Biochemical and Biophysical Research Communications 443 (2014) 894–898

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Genetically encoded red fluorescent copper(I) sensors for cellular copper(I) imaging

Junyi Liang [a,*], Lele Guo [b], Yin Ding [c], Lei Xia [a], Yan Shen [b], Meng Qin [a], Qiang Xu [b], Yi Cao [a], Wei Wang [a]

[a] National Laboratory of Solid State Microstructure, Department of Physics, Nanjing University, Nanjing, Jiangsu 210008, China
[b] State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing, Jiangsu 210008, China
[c] Sate Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing, Jiangsu 210093, China

ARTICLE INFO

Article history:
Received 28 November 2013
Available online 28 December 2013

Keywords:
Fluorescent protein
Copper(I) sensor
Protein engineering
Biosensor design

ABSTRACT

Copper ranks among the most important metal ions in living organism, owing to its key catalytic effect in a range of biochemical processes. Dysregulation of *in vivo* copper(I) metabolism is extremely toxic and would cause serious diseases in human, such as Wilson's and Menkes. Thus, it would be highly valuable to have a proper approach to monitor the dynamics of copper(I) *in vivo*, as it is directly related to the onset of human copper(I)-related diseases. Under these circumstance, developing fluorescent protein based copper(I) sensors is highly demanded. However, these established sensors are mostly based on green or yellow FPs. Fluorescent copper(I) sensors with a spectra in the red range are more desirable due to lower phototoxicity, less auto-fluorescent noise and better penetration of red light. In the present work, we grafted a special red FP into three different location of a copper(I) binding protein, and generate a series of red fluorescent copper(I) sensors. Despite their limited *in vivo* sensitivity toward copper(I), these sensors are viable for cellular copper(I) imaging. Furthermore, these red fluorescent copper(I) sensors are a good starting point to develop superior copper(I) biosensors capable of imaging copper(I) fluctuations within a truly biologically relevant concentration, and further effort to realize this endeavor is under way.

1. Introduction

Copper, as a trace element, is an essential metal ion that is required by nearly every living organisms, because its biochemical role as catalyst in a variety of biological processes, ranging from oxygen transportation, hormone maturation, signal transduction etc [1,2]. However, an excess of copper is extremely toxic, due to unfaithful competition for proteinaceous metal binging site and excessive ROS resulted from some copper catalyzed reactions [3]. As such, failure to properly control copper homeostasis could have fatal consequence and result in severe disease in human. Understandable, the development of methods to imaging *in vivo* copper is a reasonable pursuit, as this would lead to extra knowledge regarding the onset of copper-related disease in human, which would in turn provide new insights for human health.

A number of synthetic copper sensors have been developed [4–8], but most of these sensors lack a high enough affinity for copper to compete with intrinsic metalloproteins. Besides, specificity, biocompatibility, and stability are all issues yet resolved with synthetic sensors. Hence, it is much needed that alternative copper imaging methodology could be developed.

Fluorescent protein based sensors have been tremendously successful in indicating cellular metal ions metabolism due to their genetic encodability and good readability [9], ever since Dr. Roger Tsien and colleagues pioneered the field with Cameleons [10,11] for Calcium sensing. Encouraged by these pioneering researches, we and others have developed FP based copper(I) sensors which are suitable to dynamically imaging copper(I) in live cells [12–15]. These copper(I) sensors demonstrate a good sensitivity and selectivity for copper(I) imaging in cell, but these sensors are mostly constructed on green or yellow FP. So, phototoxicity, confusion with auto-fluorescence, and tissue obstruction are all potential issues to be addressed with short wavelength light [16]. As a result, it could be advantageous to construct a fluorescent copper(I) sensor with a red-shifted spectra. The red FP templates in other reported red fluorescent metal ion sensors could be a reasonable scratch line to engineer a red FP based copper(I) sensor, as these RFP templates should already possess the right general properties to be considered in a biosensor, such as photostability, brightness, maturation speed etc. Here, we adopted the red FP template developed by Dr. Robert Campbell and co-workers [17,18], and sub-cloned this RFP into three different location of a copper(I) binding protein Amt1, to deliver a series of red fluorescent copper(I) sensor.

* Corresponding author.
E-mail address: jyliang@biophy.nju.edu.cn (J. Liang).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.12.101

2. Materials and methods

2.1. Chemicals

NaCl, KCl, $ZnCl_2$, $CaCl_2$, $MgCl_2$, $NiCl_2$, $MnCl_2$, $CdCl_2$, $CoCl_2$, $FeCl_2$, $FeCl_3$, $AgNO_3$, $CuSO_4$, were from Shanghai Zhenxin Reagent Company. NaCN was from Dr. Jinlin Zou's lab at Department of Chemistry, Nanjing University. Tetrakis(acetonitrile) copper(I) hexafluorophosphate was from J&K Chemical Ltd.

2.2. Construction of expression plasmids and preparation of sensor proteins

The red fluorescent protein was sub-cloned into three different position of copper binding domain of Amt1 using AVAI respectively, which is between residue 18 and 19, between residue 41 and 42, between residue 59 and 60. Sensors are referred to as AR-18, AR-41 and AR-59, for RFP after residue 18, 41 and 59 of Amt1. All the genes were cloned into pQE80L plasmid between BamHI and KpnI sites.

2.3. Expression plasmids were transformed into Escherichia coli XL1-Blue

Each clone harboring a corresponding plasmid was grown in 2.5% LB medium containing 100 mg/L ampicillin under vigorous stirring and induced with 0.5 mM IPTG when its optical density at 600 nm reached 0.8. Protein expression continued for 8 h at 20 centigrade. Bacterial cells were harvested by centrifugation and resuspended in pre-cooled lysis buffer (20 mM Na_3PO_4, 500 mM NaCl, 4 mM DTT, pH 7.2) further lysed by sonication of 15 min. Then the cell lysate was centrifuged at 12,000 rpm for 25 min at 5 centigrade. The protein is purified using Ni^{2+}-NTA protein resin and eluted in elution buffer (20 mM Na_3PO_4, 500 mM NaCl, 500 mM imidazole, 4 mM DTT, pH 7.2).

2.4. Fluorescence measurement

The fluorescence measurement was done on a fluorescence spectrophotometer (JASCO FP-6500), with an excitation wavelength of 550 nm. Proteins' concentration was kept at 1 μM for all measurements. The molar ratio of copper(I) over sensor proteins were set at 2 and 5. The excitation and emission bandwidth were 3 and 5 nm, respectively.

The normalized fluorescence intensity refers to the fluorescence of the sensor at various wavelength divided by the maximum fluorescence intensity.

2.5. UV–Vis measurement

UV–Vis spectra of sensor protein in the absence and presence of copper(I) were taken using a UV/Vis spectrophotometer (Jasco, V-550). The sensors were kept at 20 μM in a buffer containing 200 mM Na_2H/NaH_2PO_4, 4 mM DTT (pH 7.2). For the measurement of sensor bound with copper(I), additional 100 μM copper(I) was present.

2.6. Determination of Cu(I) dissociation constant

To estimate the dissociation constant of copper(I) to sensors, 1 μM holo sensor proteins were prepared. Then different amount of NaCN is added into the samples to displace the bound Cu(I), thus returning the fluorescence. The equilibrium constants in this solution are based on NIST Critical Stability Constants of Metal complexes. And the log beta of sensors with Cu(I) was estimated using HySS2009 software.

2.7. Mammalian cell fluorescence imaging

Human embryonic kidney 293T cells were incubated at 37 centigrade under 5% CO_2 in DMEM (Hyclone) supplemented with 10% FBS. Then, the cells were seeded overnight into culture chambers to obtain 85–90% confluency. The following day, the cells were transfected with pcDNA3.1(-) carrying AR-18 or AR-41 or AR-59 or RFP template by using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After allowing a 36 h expression period, the cells were used for imaging. Images were taken with a con-focal microscope (Zeiss, LSM710) equipped with a 100-fold objective, NA 1.35.

3. Results and discussion

3.1. Design and construction of a new family of copper(I) sensors

To extend the spectra range of copper(I) sensor, we borrowed a red FP which was previously used as a template in a calcium sensor [17], and utilized the same copper(I) binding protein Amt1 as in last generation of copper(I) biosensors [12,13]. Given this red FP template being a circular permutation of original mApple, we initiated the sensor design using a strategy different from that we had used previously. Instead of inserting Amt1 into the RFP, we sub-cloned this RFP into Amt1, so as to circumvent the disadvantage of declined stability of CP construct (Fig. 1). And we envisioned that split Amt1 would reassemble in the presence of copper(I), owing to the binding of copper(I) and the formation of a tetracopper(I) cluster [19]. This enormous conformational change

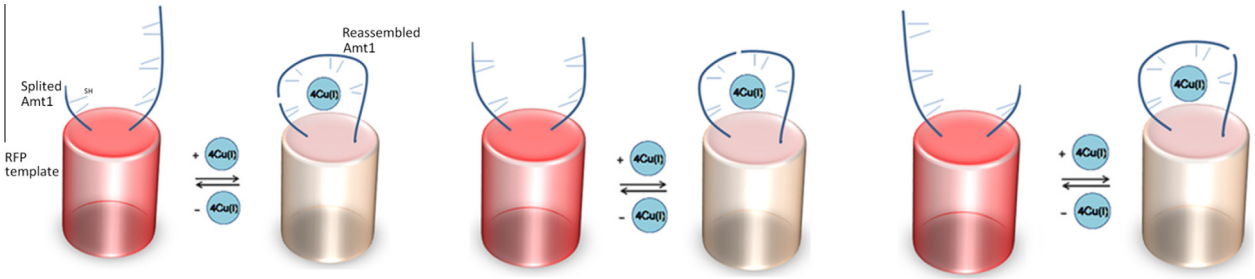

Fig. 1. Schematic of design concept for this series of copper(I) sensors. RFP template was sub-cloned into three different position of Amt1, the resulted sensors were named AR-n, where n stand for the residue in Amt1 after which RFP was inserted. Cloning site was varied to screen for the different sensitivity and *in vivo* response time. The presence of copper(I) mediate the forming of a tetracopper(I) cluster and reassembly of splited Amt1, the intra-molecular rearrangement of which would lead to a corresponding fluorescence drop.

of Amt1 could lead to intra-molecular rearrangement, which would in turn lead to a corresponding fluorescence fluctuation.

We intentionally chose to insert RFP template into several different positions of the copper(I) responding protein Amt1, to screen for the most suitable combination model of FP and Amt1, which had been proven to be an effective way to generate multiple sensors or even optimize a sensor [20,21]. As there are 4 cysteine pairs in Amt1, we employed three insertion models for sensor preparation, which is 1 cys pair-RFP-3 cys pair, 2 cys pair-RFP-2 cys pair, 3 cys pair-RFP-1 cys pair. Due to the absence of a HD

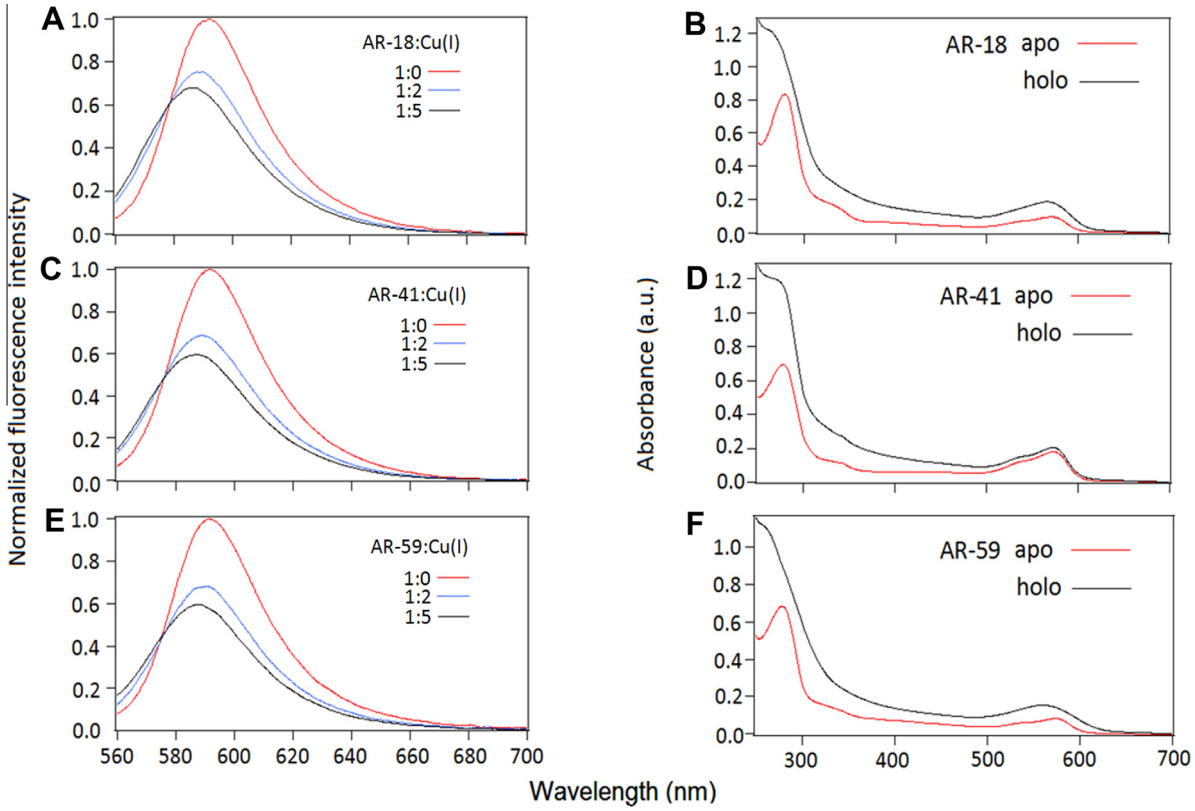

Fig. 2. Optical characterization of AR-n sensors. (protein concentration is 1 μM for left panel, 20 μM for right panel) (A, C, E) are fluorescence response to different molar of copper(I) for AR-18, AR-41 and AR-59, respectively. (B, D, F) are absorbance of sensors in the absence and presence of copper(I) for AR-18, AR-41 and AR-59, respectively.

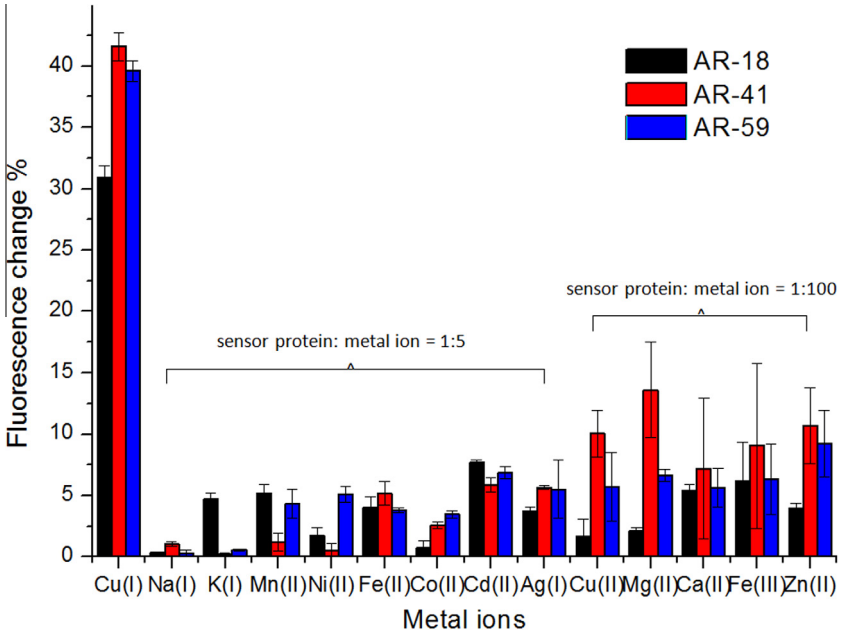

Fig. 3. Specificity of AR-n series of copper(I) sensors. Experiments were performed under the condition of 1 μM sensors with either 5 μM or 100 μM of different metal ions. Sensors' fluorescence change when the molar ratio of copper(I) over sensors being 5 is also listed. Error bars were generated from the results of two independent measurements.

crystal structure of Amt1, the detailed insertion place is selected loosely based on a computationally modeled Amt1 structure, which is between residues 18 and 19, between residues 41 and 42, and between residues 59 and 60. The resulted sensors are named AR-n, where n refers to the site after which RFP was inserted into Amt1.

3.2. Optical characterization of AR-n sensors

The sensor proteins were expressed in *E. Coli*, and then purified using Ni^{2+}-NTA protein resin column. The presence of 5 times of copper(I) was enough to result in the holo form as determined by increased absorbance at ~280 nm due to the formation of Cu(I)-thiol clusters (Fig. 2B, D, F). The emission spectra of both apo and holo form sensors were obtained by setting the excitation wavelength at 550 nm. The presence of copper(I) caused a notable decrease in the fluorescence for all these sensors. Saturation of AR-18 resulted in a 30% decrease in fluorescence intensity, while saturation of AR-41 and AR-59 resulted in a more significant response, a 40% decrease in fluorescence (Fig. 2A, C, E). Noted that, binding of copper(I) also resulted in a ~10 nm blue shift in emission maximum, which is likely due to the change in local environment near

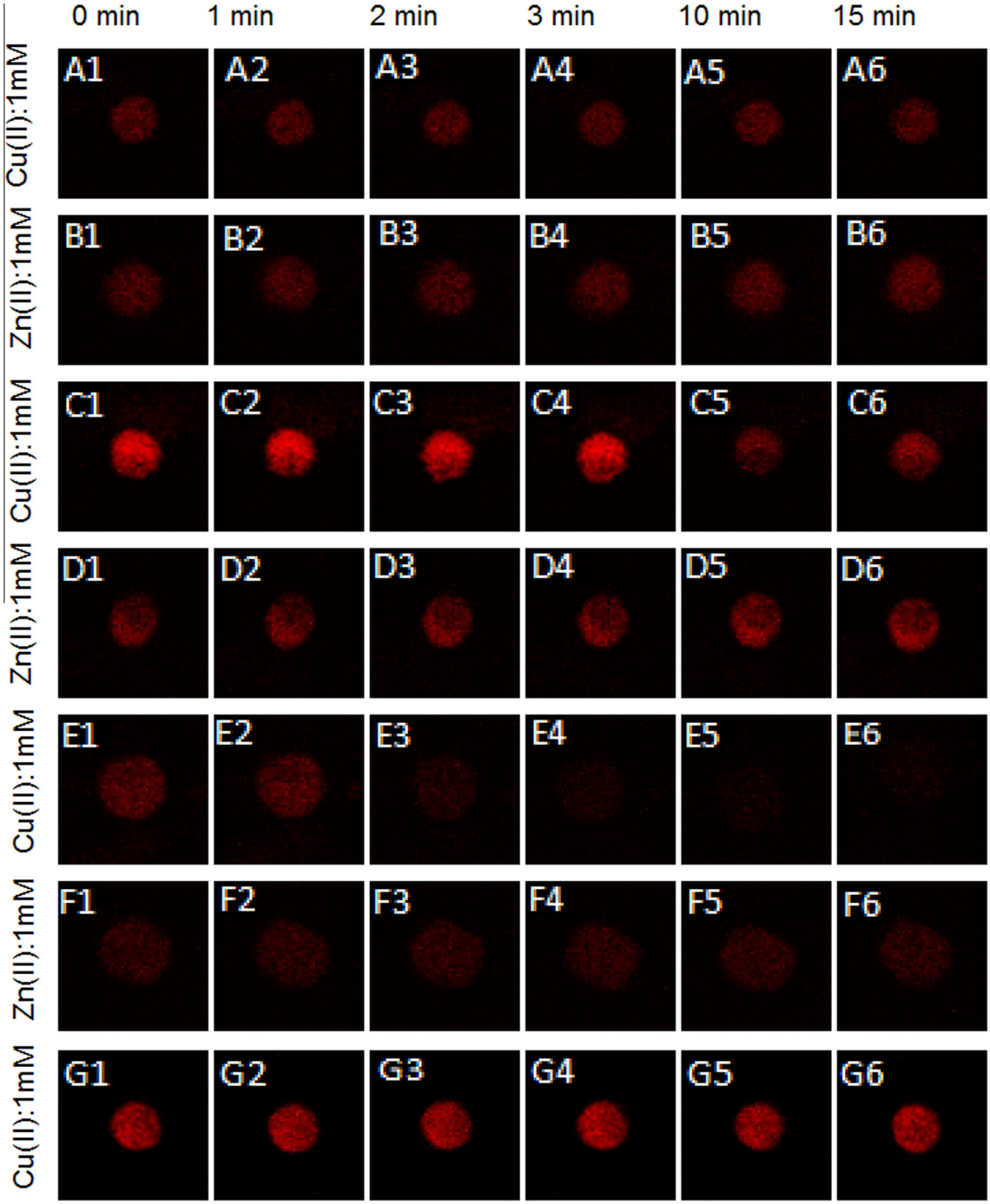

Fig. 4. *In vivo* imaging of live 293t cells expressing AR-n sensor proteins incubated with Cu(II) or Zn(II). RFP template protein expressing 293t cells incubated with Cu(II) were also monitored. (A1–A6) AR-18's time-lapse response to incubation with 1 mM Cu(II) in 293T cells. (B1–B6) AR-18's response to incubation with 1 mM Zn(II) in 293T cells, which serve as a control experiment to A series imaging. (C1–C6, D1–D6) and (E1–E6, F1–F6) are parallel experiments for AR-41 and AR-59, respectively. (G1–G6) The fluorescence change of 293t cell transfected by the plasmid encoding RFP at different time points after adding 1 mM (the length of each side: 45 μm).

the chromophore. Varying the insertion position also modulate the brightness of the sensors, apo AR-18, AR-41 and AR-59 have a relative brightness of 0.29, 0.18 and 0.19, respectively (relative to eGFP).

To receive a good estimation about the *in vivo* specificity of these sensors, we tested these sensors' response in the presence of other heavy metal ions or biologically relevant metal ions (Fig. 3). These sensors exhibit minimal response to other tested metal ions, when the sensors co-existed with 5 equiv. of the metal ions. However, in biology, the metal ions of Ca(II), Zn(II), Ma(II) etc tend to have much higher concentration. To assess AR-n sensors' immunity to this group of metal ions, we incubated AR-n sensors with 100 equiv. of these metal ions. The sensors' fluorescence response still held a reasonably good degree of indifference, even with an excess amount of these physiologically relevant metal ions (Fig. 3). Among which, AR-18 and AR-59 display a response of less than 10% change in fluorescence intensity to all tested ions, AR-41 has a slightly elevated response to magnesium ion, nevertheless, the fluorescence change is still small compared to the response to copper(I), the result of which is a testimony for the reliability of these sensors in terms of specificity and selectivity.

A high affinity is also a pre-requisite, if the sensor would be of *in vivo* usage. To characterize the affinity of our sensors to copper(I), the copper(I) bound form sensor protein were prepared by adding 1 mM $CuSO_4$ into the *E. Coli* when expressing the sensor to ensure the holo form were purified. Different concentration of NaCN was added into the protein sample to competitively displace the bound copper(I). By monitoring the recovery of the fluorescence, the log beta of AR-41 toward copper(I) was determined to be 17.9. Due to similar design strategy, AR-18 and AR-59 shall possess the same order of magnitude concerning dissociation constant for copper(I) as AR-41.

3.3. Cellular copper(I) visualization with AR-n sensors

Determined from the *in vitro* measurement of our sensors, AR-18, AR-41, and AR-59 all demonstrate a good performance for sensitively and selectively reporting copper(I). Finally, we set out to test the *in vivo* performance of our sensors in reporting cellular copper(I) fluctuations. Mammalian cell expression plasmids encoding AR-18, AR-41 and AR-59 were transfected into human embryonic kidney cancer 293T cells. Due to the expression of the sensor proteins, the 293T cells were notably red fluorescent after 24 h transfection. 1 mM $CuSO_4$ were added to the growth medium. 293T cells were then monitored using a confocal microscope, for the next 15 min (Fig. 4A, C, E). All the sensor expressing cells show a clear fluorescence drop within 15 min of copper(II) addition, proving the viability of all three sensors' *in vivo* copper(I) indicating. However, these sensors varied notably in the response time and degree.

AR-18 would take a full 15 min for the fluorescence to respond. AR-41's *in vivo* response to copper(I) is faster than AP-18, whose fluorescence reaches a maximum fluorescence drop after 10 min. While AR-59 display the fastest response to copper(I), and reach a maximum fluorescence drop within 2 min. As a parallel control experiment, the 293t cells carrying an RFP gene did not show any fluorescence change upon addition of $CuSO_4$ (Fig. 4G), providing direct evidence for a sensor specific *in vivo* fluorescence quenching effect. And it is worth mentioning that all three sensors showed no response to the same concentration of Zn(II), further confirm the good *in vivo* specificity of our sensors (Fig. 4B, D, F).

In conclusion, we have succeeded in adopting a novel red fluorescent protein to create a family of genetically encoded red fluorescent copper(I) sensors for *in vivo* copper(I) imaging. Although the spectra of these sensors have a clear advantage over previous green and yellow fluorescent copper(I) sensors, the sensitivity and response time still deserve further improvement. Nonetheless, these red fluorescent copper(I) sensors developed in this work provide the community a stepping stone to engineer superior red fluorescent copper(I) sensors for *in vivo* use in cellular culture or even in whole organism.

Acknowledgments

The authors acknowledge the support from Natural Science Foundation of Jiangsu Province and PAPD of Jiangsu Higher Education Institutions.

References

[1] B.-E. Kim, T. Nevitt, D.J. Thiele, Mechanisms for copper acquisition, distribution and regulation, Nat. Chem. Biol. 4 (2008) 176–185.
[2] N.J. Robinson, D.R. Winge, Copper metallochaperones, Annu. Rev. Biochem. 79 (2010) 537–562.
[3] D.L. Huffman, T.V. O'Halloran, Function structure, and mechanism of intracellular copper trafficking proteins, Annu. Rev. Biochem. 70 (2001) 677–701.
[4] S.C. Dodani, S.C. Leary, P.A. Cobine, D.R. Winge, C.J. Chang, A targetable fluorescent sensor reveals that copper-deficient SCO1 and SCO2 patient cells prioritize mitochondrial copper homeostasis, J. Am. Chem. Soc. 133 (2011) 8606–8616.
[5] T. Hirayama, G.C. Van de Bittner, L.W. Gray, S. Lutsenko, C.J. Chang, Near-infrared fluorescent sensor for *in vivo* copper imaging in a murine Wilson disease model, Proc. Natl. Acad. Sci. 109 (2012) 2228–2233.
[6] M.T. Morgan, P. Bagchi, C.J. Fahrni, Designed to dissolve: suppression of colloidal aggregation of Cu(I)-selective fluorescent probes in aqueous buffer and in-gel detection of a metallochaperone, J. Am. Chem. Soc. 133 (2011) 15906–15909.
[7] K.A. Price, J.L. Hickey, Z. Xiao, A.G. Wedd, S.A. James, J.R. Liddell, P.J. Crouch, A.R. White, P.S. Donnelly, The challenges of using a copper fluorescent sensor (CS1) to track intracellular distributions of copper in neuronal and glial cells, Chem. Sci. 3 (2012) 2748–2759.
[8] Y. Yang, F. Huo, C. Yin, Y. Chu, J. Chao, Y. Zhang, J. Zhang, S. Li, H. Lv, A. Zheng, D. Liu, Combined spectral experiment and theoretical calculation to study the chemosensors of copper and their applications in anion bioimaging, Sens. Actuators B 177 (2013) 1189–1197.
[9] R.Y. Tsien, The green fluorescent protein, Annu. Rev. Biochem. 67 (1998) 509–544.
[10] A. Miyawaki, J. Llopis, R. Heim, J.M. McCaffery, J.A. Adams, M. Ikura, R.Y. Tsien, Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin, Nature 388 (1997) 882–887.
[11] A. Miyawaki, O. Griesbeck, R. Heim, R.Y. Tsien, Dynamic and quantitative Ca2+ measurements using improved cameleons, Proc. Natl. Acad. Sci. 96 (1999) 2135–2140.
[12] J. Liu, J. Karpus, S.V. Wegner, P.R. Chen, C. He, Genetically encoded copper(I) reporters with improved response for use in imaging, J. Am. Chem. Soc. 135 (2013) 3144–3149.
[13] J. Liang, M. Qin, R. Xu, X. Gao, Y. Shen, Q. Xu, Y. Cao, W. Wang, A genetically encoded copper(I) sensor based on engineered structural distortion of EGFP, Chem. Commun. 48 (2012) 3890–3892.
[14] X. Yan, X. Li, S.-S. Lv, D.-C. He, A novel genetically encoded fluorescent protein as a Cu(I) indicator, Dalton Trans. 41 (2012) 727–729.
[15] S.V. Wegner, F. Sun, N. Hernandez, C. He, The tightly regulated copper window in yeast, Chem. Commun. 47 (2011) 2571–2573.
[16] N.C. Shaner, G.H. Patterson, M.W. Davidson, Advances in fluorescent protein technology, J. Cell Sci. 120 (2007) 4247–4260.
[17] Y. Zhao, S. Araki, J. Wu, T. Teramoto, Y.-F. Chang, M. Nakano, A.S. Abdelfattah, M. Fujiwara, T. Ishihara, T. Nagai, R.E. Campbell, An expanded palette of genetically encoded Ca2+ indicators, Science 333 (2011) 1888–1891.
[18] H. Hoi, T. Matsuda, T. Nagai, R.E. Campbell, Highlightable Ca2+ indicators for live cell imaging, J. Am. Chem. Soc. 135 (2012) 46–49.
[19] J.A. Graden, M.C. Posewitz, J.R. Simon, G.N. George, I.J. Pickering, D.R. Winge, Presence of a copper(I)−thiolate regulatory domain in the copper-activated transcription factor Amt1, Biochemistry 35 (1996) 14583–14589.
[20] G.S. Baird, D.A. Zacharias, R.Y. Tsien, Circular permutation and receptor insertion within green fluorescent proteins, Proc. Natl. Acad. Sci. 96 (1999) 11241–11246.
[21] T.V. Pavoor, Y.K. Cho, E.V. Shusta, Development of GFP-based biosensors possessing the binding properties of antibodies, Proc. Natl. Acad. Sci. 106 (2009) 11895–11900.