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# The Effect of pH on Green Fluorescent Protein: a Brief Review

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**Abstract**

**Green fluorescent protein (GFP) is rapidly becoming one of the most frequently employed molecular reporters. Its use in monitoring gene expression and protein localization has been well documented. Different mutational approaches have created numerous GFP variants with optimized expression, differing spectra, and differing pH sensitivity. This last characteristic, though still poorly understood mechanistically, has attracted an increasing amount of interest. To date, GFP variants have been developed with $pK_a$s ranging from 4.8 (T203I) to 8.0 (YFP-H148G/T203Y). The objective of this review is to outline both the effect of pH on GFP fluorescence and the uses of GFP to study processes in different pH environments.**

## Introduction

It is becoming increasingly difficult to peruse a biological, microbiological, or biochemical journal and avoid encountering the phrase "green fluorescent protein". The existence of the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* was reported decades ago (Shimomura *et al.* 1962, Morise *et al.* 1974). However, the cloning (Prasher *et al.* 1992) and heterologous expression of its cDNA (Chalfie *et al.* 1994) soon ignited an explosion of applications for GFP. Such applications include monitoring of gene expression (Li *et al.* 1999, Takeuchi *et al.* 1999, Wheeler *et al.* 2000), protein localization (Wang and Hazelrigg 1994, Kaether and Gerdes 1995, Lim *et al.* 1995, Harada *et al.* 2000), host-pathogen interactions (Dhandayuthapani *et al.* 1995, Valdivia *et al.* 1996), cellular dynamics (Rizzuto *et al.* 1995, Gerdes and Kaether 1996, Fricker *et al.* 1999), protein purification (Cha *et al.* 1999, Dabrowski *et al.* 1999), $Ca^{2+}$ concentration (Miyawaki *et al.* 1997, Romoser *et al.* 1997, Allen *et al.* 1999), and pH levels (Kneen *et al.* 1998, Llopis *et al.* 1998, Miesenböck *et al.* 1998, Robey *et al.* 1998).

Encoded wild-type GFP is 238 amino acids and approximately 27 kD (Prasher *et al.* 1992). It absorbs maximally at ~393 nm with a minor peak at 473 nm, and emits green light at 509 nm (Ward *et al.* 1980). Different mutational approaches have optimized expression by altering the promoter, codon usage, or ribosome binding; by eliminating splicing; or by enhancing folding. GFP variants with differing spectra have also been created, permitting multicolor microscopic visualization (Rizzuto *et al.* 1996, Palm and Wlodawer 1999). GFP fluorescence is due to the presence of a chromophore intrinsic to the primary structure, thus requiring no additional cofactors. The chromophore is a p-hydroxybenzylideneimidazolinone formed from $Ser^{65}$-$Tyr^{66}$-$Gly^{67}$. Fluorescence is acquired through the creation of the imidazolinone by nucleophilic attack of the amino group of $Gly^{67}$ on the carbonyl group of $Ser^{65}$, followed by dehydration, and then by oxidation of the hydroxybenzyl side chain of $Tyr^{66}$ by atmospheric oxygen. The beta-can crystal structure of wild-type GFP consists of an 11-stranded beta-barrel with an alpha-helix running up the axis of the cylinder. The chromophore is attached to the alpha-helix and buried in the center of the cylinder (Ormö *et al.* 1996, Tsien 1998). The *in vitro* spectral properties of GFP are influenced by temperature, ionic strength, protein concentration, and pH (Ward *et al.* 1982). Recently, interest in the pH sensitivity of GFP has led to the successful introduction of GFP as a noninvasive pH indicator. This article briefly outlines the effect of pH on GFP fluorescence and the uses of GFP to study processes in different pH environments.

## Effect of pH on GFP

The fluorescence of wild-type GFP (wtGFP) is stable from pH 6-10, but decreases at pH<6 and increases from pH 10-12 (Ward 1981, Patterson *et al.* 1997). A number of studies have demonstrated that different GFP variants display greater pH sensitivity (Kneen *et al.* 1998, Haupts *et al.* 1998, Elsliger *et al.* 1999). In denatured wtGFP, the chromophore has pH-dependent spectral characteristics due to the ionization of the $Tyr^{66}$ phenolic group (Ward *et al.* 1982). The phenolate form of the chromophore absorbs maximally at 448 nm, compared with that of 384 nm for the uncharged phenol. The $pK_a$ for this transition is 8.1 (Ward *et al.* 1980). Based on spectroscopic examination of the excited state dynamics of GFP, Chattoraj *et al.* (1996) proposed that wtGFP exists in one of two ground states, A and B, which differ in the protonation state of the chromophore. An excited-state proton transfer reaction rapidly converts state A to an intermediate state I, which is then slowly converted to state B. The existence of two ground state conformations, each with distinct spectral characteristics, has been confirmed by crystallographic research (Brejc *et al.* 1997, Palm *et al.* 1997). The phenol in $Tyr^{66}$ is uncharged in GFP variants with excitation maxima at ~395 nm (corresponding to state A), while it is in the charged phenolate form in variants with excitation maxima at 473 nm (corresponding to state B). Kneen *et al.* (1998) suggested that pH shifts the equilibrium between the GFP A and B ground states. At high pH, the phenolate

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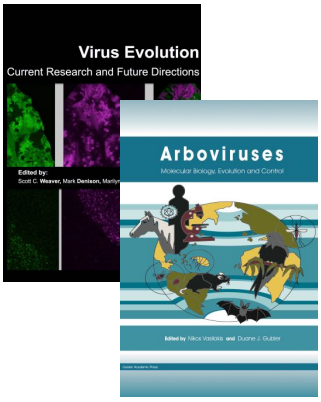


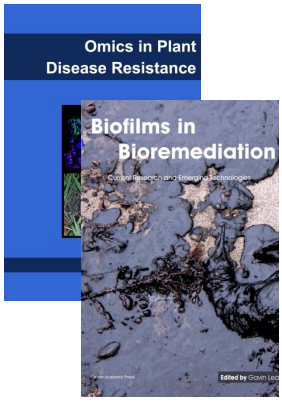


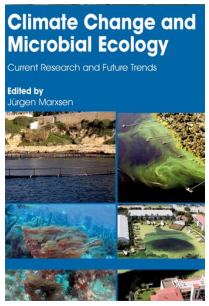


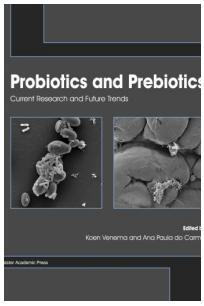


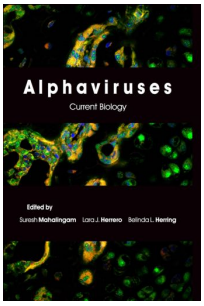



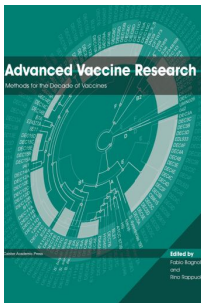


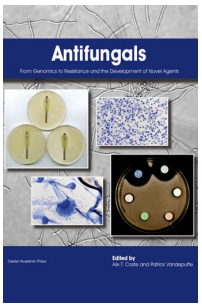


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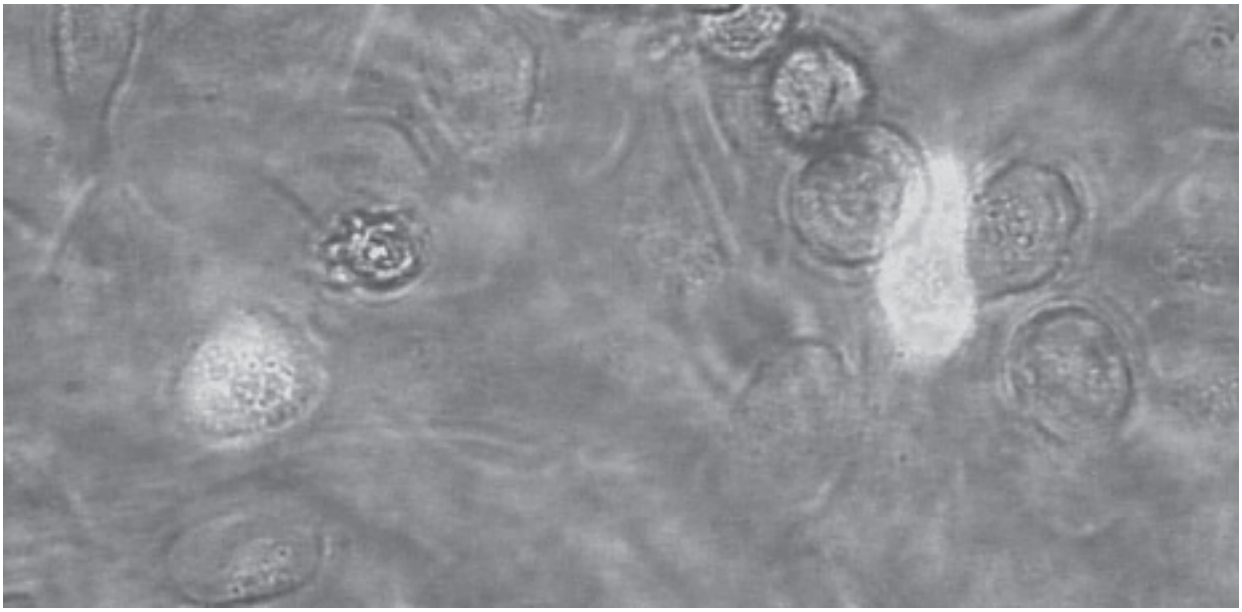

Green fluorescence demonstrating successful expression of the lysosomal hydrolase glucocerebrosidase (EC 3.2.1.45) fused to EGFP in Clontech's pEGFP-N1 vector in two HeLa cells 48 hours after transfection (source: T.N. Campbell, unpublished results, colour figure can be viewed online at www.molbio.net/v2/v2n1/01/01.html).

form of $Tyr^{66}$ is favored so that the B state is populated (excitation at 471 nm, emission at 500 nm). At low pH, the phenol form is favored so that the state A is populated and the absorbance shifts to 390 nm. The absence of fluorescence at 500 nm by excitation at 360 nm could be due to quenching of the I state or inability of the I state to convert to the B state. The authors noted that GFP pH sensitivity could involve simple protonation events at pH>5, but both protonation and conformational changes at lower pH.

As previously mentioned, different GFP variants have different pH sensitivities. Currently, GFP mutants have been developed with $pK_a$s ranging from 4.8 (T203I) to 8.0 (YFP-H148G/T203Y) (Table I). Such a range permits researchers to employ specific GFP variants to successfully study processes in either alkaline or acidic environments.

## Uses of GFP to Study Processes in Different pH Environments

Because of the relative stability of GFP fluorescence over a wide pH range, GFP can be used as a fusion tag to study various processes from subcellular dynamics to protein localization in different pH environments. Rizzuto *et al.* (1995) first demonstrated the ability of GFP to be targeted to the mitochondria, allowing the visualization of mitochondrial movement in living cells. Recently, Harada *et al.* (2000) constructed a chimera of EGFP and Wilson's disease gene product ATP7B. Although it is widely believed that ATP7B is located at the Golgi complex, the results indicated that its main localization is in the late endosomes. By combining previous data with the current results, the authors suggested that ATP7B may translocate copper from the cytosol to the late endosomal lumen, thus participating in biliary copper excretion via lysosomes. Furthermore, the disturbed incorporation of copper into the late endosomes caused by defective ATP7B may be the main defect in Wilson's disease.

Insights into molecule transport and secretion have also been obtained through the use of GFP fusions. In *Dictyostelium discoideum* cells, Hanakam *et al.* (1996) manipulated pH levels while monitoring intracellular translocation of histactophilin II fused to GFP. The results showed that histactophilin II differs from the majority of myristoylated proteins in that it translocates in a pH-dependent manner to the plasma membrane and does not require the myristoyl residue for membrane association. In leaf cells of *Nicotiana clevelandii,* Boevink *et al.* (1998) demonstrated for the first time *in vivo* brefeldin A-induced retrograde transport of Golgi membrane protein to the endoplasmic reticulum. Data also suggested that the leaf Golgi complex acts as a motile system of actin-directed stacks whose function is to pick up products from a relatively stable endoplasmic reticulum system. Wubbolts *et al.* (1996) fused GFP to the cytoplasmic tail of the class II beta chain of HLA-DR1 to visualize vesicular transport from lysosomal structures to the cell surface in human melanoma Mel JuSo cells. Results indicated that vesicles containing the chimeras did fuse with the plasma membrane and that this transport route did not intersect the earlier endosomal pathway. In one further example, Han *et al.* (1999) used a proANF/EGFP chimera expressed in rat PC12 pheochromocytoma cells to demonstrate that calcium influx into the cytoplasm rapidly alkalinizes the contents of the peptidergic secretory vesicles. This result suggests for the first time that the physical state of neuropeptides is changed in preparation for release.

The persistence of GFP fluorescence over a broad pH range has also been exploited to examine pH-induced plant pathogen gene expression. Tang *et al.* (1999)

Table I. GFP mutant $pK_a$s.

| Mutant (Common Name) | $pK_a$ | Reference(s) |
|---|---|---|
| None (wt, BioGreen) | 4.5 | Tsien 1998 |
| S65T (GFP-S65T) | 5.9, 6.0 | Kneen *et al.*1998, Elsliger *et al.*1999 |
| F64L/S65T (EGFP,GFPMut1,BioST) | 5.8, 6.0, 6.15 | Haupts *et al.*1998, Kneen *et al.*1998, Llopis *et al.*1998 |
| S65T/H148D | 7.8 | Elsliger *et al.*1999 |
| T203I | 4.8 | Kneen *et al.*1998 |
| S65G/S72A/T203Y/H231L (EYFP[a]) | 7.1 | Llopis *et al.*1998 |
| S65G/V68L/S72A/T203Y (YFP, EYFP) | 7.0 | Wachter *et al.*1998, Elsliger *et al.*1999 |
| S65G/V68L/S72A/T203Y/E222Q (YFP-E222Q) | 7.0 | Elsliger *et al.*1999 |
| S65G/V68L/S72A/H148G/T203Y (YFP-H148G) | 8.0 | Wachter *et al.*1998, Elsliger *et al.*1999 |
| S65G/V68L/S72A/H148Q/T203Y (YFP-H148Q) | 7.5 | Elsliger *et al.*1999 |
| K26R/F64L/S65T/Y66W/N146I/M153T/V163A/N164H/H231L (ECFP[a]) | 6.4 | Llopis *et al.*1998 |
| Y66H (BFP, P4, BioBlue) | 6.1 | Kneen *et al.*1998 |

[a]The authors use their own naming system with regards to the EYFP and ECFP variants. These differ in mutational composition from Clontech EYFP and ECFP.

constructed a Tn5 GFPuv-containing transposon derivative, termed mini-Tn5*gfp-km*, and used this to identify *Agrobacterium tumefaciens* genes that were upregulated in response to acidic pH. Also using a GFPuv-containing mini Tn5 transposon, Li *et al.* (1999) determined that a minimal medium of pH 5.5 was the most representative of the growth conditions that *A. tumefaciens* cells encounter in plant tissues.

A final example of the use of GFP to study processes in various pH environments is an experiment by Matsuyama *et al.* (2000) in which the authors targeted YFP-H148G to the mitochondrial matrix to examine early events that modulate caspase activation during apoptosis. Results indicated that the mitochondria-mediated alteration of intracellular pH may be an early event that regulates caspase activation in the mitochondrial pathway for apoptosis.

## GFP as a pH Indicator

The heightened pH sensitivity of GFP variants has been exploited to probe pH in living cells. Moreover, GFP has several characteristics that render it a useful pH indicator. GFP does not require ester permeation or hydrolysis of small dyes and is easily targetable to intracellular locations without leakage or migration. Furthermore, though some toxic effects of GFP have been noted (Liu *et al.* 1999), GFP is less detrimental to cells than chemical indicators requiring invasive loading procedures (Kneen *et al.* 1998, Llopis *et al.* 1998). Robey *et al.* (1998) successfully used GFPMut1 (F64L/S65T) to monitor intracellular pH of BS-C-1 (African green monkey kidney) and rabbit renal proximal tube cells in real time. Kneen *et al.* (1998) used the same mutant (F64L/S65T) to study cytoplasmic and organellar pH levels and pH regulation. Mitochondrial pH was found to be >7.5, but could not be determined accurately because of the much lower $pK_a$ (6.0) of the GFP mutant used. In HeLa cells, Llopis *et al.* (1998) employed a number of mutants ($pK_a$s 6.15-7.1) to measure cytosolic, mitochondrial, and Golgi complex pH. The enhanced yellow fluorescent protein variant (S65G/S72A/T203Y/H231L) was found to be suitable for measuring all three subcellular domains, while EGFP was suitable for cytosolic and Golgi pH measurements.

In an effort to create greater pH-sensitive GFP reporters, Miesenböck *et al.* (1998) altered key amino acids known from crystallographic studies to either be part of the proton-relay network of $Tyr^{66}$ or to change the excitation spectrum when mutated (Ormö *et al.* 1996, Brejc *et al.* 1997). Two types of reporters, termed pHluorins, were generated. The first derivative, ratiometric pHluorin, has excitation peaks at 395 nm and 475 nm and undergoes a spectral shift as pH is changed within the physiologically relevant range. The second derivative, ecliptic pHluorin, behaves as a single wavelength probe with a quench in the 475 nm excitation peak with decreasing pH. These pHluorins were used to determine Golgi complex and endosome pH. Additionally, when fused to a vesicle membrane protein, these reporters were able to monitor the release and recycling of synaptic vesicles in hippocampal neurons, as reflected by pH-induced changes in fluorescence.

## Conclusions

Since GFP maintains fluorescence over a broad pH range, it can be successfully employed as a molecular reporter in different pH environments. Outside of the pH 6-10 range, however, GFP fluorescence is modified. Spectroscopic and crystallographic data indicate that the protonation state of the chromophore is responsible for GFP pH sensitivity. A number of pH sensitive mutants have been generated, permitting GFP to be utilized as a pH indicator or to monitor exocytic/endocytic events as reflected by pH-induced changes in fluorescence. Current GFP variants have $pK_a$s ranging from 4.8 (T203I) to 8.0 (YFP-H148G/T203Y). Thus, researchers are able to employ specific variants to study processes in either alkaline or acidic environments.

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#### References

Allen, G.J., Kwak, J.M., Chu, S.P., Llopis, J., Tsien, R.Y., Harper, J.F., and Schroeder, J.I. 1999. Cameleon calcium indicator reports cytoplasmic calcium dynamics in *Arabidopsis* guard cells. Plant J. 19: 7 35-747.

Boevink, P., Oparka, K., Santa Cruz, S., Martin, B., Betteridge, A., and

Hawes, C. 1998. Stacks on tracks: the plant Golgi apparatus traffics on an actin/ER network. Plant J. 15: 441-447.

Brejc, K., Sixma, T.K., Kitts, P.A., Kain, S.R., Tsien, R.Y., Ormö, M., and Remington, S.J. 1997. Structural basis for dual excitation and photoisomerization of the *Aequorea victoria* green fluorescent protein. Proc. Natl. Acad. Sci. USA 94: 2306-2311.

Cha, H.J., Dalal, N.G., Vakharia, V.N., and Bentley, W.E. 1999. Expression and purification of human interleukin-2 simplified as a fusion with green fluorescent protein in suspended Sf-9 insect cells. J. Biotechnol. 69: 9-17.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. 1994. Green fluorescent protein as a marker for gene expression. Science 263: 802-805.

Chattoraj, M., King, B.A., Bublitz, G.U., and Boxer, S.G. 1996. Ultra-fast excited state dynamics in green fluorescent protein: multiple states and proton transfer. Proc. Natl. Acad. Sci. USA 93: 8362-8367.

Dabrowski, S., Brillowska, A., and Kur, J. 1999. Use of the green fluorescent protein variant (YFP) to monitor MetArg human proinsulin production in *Escherichia coli*. Prot. Ex. Pur. 16: 315-323.

Dhandayuthapani, S., Via, L.E., Thomas, C.A., Horowitz, P.M., Deretic, D., and Deretic, V. 1995. Green fluorescent protein as a marker for gene expression and cell biology of mycobacterial interactions with macrophages. Mol. Microbiol. 17: 901-912.

Elsliger, M-A., Wachter, R.M., Hanson, G.T., Kallio, K., and Remington, S.J. 1999. Structural and spectral response of green fluorescent protein variants to changes in pH. Biochemistry 38: 5296-5301.

Fricker, M.D. and Oparka, K.J. 1999. Imaging techniques in plant transport: meeting review. J. Exp. Bot. 50: 1089-1100.

Gerdes, H-H., and Kaether, C. 1996. Green fluorescent protein: applications in cell biology. FEBS Lett. 389: 44-47.

Han, W., Li, D., Stout, A.K., Takimoto, K., and Levitan, E.S. 1999. $Ca^{2+}$-Induced deprotonation of peptide hormones inside secretory vesicles in preparation for release. J. Neurosci. 19: 900-905.

Hanakam, F., Albrecht, R., Eckerskorn, C., Matzner, M., and Gerish, G. 1996. Myristoylated and non-myristoylated forms of the pH sensor protein histactophilin II: intracellular shuttling to plasma membrane and nucleus monitored in real time by a fusion with green fluorescent protein. EMBO J. 15: 2935-2943.

Harada, M., Sakisaka, S., Terada, K., Kimura, R., Kawaguchi, T., Koga, H., Taniguchi, E., Sasatomi, K., Miura, N., Suganuma, T., Fujita, H., Furuta, K., Tanikawa, K., Sugiyama, T., and Sata, M. 2000. Role of ATP7B in biliary copper excretion in a human hepatoma cell line and normal rat hepatocytes. Gastroenterology 118: 921-928.

Haupts, U., Maiti, S., Schwille, P., and Webb, W.W. 1998. Dynamics of fluorescence fluctuations in green fluorescent protein observed by fluorescence correlation spectroscopy. Proc. Natl. Acad. Sci. USA 95: 13573-13578.

Kaether, C. and Gerdes, H-H. 1995. Visualization of protein transport along the secretory pathway using green fluorescent protein. FEBS Lett. 369: 267-271.

Kneen, M., Farinas, J., Li, Y., and Verkman, A.S. 1998. Green fluorescent protein as a noninvasive intracellular pH indicator. Biophys. J. 74: 1591-1599.

Li, L., Li, Y., Lim, T.M., and Pan, S.Q. 1999. GFP-aided confocal laser scanning microscopy can monitor *Agrobacterium tumefaciens* cell morphology and gene expression associated with infection. FEMS Microbiol. Lett. 179: 141-146.

Lim, C.R., Kimata, Y., Oka, M., Nomaguchi, K., and Kohno, K. 1995. Thermosensitivity of green fluorescent protein fluorescence utilized to reveal novel nuclear-like compartments in a mutant nucleoporin NSP1. J. Biochem. 118: 13-17.

Liu, H-S., Jan, M-S., Chou, C-K., Chen, P-H., and Ke, N-J. 1999. Is green fluorescent protein toxic to the living cells? Biochem. Biophys. Res. Commun. 260: 712-717.

Llopis, J., McCaffery, J.M., Miyawaki, A., Farquhar, M.G., and Tsien, R.Y. 1998. Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. Proc. Natl. Acad. Sci. USA 95: 6803-6808.

Matsuyama, S., Llopis, J., Deveraux, Q.L., Tsien, R.Y., and Reed, J.C. 2000. Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activity during apoptosis. Nat. Cell Biol. 2: 318-325.

Miesenböck, G., De Angelis, D.A., and Rothman, J.E. 1998. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature 349: 192-195.

Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikura, M., and Tsien, R.Y. 1997. Fluorescent indicators for $Ca^{2+}$ based on green fluorescent proteins and calmodulin. Nature 388: 882-887.

Morise, H., Shimomura, O., Johnson, F.J., and Winant, J. 1974. Intermolecular energy transfer in the bioluminescent system of *Aequorea*. Biochemistry 13: 2656-2662.

Ormö, M., Cubitt, A.B., Kallio, K., Gross, L.A., Tsien, R.Y., and Remington, S.J. 1996. Crystal structure of the *Aequorea victoria* green fluorescent protein. Science 273: 1392-1395.

Palm, G.J. and Wlodawer, A. 1999. Spectral variants of green fluorescent protein. Methods Enzymol. 302: 378-394.

Palm, G.J., Zdanov, A., Gaitanaris, G.A., Stauber, R., Pavlakis, G.N., and Wlodawer, A. 1997. The structural basis for spectral variations in green fluorescent protein. Nature Struct. Biol. 4: 361-365.

Patterson, G.H., Knobel, S.M., Sharif, W.D., Kain, S.R., and Piston, D.W. 1997. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. Biophys. J. 73: 2782-2790.

Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G., and Cormier, M.J. 1992. Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene 111: 229-233.

Rizzuto, R., Brini, M., Pizzo, P., Murgia, M., Pozzan, T. 1995. Chimeric green fluorescent protein as a tool for visualizing subcellular organelles in living cells. Curr. Biol. 5: 635-642.

Rizzuto, R., Brini, M., De Gorgi, F., Rossi, R., Heim, R., Tsien, R.Y., and Pozzan, T. 1996. Double labelling of subcellular structures with organelle-targeted GFP mutants *in vivo*. Curr. Biol. 6: 183-188.

Robey, R.B., Ruiz, O., Santos, A.V.P., Ma, J., Kear, F., Wang, L-J., Li, C-J., Bernardo, A.A., and Arruda, J.A.L. 1998. pH-dependent fluorescence of a heterologously expressed *Aequorea* green fluorescent protein mutant: In situ spectral characteristics and applicability to intracellular pH estimation. Biochemistry 37: 9894-9901.

Romoser, V.A., Hinkle, P.M., and Persechini, A. 1997. Detection in living cells of $Ca^{2+}$-dependent changes in the fluorescence emission of an indicator composed of two green fluorescent protein variants linked by a calmodulin-binding sequence. J. Biol. Chem. 272: 13270-13274.

Shimomura, O., Johnson, F.H., and Saiga, Y. 1962. Extraction, purification and properties of Aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*. *J. Cell Comp. Physiol.* 59: 223-239.

Takeuchi, Y., Yoshizaki, G., and Takeuchi, T. 1999. Green fluorescent protein as a cell-labeling tool and a reporter of gene expression in transgenic rainbow trout. Mar. Biotechnol. 1: 448-457.

Tang, X., Lu, B.F., and Pan, S.Q. 1999. A bifunctional transposon mini-Tn*5gfp-km* which can be used to select for promoter fusions and report gene expression levels in *Agrobacterium tumefaciens*. FEMS Microbiol. Lett. 179: 37-42.

Tsien, R.Y. 1998. The green fluorescent protein. Annu. Rev. Biochem. 67: 509-544.

Valdivia, R.H. and Falkow, S. 1996. Bacterial genetics by flow cytometry: rapid isolation of *Salmonella typhimurium* acid-inducible promoters by differential fluorescence induction. Mol. Microbiol. 22: 367-378.

Wachter, R.M., Elsliger, M-A., Kallio, K., Hanson, G.T., and Remington, S.J. 1998. Structural basis of spectral shifts in the yellow-emission variants of green fluorescent protein. Curr. Biol. 6: 1267-1277.

Wang, S. and Hazelrigg, T. 1994. Implications for *bcd* mRNA localization from spatial distribution of *exu* protein in *Drosophila* oogenesis. Nature 369: 400-403.

Ward, W.W. 1981. Properties of the coelenterate green-fluorescent proteins. In: Bioluminescence and Chemiluminescence: Basic Chemistry and Analytical Applications. DeLuca, M.A. and McElroy, W.D., eds, Academic Press, New York, pp. 235-242.

Ward, W.W., Cody, C.W., Hart, R.C., and Cormier, M.J. 1980. Spectrophotometric identity of the energy transfer chromophores in *Renilla* and *Aequorea* green-fluorescent proteins. Photochem. Photobiol. 31: 611-615.

Ward, W.W., Prentice, H.J., Roth, A.F., Cody, C.W., and Reeves, S.C. 1982. Spectral perturbations of the *Aequorea* green-fluorescent protein. Photochem. Photobiol. 35: 803-808.

Wheeler, G.N., Hamilton, F.S., and Hoppler, S. 2000. Inducible gene expression in transgenic *Xenopus* embryos. Curr. Biol. 10: 849-852.

Wubbolts, R., Fernandez-Borja, M., Oomen, L., Verwoerd, D., Janssen, H., Calafat, J., Tulp, A., Dusseljee, S., and Neefjes, J. 1996. Direct vesicular transport of MHC Class II molecules from lysosomal structures to the cell surface. J. Cell Biol. 135: 611-622.