Teal fluorescent proteins: Characterization of a reversibly photoswitchable variant

Hui-wang Ai and Robert E. Campbell*
Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

ABSTRACT

Fluorescent proteins (FPs) emerged in the mid 1990s as a powerful tool for life science research. Cyan FPs (CFPs), widely used in multicolor imaging or as a fluorescence resonance energy transfer (FRET) donor to yellow FPs (YFPs), were considerably less optimal than other FPs because of some relatively poor photophysical properties. We recently initiated an effort to create improved or alternate versions of CFPs. To address the limitations of CFPs, an alternative known as monomeric teal FP1 (mTFP1) was engineered from the naturally tetrameric *Clavularia* CFP, by screening either rationally designed or random libraries of variants. mTFP1 has proven to be a particularly useful new member of the FP 'toolbox' by facilitating multicolor live cell imaging. During the directed evolution process of mTFP1, it was noticed that some earlier variants underwent fast and reversible photoisomerization. Some of the initial characterization of one particular mutant, designated as mTFP0.7, is described in this manuscript.

Keywords: fluorescent proteins, protein engineering, directed evolution, reversible photoisomerization

1. INTRODUCTION

The most intriguing feature of jellyfish and coral fluorescent proteins (FPs) is their associated visible-wavelength fluorophore which results from a self-catalyzed post-translation modification that only requires molecular oxygen.[1] Accordingly, when the gene encoding an FP is expressed in a variety of organisms ranging from bacteria to plants to mammals a functional (i.e. fluorescent) protein product is produced. The ability to *genetically encode a fluorophore* enables investigators to apply molecular biology to create FP chimeras with proteins of interest and subsequently monitor intracellular protein localization in a variety of cells and tissues using optical microscopy and related methodology.

Osamu Shimomura is credited with discovering green fluorescent protein (GFP) in 1961 when he was isolating aequorin, a calcium-dependent bioluminescent protein from the *Aequorea victoria* jellyfish collected from the sea.[2] Several decades had to pass before efforts to develop GFP as a genetically encoded tracer molecule were seriously pursued. The initial breakthrough in this regard was the cloning of the gene by Prasher in 1992.[3] Within just two more years the first reports of GFP expression in organisms other than jellyfish, including *E. Coli* and *C. elegans*, started to appear.[4, 5] However, it was soon realized that wild-type GFP is sub-optimal for live cell imaging due to drawbacks such as dual excitation peaks and poor folding efficiency. Fortunately improved variants of GFP, including the variant known as enhanced green fluorescent protein (EGFP), were soon reported.[1] In addition, mutants with new colors such as blue (BFP), cyan (CFP) and yellow (YFP) followed soon after.[6, 7] Another landmark development in the history of FPs was the discovery of GFP-like proteins in corals by Lukyanov.[8] Among the first reported coral FPs was the tetrameric red fluorescent protein known as *Discosoma* Red or DsRed (also known as dsFP583). A handful of monomeric proteins with emission wavelengths ranging from yellow to far-red (known as the "mFruit" series) have been engineered from DsRed.[9, 10]

The chromophore of wild-type GFP is autonomously generated within the protein by a process that involves a main chain cyclization, dehydration, and oxidation by molecular oxygen. In the ground state of the wild type protein, the chromophore exists as a mixture of neutral phenol (λ_{ex} = 395 nm) and anionic phenolate forms (λ_{ex} = 475 nm) (**Figure 1A and 1B, respectively**). In the excited state, the neutral phenol form deprotonates through as the process of excited-state proton transfer (ESPT) to form the phenolic anion and therefore only a single emission peak (λ_{em} = 504 nm) is observed, regardless of which form of the protein is excited. Directed evolution and engineering of *Aequorea* GFP have resulted in homologues with tyrosine-derived chromophores and red-shifted fluorescence emission (i.e. EGFP λ_{em} = 510 and EYFP λ_{em} = 529 nm) (**Figure 1B and 1E**). EYFP is significantly spectrally red-shifted because of the introduced π-π

* robert.e.campbell@ualberta.ca; phone 1 780 492-1849; fax 1 780 492-8231; www.chem.ualberta.ca/~campbell/

Small Animal Whole-Body Optical Imaging Based on Genetically Engineered Probes
edited by Alexander P. Savitsky, Robert E. Campbell, Robert M. Hoffman
Proc. of SPIE Vol. 6868, 68680D, (2008) · 1605-7422/08/$18 · doi: 10.1117/12.761423

interaction by stacking a tyrosine with the chromophore.[1] The widely used EBFP and ECFP variants were engineered by replacing Tyr66 of *Aequorea* GFP with a histidine or tryptophan to give an imidazole or indole-containing chromophore (**Figure 1C and 1D, respectively**).[1] CFP and YFP pair is the best choice of FRET partners, considering the brightness, photostability and spectral separation among all available colors. While ECFP has stood the test off time in multicolor labeling applications and as the preferred FRET donor to EYFP, its spectral properties still limit its utility in some applications: ECFP is relatively dim, has broad excitation and emission peaks, and has a multi-exponential lifetime. These problems with ECFP prompted us to attempt to engineer new monomeric FPs of cyan hues with improved spectroscopic properties.

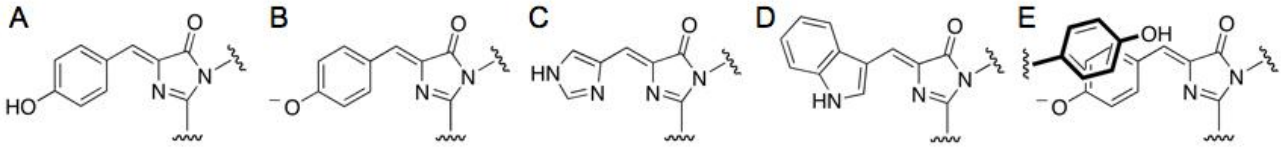

Figure. 1. The chromophore structures of **A)**. wild-type GFP in the ground state (majority). **B)**. wild-type GFP in the ground state (minority), in the excited state, or EGFP. **C)** EBFP. **D)**. ECFP. **E)**. EYFP.

A relatively recent development in the field of FPs is the discovery of variants that can be switched 'on' or 'off' or converted to a new color by irradiation with specific wavelengths of light.[11] Reversible photoswitching of individual molecules has been discovered for a number of mutants of the green fluorescent protein (GFP) since late 1990s.[12] A recent study reported that Dronpa, a mutant of a GFP-like fluorescent protein cloned from a coral *Pectiniidae*, underwent on/off photoswitching in both batch of protein or single molecular level.[13, 14] 488 and 405 nm lights can drive the protein back and forth between the bright and dark states. The protein Dronpa represents a class of fluorescent proteins that undergo reversible photoisomerization. Those proteins usually referred as 'optical highlighters' could be applied for highly novel applications such as exploring fast protein dynamics in living cells.[11]

2. METHODOLOGY

2.1. General methods

All general molecular biology were carried out as previously described.[15, 16] The cDNA sequences for all FP variants and fusion constructs were confirmed by dye terminator cycle sequencing using the DYEnamic ET kit (Amersham Biosciences). Sequencing reactions were analyzed at the University of Alberta Molecular Biology Service Unit. All filters for fluorescence screening and imaging were purchased from Chroma Technology (Rockingham, VT), Omega Filters (Brattleboro, VT) and Semrock (Rochester, NY).

2.2. Protein purification

The proteins were purified as previous.[15] *Escherichia coli* strain LMG194 was transformed with the pBAD/His B expression vector containing the FP gene of interest. A single colony was used to inoculate a 4 ml culture that was allowed to grow overnight (37 °C, 225 rpm) before being diluted into 1 L of LB medium containing ampicillin (0.1 mg/ml) and L-arabinose (0.2%). The culture was allowed to grow for 5 h before cells were harvested by centrifugation and lysed by French Press or sonification. Proteins were purified by Ni-NTA chromatography (Amersham).

2.3. Absorption measurements

Absorption spectra of mTFP0.7 were recorded on a DU-800 UV-visible spectrophotometer. Fresh protein purified by the method described above was diluted into PBS and contained in a 50 µl measuring cuvette. The absorption spectrum measured instantly was considered as in the fluorescent state. Then six Royal Blue (peak emission at 455 nm) Luxeon V LEDs (light emitting diodes) (Lumileds Lighting) with narrow beam lenses (Fraen) were used to illuminate the protein in the cuvette for 15 min after laying mineral oil on top of the solution to prevent evaporation. Then the cuvette was quickly moved into the spectrophotometer and the spectrum recorded was considered as in the dark state.

2.4. Kinetics

On a clean plastic surface, 5 µl protein of mTFP0.7 was added as a drop, followed by laying 20 µl mineral oil to prevent evaporation. The LEDs described above were used to bleach the protein drop. The intensity of light was adjusted

to 6 mW/cm^2, 3.3 mW/cm^2, 2 mW/cm^2 or 1 mW/cm^2 by connecting different numbers of LEDs or changing the distances between LEDs and the protein drop. Much weaker light (~ 0.04 mW/cm^2) bandpass-filtered from a Xe-arc lamp was used to record the fluorescence. The LEDs could be switched on and off at computer-controlled intervals. An Image Pro Plus macro (Media Cybernetics) was used to automate acquisition and processing. For each measurement, fluorescence images were acquired following a series of programmed intervals of intense illumination.

To measure the temperature dependence of fluorescence recovery of mTFP0.7, a glass well bathed by a temperature-controllable water circulating system was used. 500 μl protein solution was contained in the well for 15 min at a certain temperature, followed by illuminating the solution by LEDs for 15 min. Same system described above was used to record the fluorescence recovery of the solution.

All curves were fitted by OriginLab 7.0 to derive the rates of reactions.

3. RESULTS AND DISCUSSION

3.1. The structure-guided evolution of cyan/teal fluorescent proteins

Recently, we reported a new monomeric teal fluorescent protein (mTFP1) and tested it for live cell imaging and other applications.[15, 17] When this project was initiated in 2003, there were 3 reported naturally occurring fluorescent proteins with tyrosine-derived chromophores and λ_{em} < 490 nm.[8] They were *Clav*CFP (a.k.a. cFP484) from *Clavularia sp.,* dsFP483 from *Discosoma striata,* and amFP486 from *Anemonia majano*. Later on, another protein, mcCFP477 from *Montastrea cavernosa*, was also reported.[18] Although commonly referred to as a 'CFP', MiCy (*Acropara sp.)* has a λ_{em} = 495 nm (Karasawa et al. 2004). The naturally occurring CFPs have quantum yields (QY) of 0.24-0.48 and extinction coefficients (EC) of 24,000-40,000M^{-1}cm^{-1}. They all have 4° structure with some reported to be trimers and some tetramers. Therefore they are not suitable for use as non-perturbing genetic fusion partners.

A sequence alignment revealed that cFP484 is the least divergent from a hypothetical 'consensus' sequence. In addition, cFP484 has been reported to have the highest fluorescence brightness and the fewest cysteine residues. In consideration of these factors we chose to use the cFP484 amino acid sequence as the starting point for the directed evolution process.

At that time, we did not have any crystal structure of cFP484 or one of its cyan homologues. We therefore used the DsRed crystal structure[19] to model the cFP484. We rationally designed and synthesized a gene library with a theoretical diversity of approx. 5×10^5 cFP484 variants. The most important feature of this library is the semi-degenerate codons encoding potential 'tetramer-breaking' mutations at three external positions sitting on the A-B interface of the modeling structure; and semi-degenerate codons encoding potential 'rescuing' mutations at eleven internal positions of the modeling structure.

The synthetic gene library was used to transform *E. coli* and colonies were screened for fluorescence brightness. The bright dimeric protein dTFP0.1 was selected and subjected for further evolution. The remaining dimer interface of the improved version dTFP0.2 was disrupted to provide the monomeric version mTFP0.3. Interface disruption was achieved by mutating two residues on the A-C interface of the modeled tetrameric structure. Following multiple successive rounds of screening libraries, generated by random or site-targeted mutagenesis, for variants with improved brightness and high 480/530 nm emission ratio, we eventually arrived at mTFP0.7 that has high fluorescence brightness (**Table 1**). However, when we attempted to image an mTFP0.7–beta-actin fusion in live HeLa cells by confocal microscopy, we discovered that the fluorescent signal rapidly disappeared upon illumination with the 458 nm laser.[15]

To screen for photostable variants we constructed an array of six 460 nm LEDs (Lumileds, San Jose, CA, U.S.A.) that provides more intense light than the excitation used in previous screening. The new light source is sufficient to photoconvert mTFP0.7 expressed in a colony of *E. coli*. The fluorescence of libraries of mTFP0.7 variants expressed in bacterial colonies was digitally imaged during exposure to intense illumination and colonies with decreased propensity to photobleach were identified. Following several rounds of selection for variants that were photostable, bright and retained a high 480/530 nm emission ratio, we arrived at mTFP1 that has a total of 31 amino acid replacements relative to the wild-type protein.

mTFP1 displays red-shifted spectral profiles (excitation and emission maxima at 462 and 492 nanometers, respectively) when compared to other cyan fluorescent proteins, such as ECFP.[15] So the color 'teal' was used to refer this

protein. Whereas ECFP has a tryptophan-derived chromophore, mTFP1 contains the tyrosine-derived chromophore. Not surprisingly, fluorescence decay of mTFP1 could be fitted monoexponentially.[15] Also the difference in chromophore structure results in narrowed emission profile relative to ECFP, which is useful for reducing bleed-through in multi-color experiments. We have demonstrated that mTFP1 provides an excellent alternative to other ECFP derivatives as a FRET donor to either yellow or orange fluorescent proteins.[15] mTFP1 showed robust ability to use as a fusion partner to a variety of proteins.[17] Considering its low pKa, high brightness and high photostability, mTFP1 is one of the most optimal fluorescent proteins for live cell imaging (**Table 1**).[15]

Table 1. Fluorescent properties of different fluorescent proteins

	Absorbance or excitation (nm)	Emission (nm)	EC[a]*10^{-3} ($M^{-1}cm^{-1}$)	QY[b]	Brightness[c] ($mM^{-1}cm^{-1}$)	pKa	Relative $t_{0.5}$ for bleaching[d]
ECFP	433/451[e]	475/504[e]	33/30[e]	0.41	13/12[e]	4.7	64
mTFP0.7	453	488	60	0.50	30	4.0	<0.10
mTFP1	462	492	64	0.85	54	4.3	110
EGFP	488	507	56	0.60	34	6	174

a Extinction coefficient. **b** Quantum yield. **c** Product of EC*10^{-3} and QY. **d** Time to bleach from an initial emission rate of 1000 photons/s/molecule to 500 photons/s/molecule. **e** Wavelength maxima, EC, and brightness of both 'humps' of ECFP are quoted.

3.2. Photophysical characterization of mTFP0.7

When mTFP0.7 was used for live cell imaging in conjunction with confocal fluorescence microscopy, the fluorescence bleached so quickly that the acquisition of even a single image was unsuccessful. Upon careful investigation of this phenomenon, we found that mTFP0.7 underwent a light-induced reversible transition from the fluorescent state to a 'bleached' state. That is, the cyan fluorescent state diminished when illuminating the sample with 458 nm light, but was slowly and spontaneously replenished in the dark. To characterize the two states, we measured the UV-vis absorbance of the same protein solution in both states (**Figure 3A**). The freshly purified 'bright' state of the protein absorbs lights with a 453 nm peak while the 'dark' state with the absorption maximum at 376 nm. When exiting the 'dark' protein at 376 nm, the protein shows no detectable fluorescence. Previous studies of GFP chromophores have demonstrated that the protonated form of GFP absorbs at 395 nm maximally. We suspected that the 376 nm-absorbing 'dark' state of mTFP1 corresponds to a protonated chromophore state and the photobleaching of mTFP0.7 involves a protonation event. Another phenomenon we noticed was the bleached protein took about 30 minutes to relax back to the initial fluorescent state in the room temperature. Because of the relatively slow rate of recovery, certainly much slower than expected for a simple deprotonation event, we speculated that there should be additional molecular rearrangments or reactions involved. A reasonable hypothesis was that the photobleaching (and recovery) were associated with cis-trans isomerization about the double bond between what was formerly the Cα-Cβ of Tyr67. In a subsequent collaboration with S. James Remington of the University of Oregon, the X-ray crystal structures of mTFP0.7 in both the 'bright' and 'dark' states were determined.[20] The crystal structures clearly supported the cis-trans isomerization hypothesis (**Figure 2**). In addition, the hydrogen bonding of the chromophore phenol oxygen and the hydrophobicity of the chromophore environment correlated with our previous protonation/deprotonation proposal. That is, the chromophore was protonated in the 'dark' trans state and deprotonated in the 'bright' cis state.

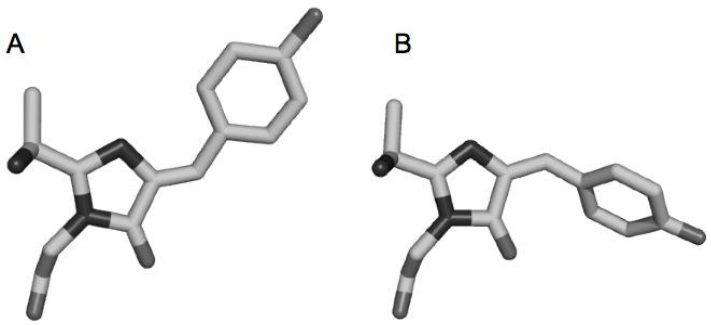

Figure. 2. The chromophore structures of mTFP0.7 in **A)** fluorescent state and **B)** dark state. The structures were drawn from PDB 2OTB and 2OTE.

In order to further characterize the isomerization process, we measured the kinetics of bleaching and recovery by imaging the fluorescent changes of protein drops under mineral oil. The intensity of light used to bleach the protein was controlled by connecting different numbers of LEDs or changing the distances between LEDs and samples. Low intensity filtered light from a Xe-arc lamp was used to record the fluorescence. The bleaching curves under 6 mW/cm^2, 3.3 mW/cm^2, 2 mW/cm^2 and 1 mW/cm^2 were recorded respectively (**Figure 3B**). The recovery processes for each protein drop bleached by different light intensities were recorded as well. Under the measured light intensity range, the recovery processes showed little kinetic difference. But the rates of bleaching reaction were strongly dependent on the intensity of light. Both the fluorescence loss and recovery could be fitted as first order reaction. When plotting the rates of fluorescence loss with the light intensities, strong linear correlation was shown (**Figure 3C**). The detailed mathematical analysis showed the half-life of the bleaching reaction to be 66 seconds per mW/cm^2 when illuminated with the Royal Blue (peak emission at 455 nm) Luxeon V LEDs. Those numbers could only be used to estimate the rate of reaction under light intensity ranging in mW/cm^2, because the light effect is most likely to be non-linear in much higher light intensity condition, e.g. > 1 W/cm^2.

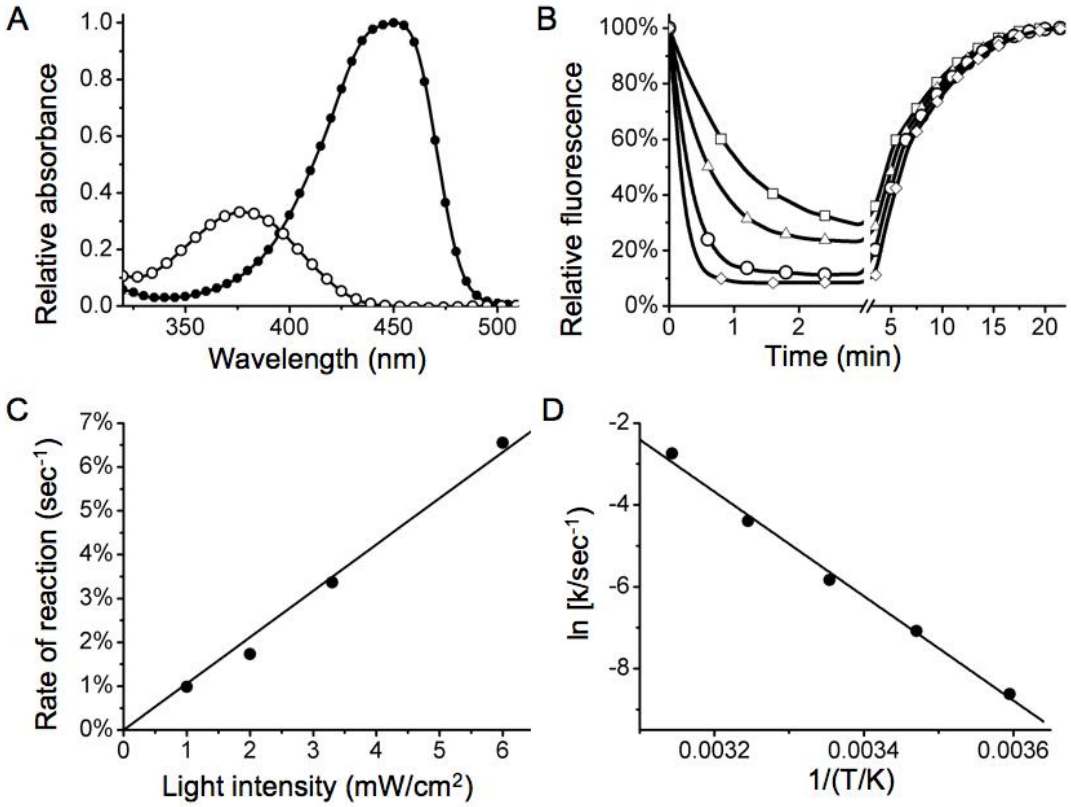

Figure. 3. **A)** The absorbance of mTFP0.7 in the fluorescent state and dark state. **B)** The fluorescence loss of mTFP0.7 under different light intensities (from top to bottom: 6, 3.3, 2 and1 mW/cm^2) and the respective recovery. **C)** The dependence of the rates of fluorescence loss of mTFP0.7 on light intensities. (Linear fit: y = 1.06% x, R=0.994) **D)** The Arrhenius plot of the fluorescence recovery of mTFP0.7. (Linear fit: y = -12757x + 37.147, R = 0.997)

To characterize the fluorescence recovery reaction, a bleached solution was incubated at a variety of temperatures and the fluorescence was recorded over time. The resulting curves were fitted as first order reactions in OriginLab 7.0. The rates of reaction showed strong dependence on temperature (**Table 2**). For example, the half-life of the reaction is 300 longer at 5 °C than at 45 °C. The rates of reaction were calculated by fitting the curves and are shown in **Table 2**. Using the rates of reaction and the corresponding temperatures, the activation energy of the trans-cis isomerization was identified as ~ 106 kJ/mol (**Figure 3D**). The energy barrier for mTFP0.7 trans-cis relaxation is significantly higher than the barrier of ~71 kJ/mol determined for the KFP cis-trans relaxation.[21] Correspondingly the KFP cis-trans relaxation (half-life to be ~ 70 s at room temperature) is much faster than mTFP0.7 trans-cis isomerization.[21]

Table 2. The kinetics of the fluorescence recovery of mTFP0.7 at different temperatures

Temperature	Rate of reaction **k** (sec^{-1})	Half-life $\mathbf{t_{1/2}}$ (min)	ln**k**	1/**T**
5 °C	1.80E-04	64.2	-8.62	0.0036
15 °C	8.40E-04	13.7	-7.08	0.0035
25 °C	2.91E-03	4.0	-5.84	0.0034
35 °C	1.23E-02	0.9	-4.40	0.0032
45 °C	6.43E-02	0.2	-2.74	0.0031

3.3. Prospects for use of mTFP0.7 in live cell imaging

mTFP0.7 undergoes fast photobleaching that limits its use in conventional microscope. However, like Dronpa and its mutants, mTFP0.7 may find application in some specialized experiments.[13, 22] For example, simultaneous 400 nm and 458 nm irradiation of mTFP0.7 might produce significant fluorescence by keeping a significant population of the molecules in the 'bright' state. This approach could be used to spatially limit the volume of excited fluorophores, providing some of the advantages of 2-photon excitation, such as improved spatial resolution (z-axis) and optical sectioning in confocal microscopy. Further more, this protein could be applied for other super resolution microscopy techniques, such as RESOLFT[23] and PALM[24].

CONCLUSION

mTFP1, a valuable addition to the fluorescent protein 'toolbox', is the brightest and most photostable fluorescent protein in its respective color class. The new reversibly photoswitchable mTFP0.7 has proven to be transiting between a fluorescent state with a cis and deprotonated chromophore, and a dark state with a trans and protonated chromophore. We expect that further development of this variant may lead to yet another useful addition to the FP 'toolbox' for use in live cell imaging.

ACKNOWLEDGMENTS

This research was made possible with support from the University of Alberta, the Canadian Foundation for Innovation, the Natural Sciences and Engineering Research Council (NSERC) of Canada, and an Alberta Ingenuity New Faculty Grant. The authors thank the University of Alberta Molecular Biology Service Unit for analysis of DNA sequencing reactions. REC holds a Canada Research Chair in Bioanalytical Chemistry. Finally, the authors would like to gratefully acknowledge the invaluable contributions of S. James Remington, J. Nathan Henderson, and Michael W. Davidson to this and other collaborative projects.

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