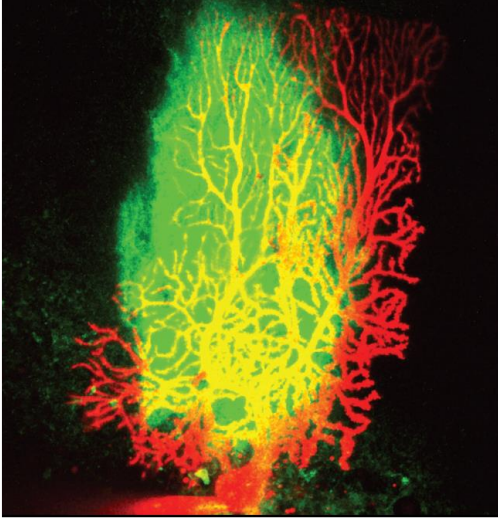


## Site map for translation of

Hires SA, Zhu Y, Tsien R (2008). Optical measurement of synaptic glutamate spillover and reuptake by linker optimized glutamate-sensitive fluorescent reporters

[Proc Natl Acad Sci USA 105:4411–4416](#)

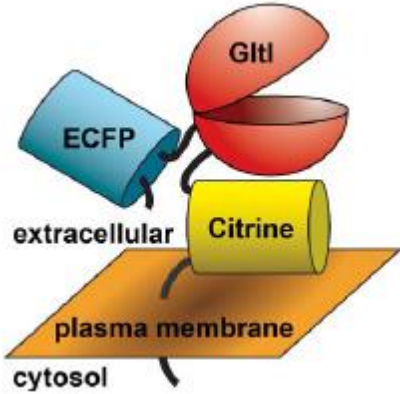
### Front page

Text	Graphics
<p><b>Significance</b> Nerves typically communicate with muscles and other nerves through chemicals (neurotransmitters) that are released at the nerve tips and are sensed by cell surfaces that lie immediately adjacent to them. In the brain, however, neurotransmitters are sometimes released to a larger neighborhood of cells. This is often true of the neurotransmitter glutamate, the most abundant neurotransmitter in the central nervous system [1].</p> <p>To understand the action of glutamate in neurotransmission and neural plasticity, it is essential to know how its concentration changes during the course of neural stimulation and afterwards. Hires et al (2008) devised a method of measuring glutamate in real time and found that at least with certain neurons, glutamate concentration rises and falls rapidly.</p> <p><b>Contents</b> <a href="#">Abstract</a> <a href="#">Introduction</a> Experiments:  <ul style="list-style-type: none"> <li><a href="#">Construction of glutamate sensors</a></li> <li><a href="#">Initial testing of sensors in vitro</a></li> <li><a href="#">Optimization of sensor characteristics</a></li> <li><a href="#">Expression of sensor in living cells</a></li> <li><a href="#">Glutamate measurement in dendrites49</a></li> <li><a href="#">Glutamate measurement in response to frequency of action potentials</a></li> </ul> Implications:  <ul style="list-style-type: none"> <li><a href="#">Glutamate function</a></li> <li><a href="#">Sensor design</a></li> </ul> </p>	 <p>Visualization of glutamate (green fluorescence) in cerebellum slice, using a different fluorophore than Hires et al. From Okubo Y &amp; Iino M, cover of J Physiol (2011) 589 #3.</p>

## Abstract



Text	Graphics
[Translate abstract]	No graphic

## Introduction

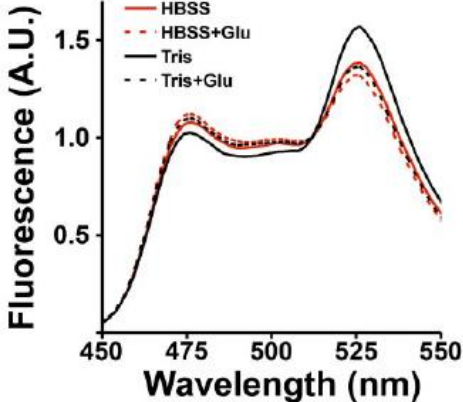
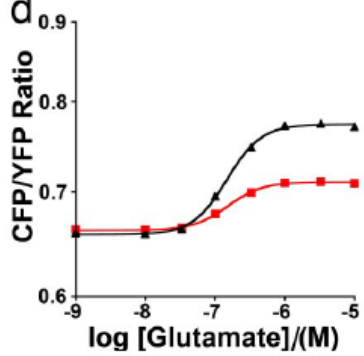
Text	Graphics
<p>The classical junction between one nerve and another or one nerve and a muscle is the synapse. Neurotransmitter is released from the cell on one side of the synapse and detected by a receptor on the membrane on the opposite side. The neurotransmitter glutamate also excites other nearby cells, by spillover of glutamate from the synapse [1].</p> <p>To understand how glutamate spillover works, it would be helpful to be know how much glutamate is present and at what times during and after nervous stimulation.</p>	<p>[Simple synapse with neurotransmitter spilling out of the synaptic cleft and interacting with neighboring cells]</p>
<p>[Principle behind measurement of glutamate through a glutamate-binding protein that fluoresces blue-green or yellow, depending on the binding of glutamate.</p> <p>Explanation of Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP) and principle behind fluorescence resonance energy transfer [2].</p> <p>Nature of glutamate periplasmic binding protein (GltI), how it changes its shape upon binding glutamate [need ref].]</p>	 <p>[need to improve on this picture to show +/- glutamate and energy transfer. Remove plasma membrane.]</p>

1. Pál B (2018). Involvement of extrasynaptic glutamate in physiological and pathophysiological changes of neuronal excitability. [Cell Molec Life Sci 75:2917-2949](#).
2. Deuschle K, Okumoto S, Fehr M, Looger LL, Kozhukh L, Frommer WB (2005). Construction and optimization of a family of genetically encoded metabolite sensors by semirational protein engineering. [Prot Sci, 14:2304–2314](#).

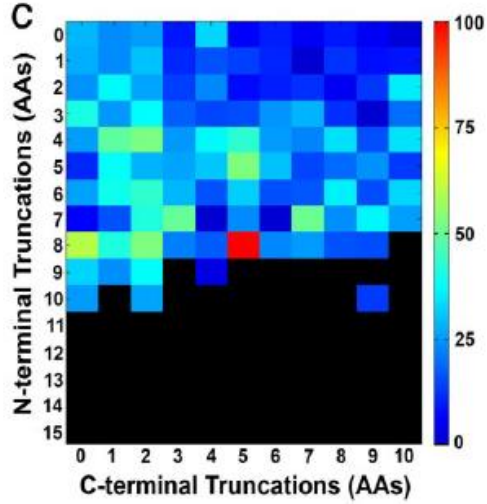
## Experiment: Construction of glutamate sensors

Text	Graphics
<p>[Two sensors: one soluble, one attached to cell membrane.</p> <p>Soluble: Good for characterizing sensor in the test tube.</p> <p>Genes encoding CFP, GltI, and Citrine (a form of YFP) fused together. Preceded by six histidine residues strung one after the other (His-His-His-His-His-His = His6). Link to use of <a href="#">His tags in protein purification</a>.</p> <p>This was accomplished by cloning into a special-purpose plasmid, <a href="#">pRSETB</a>, used to express large amounts of protein in E. coli.]</p>	<p>Soluble:</p>  <p>The diagram shows a linear protein construct. It starts with a label 'His6-' followed by a blue box labeled 'CFP', a red box labeled 'GltI', and a yellow box labeled 'Citrine'.</p>
<p>[Membrane bound: Good for placing sensor where it can detect extra-synaptic glutamate.</p> <p>Same three genes fused, preceded by <a href="#">signal peptide</a> (from Immunoglobulin = Ig) to direct the protein to the membrane) and a protein to provide <a href="#">transmembrane regions</a> so the sensor will get stuck in the membrane. The protein used for this purpose is Platelet-Derived Growth Factor Receptor (PDGFR), but the nature of the protein is not important.</p> <p>This was accomplished by cloning into a special purpose plasmid, <a href="#">pDISPLAY</a>, used to express proteins on the surface of mammalian cells.]</p>	<p>Membrane-bound:</p>  <p>The diagram shows a linear protein construct. It starts with a label 'Ig-s-' followed by a blue box labeled 'CFP', a red box labeled 'GltI', a yellow box labeled 'Citrine', and a grey box labeled 'PDGFR'.</p>

**Experiment: Initial testing of sensors in vitro**

Text	Graphics
<p><u>Significance</u>                      [Fluorescence energy transfer emission from soluble glutamate sensor produced as <a href="#">described above</a> measured +/- glutamate. Show how to calculate ratio of CFP/YFP.]</p>	 <p>[Get rid of Tris lines (may have to trace), label red lines + glutamate and - glutamate. Make horizontal color bar for wavelength, superimpose on graph. Point to downward difference in emission at 526 nm from YFP and upward difference at 476 nm from CFP in response to glutamate. Draw lines to Y axis]</p>
<p>[CFP/YFP ratio is sensitive to level of glutamate. Show that above graph represents extremes of graph to right. Problem: glutamate binding too good. No change in range of glutamate that is physiologically relevant. Need to improve. Also maximum response is about 0.                      Omit Fig. 1e.]</p>	 <p>[Erase "d". Erase black line. Expand Y axis. Change X axis to <math>10^{-9}</math>, <math>10^{-8}</math>, etc, no log. Arrow at <math>\frac{1}{2}</math> maximal increase with line to X axis (150 nM) = <math>K_D</math>. Label maximum response = +7%.]</p>

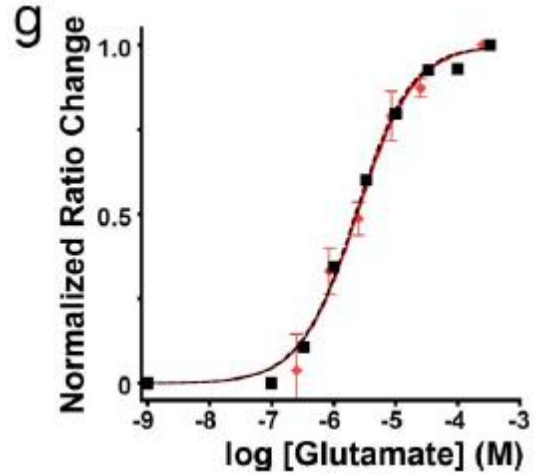
### Experiment: Optimization of sensor characteristics

Text	Graphics
<p>[Need to decrease affinity of glutamate binding protein for glutamate. Site-specific mutagenesis: S73T had <math>K_D</math> of 2.5 <math>\mu</math>M (17x decrease in affinity).</p>	<p>[Picture of GltI bound to glutamate from PDB. Highlight Ser-73.]</p>
<p>[Need to increase response magnitude. Truncate GltI(S73T) on N terminus and C terminus.]</p>	<p>[Need figure for truncation of sequence. Use graphical convention like that below.]</p>
<p>[Measure maximum response to glutamate as in Fig. 1d of all 176 possible truncation combinations. One big winner: 8 amino acids truncated on N terminus, 5 amino acids on C terminus. 44% maximum response to glutamate.</p> <p>Omit Fig. 2e</p>	 <p>Heatmap showing the response of various truncations of GltI(S73T) to glutamate. The Y-axis is 'N-terminal Truncations (AAs)' (0 to 15) and the X-axis is 'C-terminal Truncations (AAs)' (0 to 10). A color scale on the right indicates response percentage from 0 (blue) to 100 (red). A red box highlights the combination of 8 N-terminal and 5 C-terminal truncations, indicating a 44% response. A line is drawn from this red box to the X and Y axes.</p> <p>Show truncations graphically, like:</p> <pre>       _____      _____     _____    _____   _____  _____ </pre> <p>Label color bar with absolute percentages: 7.1% = blue, 44% = red. Draw line from red box to X- and Y-axes</p>

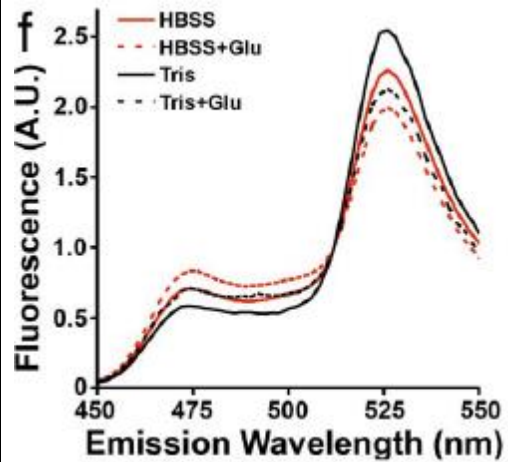
Performance of final optimized sensor with the following modifications (as [described above](#)): S73T, -8N, -5C.

Optimized range of sensitivity to glutamate.

Optimized range of fluorescent response +/- glutamate.

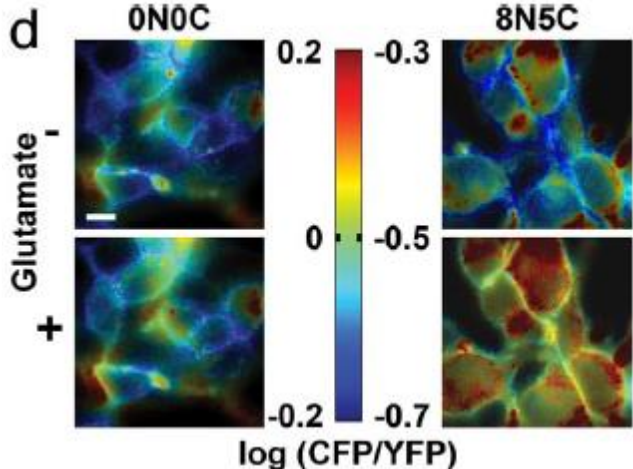


Change X-axis to  $10^{-9}$  etc. Draw line from midpoint down to X axis, label  $K_D = 2.5\mu\text{M}$ .



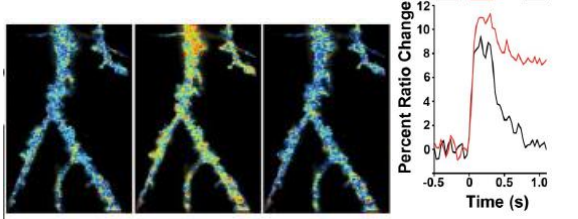
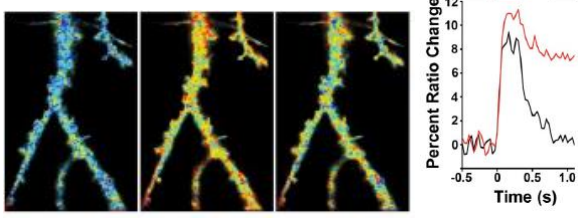
Color bar. Erase black. Lines to Y axis. Maximum response = 44%

### Experiment: Expression of sensor in living cells

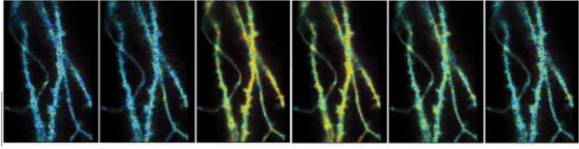
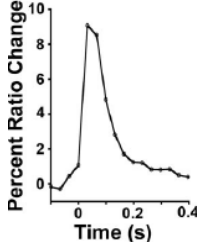
Text	Graphics
[Brief explanation of transfection] [Brief explanation of HEK293 cells]	[Cartoon of plasmid into cell, regrown to dendrite]
[Membrane bound response in HEK293 cells to known amount of glutamate (100 $\mu$ M), using original sensor and optimized sensor.]	 <p data-bbox="764 898 1365 972">[Change log scale to linear. Change "0N0C" to "original", "8N5C" to "optimized"]</p>



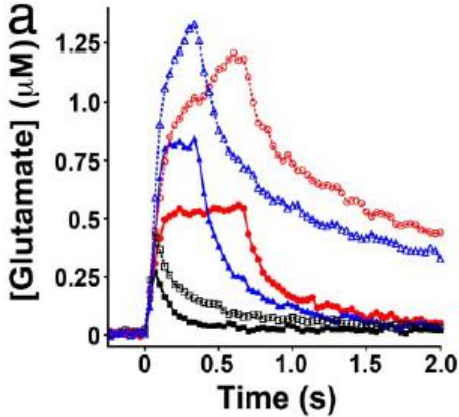
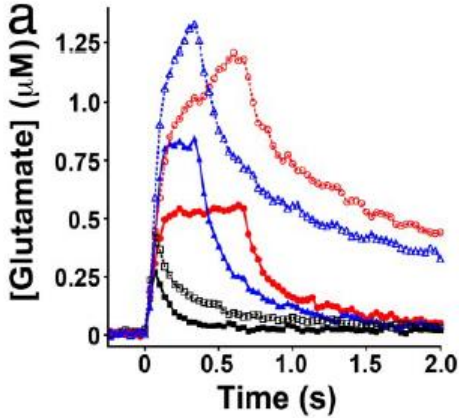
## Experiment: Glutamate measurement in dendrites

Text	Graphics
[Transfect membrane-bound sensor gene into neuron cells from rat hippocampus (significance of hippocampus?).]	[graphic of brain, hippocampus, cartoon of neural cell culture]
[Stimulate cells]	[graphic of external voltage set up]
<p>[CFP/YFP fluorescence from Membrane-bound glutamate sensor in dendrites stimulated for 0.3 seconds (10 action potentials). Record before, during, after. Transient increase in CFP/YFP =&gt; transient increase in glutamate.</p> <p>Quantitated and averaged over all dendrite surface.</p>	 <p>-----      - - - - -      ----- 0.3 sec stimulation</p> <p>Erase red line in graph Add color bar</p>
<p>[Concentration of neurotransmitter affected not only by release but also reuptake. Cocaine works largely by inhibiting the reuptake of the neurotransmitter dopamine.</p> <p>[+/- inhibitor of glutamate reuptake: DL-threo-<math>\beta</math>-benzyloxyaspartate (TBOA)]</p>	[cartoon of neurotransmitter reuptake, +/- drug that blocks reuptake]
[Same conditions as before, but with TBOA added to block glutamate reuptake. Glutamate spike persists.]	 <p>-----      - - - - -      ----- 0.3 sec stimulation</p> <p>Leave red line intact</p>

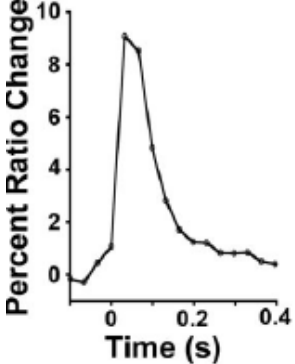
## Experiment: Glutamate measurement in dendrites

Text	Graphics														
<p>[Same as above, but for just <u>one</u> action potential (averaged over 30 separate repetitions). No TBOA. Shows mostly over after 0.1 seconds. Glutamate transient.</p>	<p style="text-align: center;">Stimulus ↓</p>  <p style="text-align: center;">Time (seconds)</p> <p>Add color bar</p>  <table border="1"><caption>Approximate data from the Percent Ratio Change graph</caption><thead><tr><th>Time (s)</th><th>Percent Ratio Change</th></tr></thead><tbody><tr><td>0.00</td><td>0</td></tr><tr><td>0.02</td><td>1</td></tr><tr><td>0.05</td><td>9</td></tr><tr><td>0.10</td><td>3</td></tr><tr><td>0.20</td><td>1</td></tr><tr><td>0.40</td><td>0.5</td></tr></tbody></table>	Time (s)	Percent Ratio Change	0.00	0	0.02	1	0.05	9	0.10	3	0.20	1	0.40	0.5
Time (s)	Percent Ratio Change														
0.00	0														
0.02	1														
0.05	9														
0.10	3														
0.20	1														
0.40	0.5														

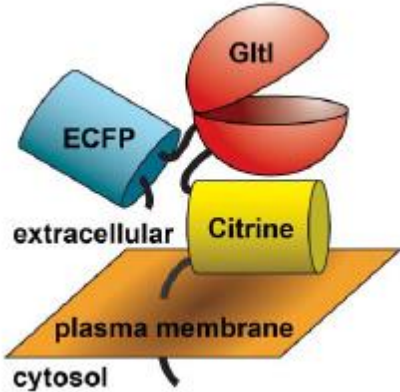
### Experiment: Glutamate measurement in response to frequency of action potentials

Text	Graphics
<p>[Nerves may fire at different frequencies. How does that affect the concentration of spillover glutamate?</p> <p>Three conditions:</p> <ul style="list-style-type: none"> <li>- 1 action potential</li> <li>- 10 action potentials spaced by 67 msec</li> <li>- 10 action potentials spaced by 33 msec</li> </ul> <p>Glutamate accumulates to higher level when time of reuptake is smaller.</p>	 <p>[Erase lines with open symbols. Label lines: black = 1 action potential; red = 10 action potentials, 1 per 67 msec; blue = 10 action potentials, 1 per 33 msec.]</p>
<p>[The idea that the difference in glutamate levels is due to a shorter period of reuptake can be tested by blocking reuptake with TBOA.</p> <p>When reuptake blocked, magnitude of glutamate spike for 10 action potentials no longer depends on frequency]</p>	 <p>[Erase lines with closed symbols. Label lines:</p>

## Implications: Glutamate function

Text	Graphics																		
<p>[Glutamate concentration rises rapidly at surface of dendrite and disappears rapidly – 10's of milliseconds-- unless reuptake inhibited.</p> <p>Since magnitude of glutamate depends on frequency of stimulation, neighboring cells may be able to respond to the frequency at which a nerve is firing.]</p>	 <p>The graph plots Percent Ratio Change on the vertical axis (0 to 10) against Time in seconds on the horizontal axis (0 to 0.4). The curve starts at 0, rises to a peak of approximately 9 at 0.05 seconds, and then decays rapidly, reaching about 2 at 0.15 seconds and stabilizing near 1 by 0.4 seconds.</p> <table border="1"><thead><tr><th>Time (s)</th><th>Percent Ratio Change</th></tr></thead><tbody><tr><td>0.00</td><td>0</td></tr><tr><td>0.02</td><td>1</td></tr><tr><td>0.05</td><td>9</td></tr><tr><td>0.10</td><td>4</td></tr><tr><td>0.15</td><td>2</td></tr><tr><td>0.20</td><td>1.5</td></tr><tr><td>0.30</td><td>1</td></tr><tr><td>0.40</td><td>0.5</td></tr></tbody></table>	Time (s)	Percent Ratio Change	0.00	0	0.02	1	0.05	9	0.10	4	0.15	2	0.20	1.5	0.30	1	0.40	0.5
Time (s)	Percent Ratio Change																		
0.00	0																		
0.02	1																		
0.05	9																		
0.10	4																		
0.15	2																		
0.20	1.5																		
0.30	1																		
0.40	0.5																		

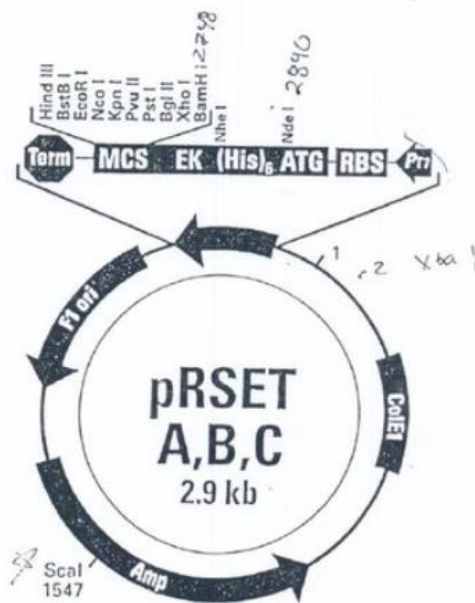
## Implications: Sensor design

Text	Graphics
<p>[Two means of optimization: mutagenesis of glutamate binding protein to reduce affinity and truncation of the protein to increase +/- glutamate difference.</p> <p>Optimized sensor far better than original, but difficult to predict best way to truncate the glutamate binding protein to achieve optimization.]</p>	 <p>The diagram illustrates a sensor protein structure. It features a blue cylindrical component labeled 'ECFP' and a red, bowl-shaped component labeled 'GltI'. These two components are connected to a yellow cylindrical component labeled 'Citrine'. The 'Citrine' component is embedded in an orange horizontal plane representing the 'plasma membrane'. The region above the membrane is labeled 'extracellular', and the region below is labeled 'cytosol'.</p>

## Protein purification through His tags

Text	Graphics
[Explanation of figure]	[Graphic of His-tag-mediated purification]

## Overexpression of protein using pRSETB

Text	Graphics
<p>[Explanation of promoter(P<sub>T7</sub>), ribosome binding site (RBS), <a href="#">His-tag</a> (His<sub>6</sub>), EK [???], Multiple cloning sites (MCS), transcriptional terminator (Term), rest of plasmid for replication and selection in E.coli.]</p>	 <p>The diagram illustrates the pRSETB plasmid, a 2.9 kb circular vector. Key features include:</p> <ul style="list-style-type: none"><li><b>Term</b>: Transcriptional terminator.</li><li><b>MCS</b>: Multiple cloning sites, with various restriction enzyme sites listed above: Hind III, BstBI, EcoRI, Nco I, Kpn I, Pvu II, Pst I, Bgl II, Xho I, BamHI, and Mho I.</li><li><b>EK (His)<sub>6</sub> ATG</b>: Expression cassette containing an EK tag, a 6-histidine tag, and an ATG start codon.</li><li><b>RBS</b>: Ribosome binding site.</li><li><b>P<sub>T7</sub></b>: T7 promoter.</li><li><b>ori</b>: Origin of replication.</li><li><b>ColEI</b>: Colony formation in E. coli.</li><li><b>Amp</b>: Ampicillin resistance gene.</li><li><b>Scal 1547</b>: A specific restriction site.</li></ul> <p>Handwritten annotations include '1', '2', and 'x ka!' near the P<sub>T7</sub> promoter region.</p>

## Directing proteins to a membrane by signal peptides

Text	Graphics
[Explanation of figure]	[graphic of signal peptide and passage through membrane]



## Transmembrane regions of proteins

Text	Graphics
[Explanation of figure]	[Graphic of transmembrane region]

## Expression of proteins on the surface of mammalian cells through pDISPLAY

Text	Graphics
<p>[Explanation of promoter (<math>P_{CMV}</math>), <a href="#">signal peptide</a> (IgK Leader), HA [???], Multiple cloning sites, <a href="#">transmembrane region</a> (PDGFR), <a href="#">poly-A tail</a> (BGH pA), rest of plasmid for replication and selection in E.coli.]</p>	<p>The diagram illustrates the pDisplay™ 5.3 kb plasmid. It features a circular structure with the following components: a T7 promoter (yellow arrow), an Igκ Leader (blue box), an HA tag (blue box), a multiple cloning site (MCS) containing restriction sites (Sfi I, Bgl II, Xma I, Sma I, Sac II, Pst I, Sal I, Acc I), a PDGFR Transmembrane Domain (blue box), a BGH pA-pUC ori (yellow arrow), a Kan/Neo resistance gene (red box), a Psv40/ori (yellow arrow), and an Ampicillin resistance gene (yellow arrow). The CMV promoter (green arrow) is also indicated.</p>