCTCAGGAAGCCCCTAGAGAACCTGGGAATGACCGACATGTTCAGAC
AGTTTCAGGCTGACTTCACGAGTCTTTCAGACCAAGAGCCTCTCCACGTCGCGCAGGCGCTGCAGAAAGTGAAGATCGAGGTGAACGAGAGTGGCAC
GGTGGCCTCCTCATCCACAGCTGTCATAGTCTCAGCCCGCATGGCCCCCGAGGAGATCATCATGGACAGACCCTTCCTCTTTGTGGTCCGGCACAAC
CCCACAGGAACAGTCCTTTTCATGGGCCAAGTGATGGAACCC-TAG-GGA-TCC-ACC-GTC
                                            --- ---
                                            BamHI
```

gBlock sequence to clone human PAI-1 into ppHl-1 (accession number: NM_000602.4)
Design notes: (1) The same coding sequence above was used, but without the stop codon to clone into ppHl-N1 using BamHI and HindIII.

```
US Fill HindIII Kozak Seq 5-PAI>>>

------- ------ --------
CGAGCTC-AAGCTT-GCCGCCACC-
ATGCAGATGTCTCCAGCCCTCACCTGCCTAGTCCTGGGCCTGGCCCTTGTCTTTGGTGAAGGGTCTGCTGTGCACCATCCCCCATCCTACGTGGCCC
ACCTGGCCTCAGACTTCGGGGTGAGGGTGTTTCAGCAGGTGGCGCAGGCCTCCAAGGACCGCAACGTGGTTTTCTCACCCTATGGGGTGGCCTCGGT
GTTGGCCATGCTCCAGCTGACAACAGGAGGAGAAACCCAGCAGCAGATTCAAGCAGCTATGGGATTCAAGATTGATGACAAGGGCATGGCCCCCGCC
CTCCGGCATCTGTACAAGGAGCTCATGGGGCCATGGAACAAGGATGAGATCAGCACCACAGACGCGATCTTCGTCCAGCGGGATCTGAAGCTGGTCC
AGGGCTTCATGCCCCACTTCTTCAGGCTGTTCCGGAGCACGGTCAAGCAAGTGGACTTTTCAGAGGTGGAGAGAGCCAGATTCATCATCAATGACTG
GGTGAAGACACACACAAAAGGTATGATCAGCAACTTGCTTGGGAAAGGAGCCGTGGACCAGCTGACACGGCTGGTGCTGGTGAATGCCCTCTACTTC
AACGGCCAGTGGAAGACTCCCTTCCCCGACTCCAGCACCCACCGCCGCCTCTTCCACAAATCAGACGGCAGCACTGTCTCTGTGCCCATGATGGCTC
AGACCAACAAGTTCAACTATACTGAGTTCACCACGCCCGATGGCCATTACTACGACATCCTGGAACTGCCCTACCACGGGGACACCCTCAGCATGTT
CATTGCTGCCCCTTATGAAAAAGAGGTGCCTCTCTCTGCCCTCACCAACATTCTGAGTGCCCAGCTCATCAGCCACTGGAAAGGCAACATGACCAGG
CTGCCCCGCCTCCTGGTTCTGCCCAAGTTCTCCCTGGAGACTGAAGTCGACCTCAGGAAGCCCCTAGAGAACCTGGGAATGACCGACATGTTCAGAC
AGTTTCAGGCTGACTTCACGAGTCTTTCAGACCAAGAGCCTCTCCACGTCGCGCAGGCGCTGCAGAAAGTGAAGATCGAGGTGAACGAGAGTGGCAC
GGTGGCCTCCTCATCCACAGCTGTCATAGTCTCAGCCCGCATGGCCCCCGAGGAGATCATCATGGACAGACCCTTCCTCTTTGTGGTCCGGCACAAC
CCCACAGGAACAGTCCTTTTCATGGGCCAAGTGATGGAACCC-GGA-TCC-ACC-GTC
                                           --- ---
                                           BamHI
```

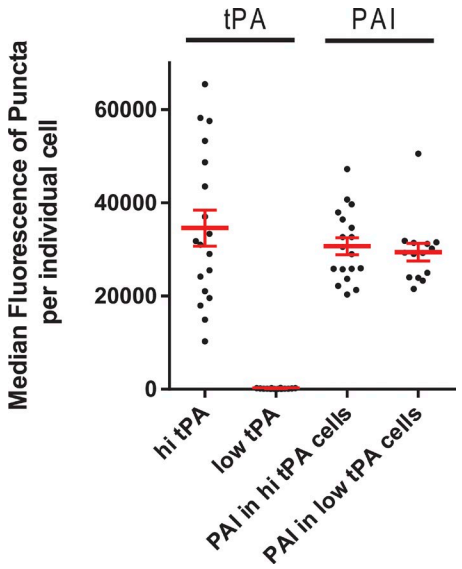


Figure S1. **tPA has no effect on mean PAI immunoreactivity per punctum.** PAI-1 immunofluorescence in puncta from cells with or without tPA-immunoreactivity was quantitated with ImageJ from images from the experiment in Fig. 1. The median PAI-immunofluorescence and tPA-immunofluorescence/punctum was calculated for each cell: high tPA (n = 18 cells) and low tPA (n = 14 cells). Means ± SEM are indicated.

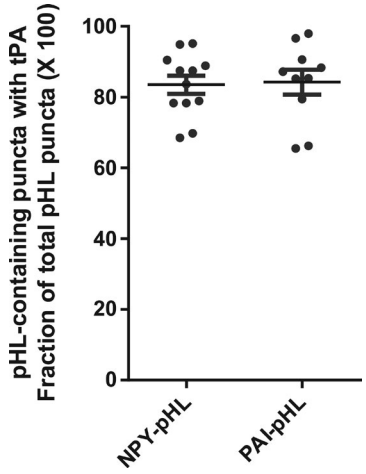


Figure S2. **Coexpression of untagged tPA with PAI-pHL or NPY-pHL.** Cells transfected with DNA encoding PAI-pHL and unlabeled human tPA at a ratio of 1:2 (PAI-pHl:tPA), or NPY-pHL and unlabeled human tPA again at a ratio of 1:2 (NPY-pHL:tPA), were fixed and permeabilized as in Fig. 1. Cells were incubated with rabbit anti–mouse tPA, followed by an Alexa Fluor 546–labeled anti-rabbit secondary antibody, and then imaged by confocal microscopy. PAI-pHL and NPY-pHL fluorescence was imaged directly. Shown are the fractions of pHL-containing puncta that also contain tPA. Each point represents a single cell. Cells and puncta analyzed in each group: transfected NPY-pHL + tPA, *n* = 1,246 NPY-pHL–containing puncta in 12 cells; transfected PAI-pHL + tPA, *n* = 1,004 PAI-pHL–containing puncta in 10 cells. In transfected cells expressing NPY-pHl, 84% of granules also overexpressed tPA. In transfected cells expressing PAI-pHl, 85% of PAI-pHl–containing puncta also contained tPA. With respect to total puncta, in cells transfected with NPY-pHL + tPA, 12.65 ± 1.99% (mean ± SEM) contained NPY-pHL only, 23.11 ± 3.12% contained tPA only, and 64.25% ± 3.4% contained both proteins. In cells transfected with PAI-pHL + tPA, 12.77 ± 2.76% contained PAI-pHL only, 17.39 ± 1.63% contained tPA only, and 69.85 ± 3.70% contained both proteins.

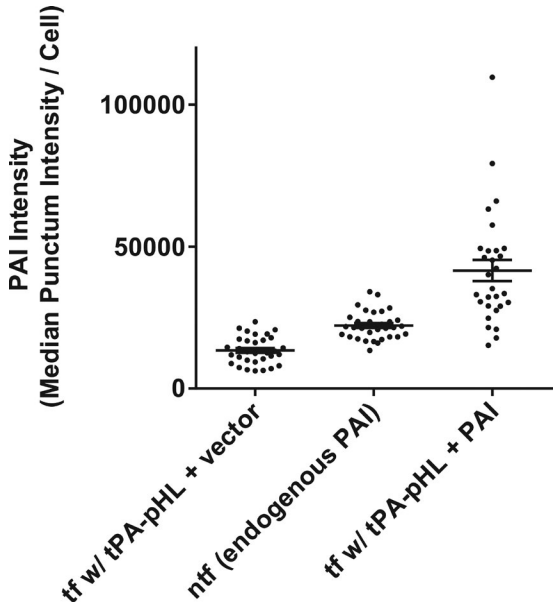


Figure S3. **Extent of PAI overexpression: transfected versus endogenous PAI.** Cells transfected with DNA encoding tPA-pHL and either unlabeled human PAI or empty vector (1:2 ratio) were fixed and permeabilized as in Fig. 1. Cells were incubated with rabbit anti–human PAI-1, followed by an Alexa Fluor 546–labeled anti-rabbit secondary antibody, and then imaged by confocal microscopy. tPA-pHL was imaged directly. Shown are the median intensities per cell of PAI immunofluorescence, each point representing one cell, with mean ± SEM indicated for the group. PAI fluorescence in cells with empty vector (endog. PAI) and tPA-pHL 13,437 ± 858; PAI fluorescence in nontransfected cells (endogenous PAI) 22,172 ± 867; PAI fluorescence in cells transfected with untagged PAI + tPA-pHL 41,555 ± 3,703. Cells and puncta analyzed in each group: transfected tPA-pHL with endogenous PAI, *n* = 1,934 puncta in 32 cells; nontransfected cells, endogenous PAI, *n* = 1,492 puncta in 32 cells; transfected tPA-pHL + transfected human PAI, *n* = 2,135 puncta in 29 cells.

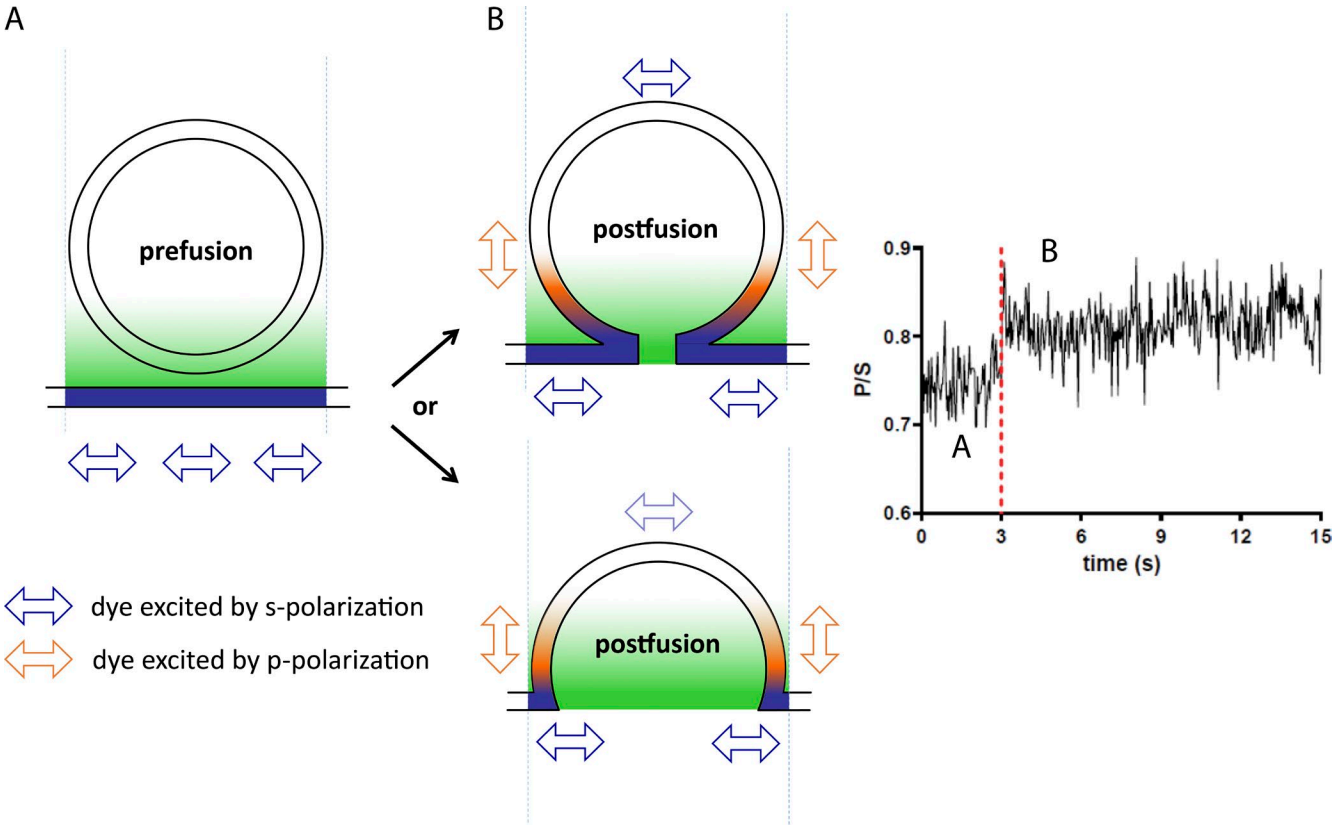
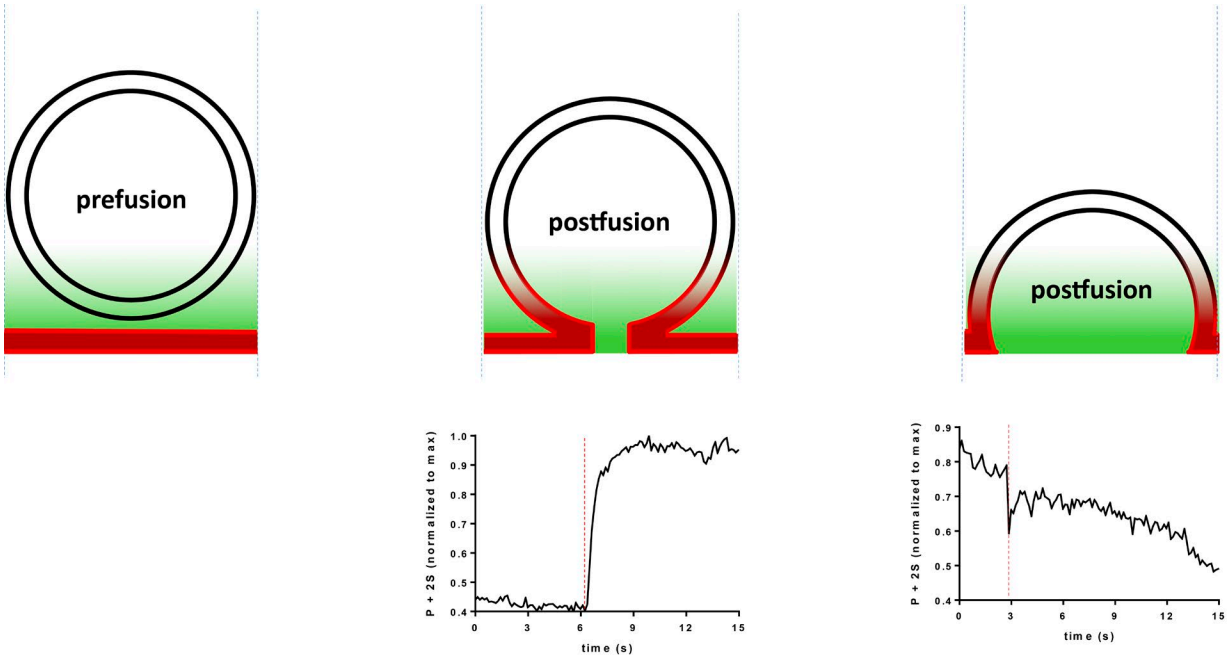


Figure S4. **Illustration of the *P*/*S* response after secretory granule fusion in pTIRFM.** The transition dipole moment of lipophilic dye DiD is oriented as indicated by the arrowheads and by color (blue, S-polarized dye; orange, P-polarized dye). The schematic is oriented along the *y*-*z* plane; S-polarized dye is oriented along the *y* axis, and P-polarized dye is oriented along the *z* axis. The green gradient depicts the rapidly decaying evanescent field. Prefusion (A), the transition dipole moment of DiD in the plasma membrane is oriented parallel to the glass interface. Postfusion (B), membrane curvature increases the amount of DiD with its transition dipole moment oriented perpendicularly to the glass interface. Thus, the *P*/*S* ratio increases when a membrane is curved within a region of interest. The increase occurs for both narrow-neck and wide-neck fusion pores. An example is shown on the right of a long-lived fusion pore resulting from the fusion of a tPA-pHl–containing granule.



Prefusion
Baseline fluorescence from DiD-labeled plasma membrane

Narrow fusion pore
More fluorescent membrane within the region with highest evanescent field excitation. P + 2S increase.

Wide fusion pore
Less fluorescent membrane within the region with highest evanescent field excitation. P + 2S decrease.

Figure S5. **Illustration of the *P* + 2*S* response after secretory granule fusion in pTIRF.** The figure shows a schematic along the *y-z* plane; S-polarized dye is oriented along the *y* axis, and P-polarized dye is oriented along the *z* axis. The extent of the rapidly decaying evanescent field is indicated by the green gradient within blue dotted lines that indicate a region of interest. The fluorescence of the DiD-labeled membrane is indicated by red coloration. A narrow fusion pore neck (left) will have more membrane close to the glass interface in a region of high evanescent field excitation compared with before fusion. Thus, a narrow fusion pore will increase the total amount of DiD (*P+2S*) within the region of interest that is excited by the evanescent field. In contrast, a wide fusion pore will have less membrane in the region of high evanescent field excitation compared with before fusion. Thus, a wide fusion pore will decrease the total amount of membrane labeled with DiD (*P+2S*) that is excited by the evanescent field. Examples of fusion pores resulting in an increase in *P+2S* (left, fusion of a granule containing PAI-pHl + unlabeled tPA resulting in a long-lived fusion pore) and a decrease in *P+2S* (right, fusion of a PAI-pHl–containing granule without tPA with a short-lived fusion pore).