The Optical Properties for Bilirubin-Inducible Fluorescent Protein (holoUnaG)

Chang Ho Kim[1,2], Euihyun Lee[1,2], Bonghwan Chon[1,2], Jiwoong Kwon[1,2], Sang-Hee Shim[*,1,2], Minhaeng Cho[*,1, 2]

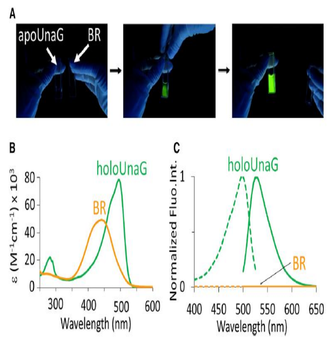
1. Center for Molecular Spectroscopy and Dynamics, Institute for Basic Science (IBS), Seoul 02841, Republic of Korea

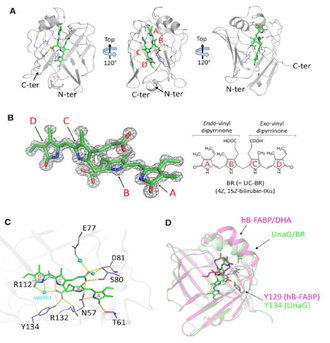
2. Department of Chemistry, Korea University, Seoul 02841. Republic of Korea

ABSTRACT

Bilirubin-inducible fluorescence protein (holoUnaG) was recently discovered and identified from eel muscle by the Miyawaki group. Fluorescence proteins like GFP have been used in a variety of biomedical researches. HoloUnaG of which size is smaller GFP is likely to be of use in studying biomedical and super-resolution researches. However, the optical and spectroscopic properties of holoUnaG have not been studied yet. For instance, even its fluorescence lifetime has not been clearly measured yet. Recently, we have carried out fluorescence lifetime measurement of holoUnaG *in vitro* and found that there are two fluorescence decay components, 2.2~2.3 ns and 0.8~0.9 ns. The relative amplitudes of these two components are about 80 and 20 %, respectively. It is believed that the slow component with 2.2~2.3 ns decay constant corresponds to the excited state lifetime of holoUnaG, whereas the fast component with 0.8~0.9 ns decay constant is from photo-damaged bilirubin or photo-isomerized bilirubin. We have also investigated the origin of significantly enhanced fluorescence intensity of holoUnaG as compared to those of either bovine serum albumin (BSA)-bilirubin complex or bilirubin molecule itself. Using electronic circular dichroism spectroscopy and quantum chemistry calculation methods, we were able to elucidate the mechanisms of fluorescence enhancement and extended lifetime of holoUnaG.

Fluorescence Protein (HoloUnaG, Protein-Encapsulated Bilirubin)





<Atsushi Miyawaki et al. Cell, 2013, 153, 1602–1611 (HoloUnaG)>

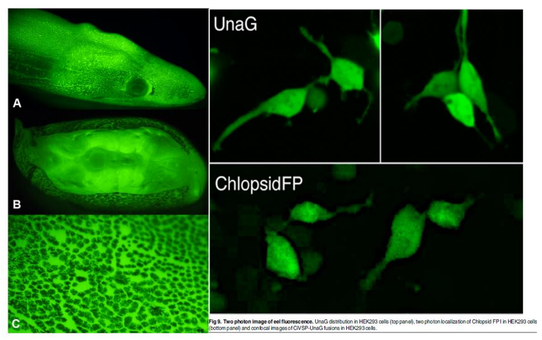
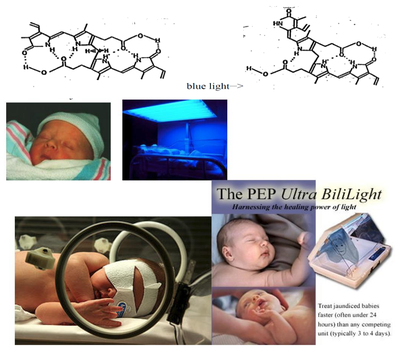


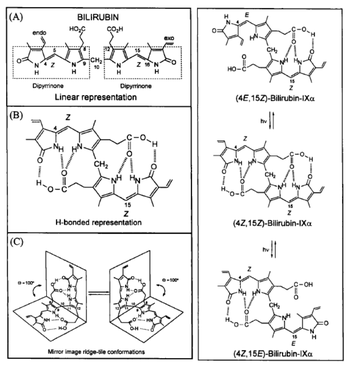
Fig 8. Two photon image of eel fluorescence. UnaG distribution in HEK293 cells (top panel), two photon localization of Chlopsid FP1 in HEK293 cells (bottom panel) and confocal images of CVSP-UnaG fusions in HEK293 cells.

<David F. Gruber et al. PLOS ONE, 2015>

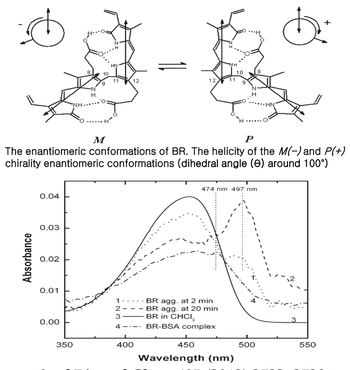
Bilirubin Molecule's Structure and Optical Properties



<Photoisomerization of Bilirubin, 신생아 황달 치료 방법>



<Photochemistry and Photobiology, 1999, 70(6), 941-928>

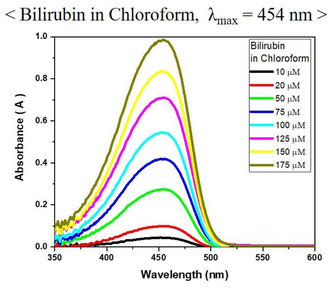


The enantiomeric conformations of BR. The helicity of the *M(−)* and *P(+)* chirality enantiomeric conformations (dihedral angle (θ) around 100°)

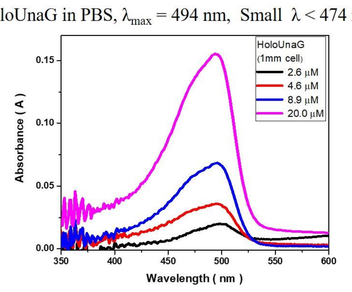
<Anal Bioanal Chem 405 (2013) 2723–2728>

UV Spectrum (Bilirubin in Chloroform, HoloUnaG in PBS, Bilirubin in PVA)

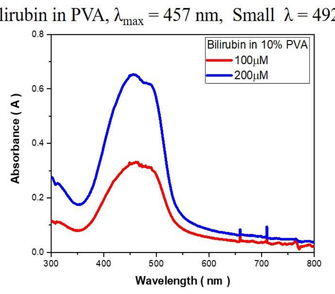
< Bilirubin in Chloroform, λ_{max} = 454 nm >



<HoloUnaG in PBS, λ_{max} = 494 nm, Small λ < 474 nm >

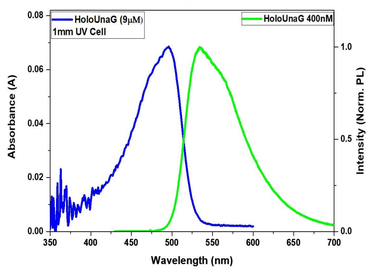


<Bilirubin in PVA, λ_{max} = 457 nm, Small λ = 492 nm >

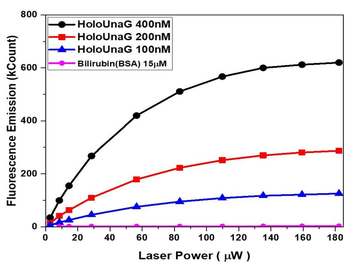


The Fluorescence Spectrum and PL Intensity for HoloUnaG

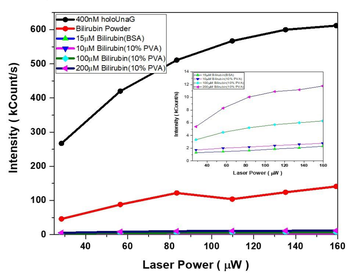
The Absorbance and Fluorescence Spectrum of HoloUnaG



HoloUnaG PL Intensity vs. Laser Power (488nm, 40MHz)

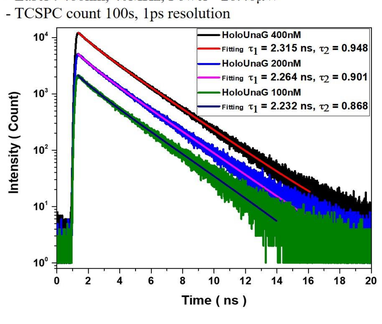


PL Intensity of HoloUnaG, Bilirubin Powder and Bilirubin(BSA)or (in PVA)

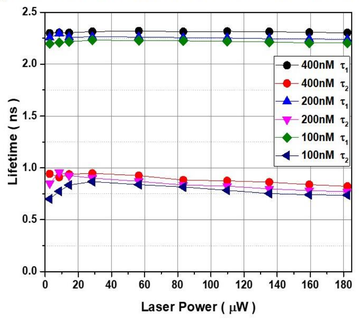


The Fluorescence Lifetime for HoloUnaG

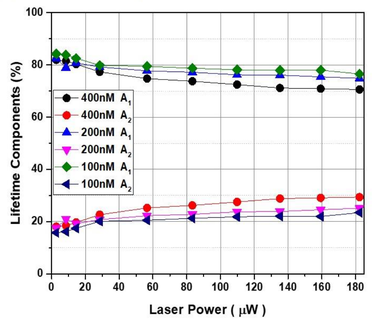
TCSPC Time Decay Spectrum of HoloUnaG in PBS
- Laser : 488nm, 40MHz, Power =28.48μW
- TCSPC count 100s, 1ps resolution



(a) Fluorescence Lifetime of HoloUnaG in PBS



(b) Lifetime Components Percent of HoloUnaG

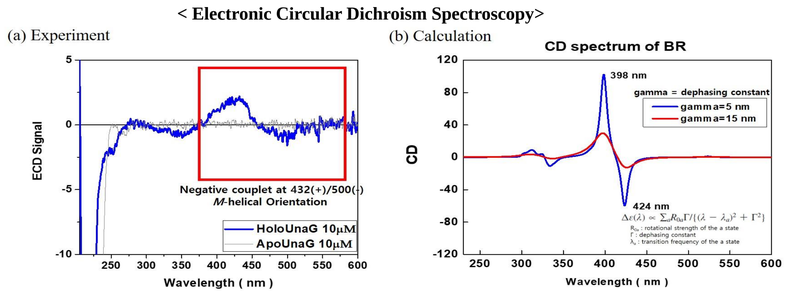


➢ **Why I : Why does holoUnaG has significantly enhanced fluorescence intensity on the major lifetime with 2.2~2.3 ns slow decay ?**

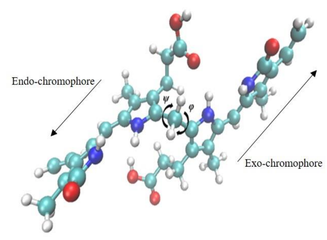
➢ **Why & What II : The fluorescence's lifetime of holoUnaG is 2.2~2.3 ns and 0.8~0.9 ns. Two components are about 80 and 20 %, respectively. Why does holoUnaG has the second order decay for lifetime and what mean is the lifetime of fast decay ?**

Quantum Chemistry Calculation for HoloUnaG (Clue for Why I))

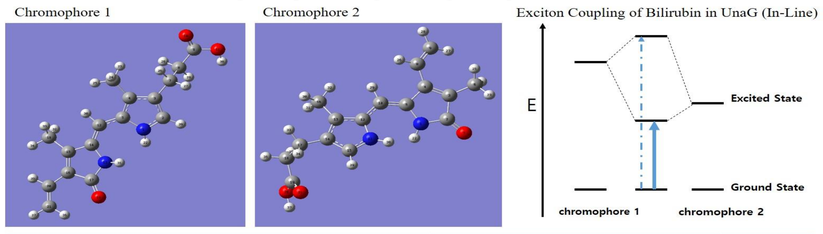
< Electronic Circular Dichroism Spectroscopy>



< Two Chromophore of Bilirubin in UnaG>

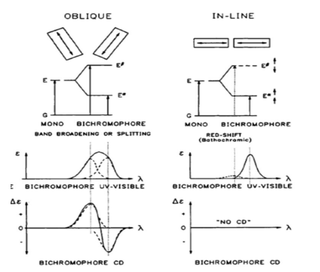


< Transition Wavelength / Dipole Strength through Computational Calculation>



Original structure in HoloUnaG	Chromophore1 transition wavelength / dipole strength	Chromophore2 transition wavelength / dipole strength	Angle
	392.36 nm / 12.4451	412.82 nm / 7.7857 (291.52 nm / 2.7945)	150.6 (146.07)

< Exciton Coupling for Bichromophore>

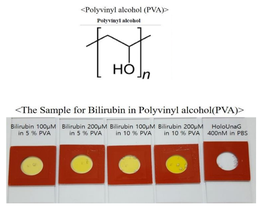


<Exciton Splitting Diagram (upper) and Predicted UV-visible and CD Spectra>

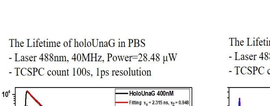
<J. Am. Chem. Soc., 1994, 116, 42–59>

Optical Properties for Increased Rigidness of Bilirubin (Clue for Why I))

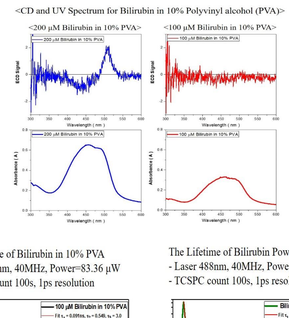
<Polyvinyl alcohol (PVA)>



<The Sample for Bilirubin in Polyvinyl alcohol(PVA)>



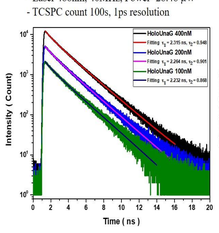
<CD and UV Spectrum for Bilirubin in 10% Polyvinyl alcohol (PVA)>



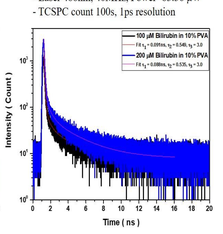
<The Fluorescence vs. Laser Power (Picosecond Laser with 488nm and 40 MHz)>

Laser Power (μW)	HoloUnaG 400nM in PBS	Bilirubin Powder	Bilirubin 15μM (BSA) in PBS	Bilirubin 10μM in10% PVA	Bilirubin 100 μM in 5% PVA	Bilirubin 100 μM in 10% PVA	Bilirubin 200 μM in 5% PVA	Bilirubin 200 μM in 10% PVA
	PL(kCount/s)	PL(kCount/s)	PL(kCount/s)	PL(kCount/s)	PL(kCount/s)	PL(kCount/s)	PL(kCount/s)	PL(kCount/s)
28.48	267	46.07	1.30	1.74	2.44	3.34	2.92	5.40
56.48	420	88.10	1.47	2.01	3.25	4.50	4.18	8.29
83.36	511	122	1.64	2.20	3.72	5.22	4.80	10.05
109.80	567	104	1.88	2.41	4.26	5.71	5.50	10.90
135.20	600	124	2.07	2.60	4.58	6.01	6.03	11.19
159.30	612	141	2.31	2.79	4.90	6.29	6.58	11.78

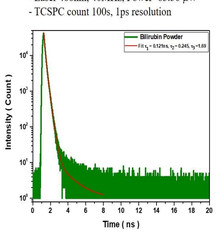
The Lifetime of holoUnaG in PBS
- Laser 488nm, 40MHz, Power=28.48 μW
- TCSPC count 100s, 1ps resolution



The Lifetime of Bilirubin in 10% PVA
- Laser 488nm, 40MHz, Power=83.36 μW
- TCSPC count 100s, 1ps resolution



The Lifetime of Bilirubin Powder
- Laser 488nm, 40MHz, Power=83.36 μW
- TCSPC count 100s, 1ps resolution

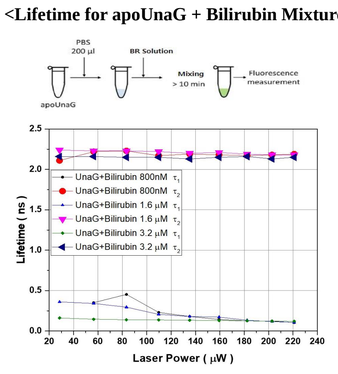


The Lifetime & Components Percent of HoloUnaG, Bilirubin Powder, Bilirubin (BSA) and Bilirubin in 10% PVA

Laser Power (μW)	HoloUnaG 400nM in PBS							Bilirubin Powder						
	A1	τ1 (ns)	A2	τ2 (ns)	χ²	-	-	A1	τ1 (ns)	A2	τ2 (ns)	A3	τ3 (ns)	χ²
56.48	14.57 (74.8%)	2.321	4.92 (25.2%)	0.925	1.05			21.48 (68.2%)	0.122	10.02 (31.8%)	0.249	0.020 (0.06%)	1.530	1.298
Laser Power (μW)	Bilirubin 15 μM (BSA) in PBS							Bilirubin 10 μM in 10% PVA						
	A1	τ1 (ns)	A2	τ2 (ns)	χ²	-	-	A1	τ1 (ns)	A2	τ2 (ns)	χ²	-	-
56.48	0.132 (95.6%)	0.106	0.006 (4.4%)	1.456	1.005			0.147 (94.2%)	0.103	0.009 (5.8%)	2.45	1.029		
Laser Power (μW)	Bilirubin 100 μM in 10% PVA							Bilirubin 200 μM in 10% PVA						
	A1	τ1 (ns)	A2	τ2 (ns)	A3	τ3 (ns)	χ²	A1	τ1 (ns)	A2	τ2 (ns)	A3	τ3 (ns)	χ²
56.48	0.967 (92.1%)	0.087	0.064 (6.1%)	0.389	0.019 (1.8%)	2.8	1.015	2.380 (94.3%)	0.092	0.106 (4.2%)	0.491	0.037 (1.5%)	3.04	1.025

Lifetime of ApoUnaG + Bilirubin / Bleaching of HoloUnaG (Clue for Why II))

<Lifetime for apoUnaG + Bilirubin Mixture>



<The Original Lifetime of HoloUnaG is 2.2~2.3 ns>

<Lifetime and Components of HoloUnaG on Bleaching (Photo- Damage or Isomerization of Bilirubin)>

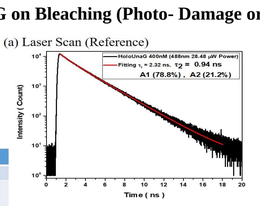
Scan Range 25μmx25μm Power 100% (472, 480, 488nm) <Bleaching>

ROI Laser Power 10% (488nm,28.48 μW) <ROI Measurement after Bleaching>

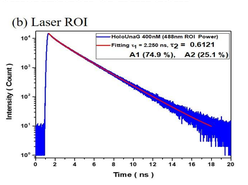
The Lifetime and Components of HoloUnaG 400nM on Bleaching (Photo- Damage or Isomerization of Bilirubin)

	Laser Power (μW)	The Lifetime of HoloUnaG 400nM in PBS				
		A_1 (A_1(%))	τ_1 (ns)	A_2 (A_2(%))	τ_2 (ns)	χ^2
(a) Laser Scan (Reference)	28.48	9.579 (78.8%)	2.321	2.580 (21.2%)	0.939	1.005
(b) Laser ROI	28.48	10.90 (74.9%)	2.250	3.660 (25.1%)	0.612	1.117
Bleaching Time	472nm, 480nm, 488nm 100% Power Bleaching (Scan Range: 25 x 25 μm)					
	PL(kCount)	A_1 (A_1(%))	τ_1 (ns)	A_2 (A_2(%))	τ_2 (ns)	χ^2
(c) On 1 min.	506	8.050 (69.1%)	2.380	3.600 (30.9%)	0.906	1.029
ROI after Bleaching	After Bleaching, ROI on Center for Scan Range with 488nm (28.48μW)					
	PL(kCount)	A_1 (A_1(%))	τ_1 (ns)	A_2 (A_2(%))	τ_2 (ns)	χ^2
(d) After 1 min.	487	8.900 (74.7%)	2.260	3.008 (25.3%)	0.621	1.055

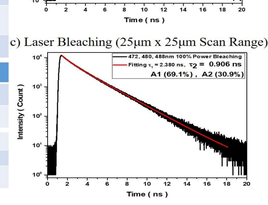
(a) Laser Scan (Reference)



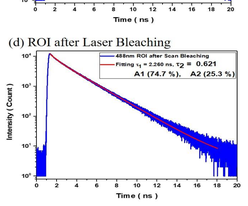
(b) Laser ROI



c) Laser Bleaching (25μm x 25μm Scan Range)



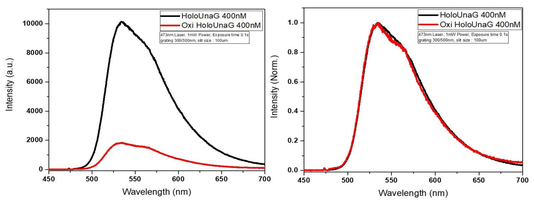
(d) ROI after Laser Bleaching



<The Original Lifetime of HoloUnaG is 2.2~2.3 ns> <The Fast Component with 0.8~0.9 ns Decay is from Photo-Damaged or Photo-Isomerized Bilirubin (photosensitive component)>

The Fluorescence and Lifetime of Oxidized HoloUnaG (Clue for Why II))

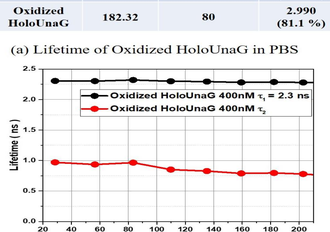
<The Fluorescence of HoloUnaG (Ref) and Oxidized HoloUnaG (UV Lamp 1day)>



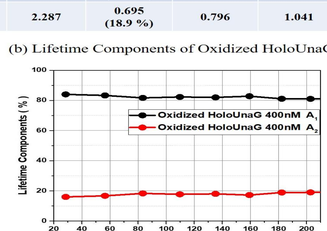
The Lifetime of Oxidized HoloUnaG 400nM in PBS (Laser: 488nm, 40MHz, TCSPC count: 100s, 1ps resolution)

Sample	Laser Power (μW)	PL (kCount/s)	Lifetime for HoloUnaG 400nM in PBS				
			A1	τ1 (ns)	A2	τ2 (ns)	χ²
Ref. (non) HoloUnaG	56.48	420	14.566 (74.8 %)	2.321	4.918 (25.2 %)	0.925	1.050
Oxidized HoloUnaG	56.48	56	2.080 (83.3 %)	2.302	0.417 (16.7 %)	0.935	1.011
Oxidized HoloUnaG	182.32	80	2.990 (81.1 %)	2.287	0.695 (18.9 %)	0.796	1.041

(a) Lifetime of Oxidized HoloUnaG in PBS



(b) Lifetime Components of Oxidized HoloUnaG



➢ **Conclusion for Why I : The enhanced fluorescence of holoUnaG is from the exciton coupling of in-line structural conformation of bilirubin with increased rigidness from protein-encapsulation.**

➢ **Conclusion for Why II : The original lifetime of holoUnaG is 2.2~2.3ns and the lifetime of fast decay (0.8~0.9 ns) is caused photo-damaged bilirubin or photo-isomerized bilirubin.**