

Development and optimization of heavy metal lead biosensors in biomedical and environmental applications

Tai-Jay Chang^{a,b}, Wei-Qun Lai^{c,d}, Yu-Fen Chang^e, Chia-Lin Wang^{a,b}, De-Ming Yang^{c,d,*}

^aLaboratory of Genome Research, Basic Research Division, Department of Medical Research, Taipei Veterans General Hospital, Taipei, Taiwan, ROC; ^bSchool of Biomedical science and Engineering, National Yang Ming Chiao Tung University, Taipei, Taiwan, ROC; ^cMicroscopy Service Laboratory, Basic Research Division, Department of Medical Research, Taipei Veterans General Hospital, Taipei, Taiwan, ROC; ^dInstitute of Biophotonics, School of Biomedical Science and Engineering, National Yang Ming Chiao Tung University, Taipei, Taiwan, ROC; ^eLumiSTAR Biotechnology, Inc., Taipei, Taiwan, ROC

Abstract: The detrimental impact of the heavy metal lead (Pb) on human health has been studied for years. The fact that Pb impairs human body has been established from countless painful and sad historical events. Nowadays, World Health Organization and many developmental countries have established regulations concerning the use of Pb. Measuring the blood lead level (BLL) is so far the only way to officially evaluate the degree of Pb exposure, but the so-called safety value (10 µg/dL in adults and 5 µg/dL in children) seems unreliable to represent the security checkpoint for children through daily intake of drinking water or physical contact with a lower contaminated level of Pb contents. In general, unsolved mysteries about the Pb toxicological mechanisms still remain. In this review article, we report on the methods to prevent Pb poison for further Pb toxicological research. We establish high-sensitivity Pb monitoring, and also report on the use of fluorescent biosensors such as genetically-encoded fluorescence resonance energy transfer-based biosensors built for various large demands such as the detection of severe acute respiratory syndrome coronavirus 2. We also contribute to the development and optimization of the FRET-based Pb biosensors. Our well-performed version of Met-lead 1.44 M1 has achieved a limit of detection of 10 nM (2 ppb; 0.2 µg/dL) and almost 5-fold in dynamic range (DR) supported for the real practical applications—that is, the in-cell Pb sensing device for blood and blood-related samples, and the Pb environmental detections in vitro. The perspective of our powerful Pb biosensor incorporated with a highly sensitive bio-chip of the portable device for quick Pb measurements will be addressed for further manipulation.

Keywords: Blood lead level; Environmental Pb detection, biosensor; Fluorescence resonance energy transfer; Fluorescent biosensors; Genetically-encoded fluorescent protein biosensors, in-cell Pb biosensing; Heavy metal lead

1. HIDING DANGER OF LEAD

Lead (Pb), as a heavy metal, has been used by humans in various fields. For example, it can be used as the protective apron for radiation shielding, and can be used as the adulteration ingredient for sweet-tasted wine in the history of ancient Rome Empire.¹ For more than 70 years, Pb-containing water pipelines have been used in Taiwan for various purposes. Now, Pb-contaminated drinking water exist in many countries and Pb-contaminated irrigation water in some countries.^{2,3} However, Pb-containing materials have been used by humans for some special purposes, such as in the traditional Chinese medicine, in the ingredients

for painting the wall and glasses, in hair dyes, and in toys' coloring. The lead content in Petrol and Diesel are used in some urban environments to balance the outcome between the amount of Pb-absorbed from environmental exposure and the health deficits occurred due to less notice of Pb, as reported by Dr. Clair C. Patterson.⁴ Dr. Patterson was the pioneer to estimate the aging of the Earth by determining the isotopic composition of Pb.⁵ His findings reported on the detrimental impact of Pb released from Pb-containing gasoline into the air we breathe. However, researchers took a long time to become aware of the horrific impact of Pb on humans (Table 1). Without significant symptoms found under chronic poison of low-level Pb¹³, the leaded gasoline would be used freely without control, and safety regulations are required in our environment.¹⁴

2. KNOWING EXPOSURE STATUS OF PB

Nowadays, regulations for preventing the invasive toxicity of Pb to humans have been set up by World Health Organization (WHO) and by many developed countries (Table 2). Briefly, the test of blood lead level (BLL) is so far the only effective way to understand the status of Pb exposure in human body (Fig. 1). BLL represents the amount of Pb detected in the blood. According to previous evidence found from Pb-affected patients (adults or young children) with various symptoms, BLL is

* Address correspondence. Dr. De-Ming Yang, Microscopy Service Laboratory, Basic Research Division, Department of Medical Research, Taipei Veterans General Hospital, 201, Section 2, Shi-Pai Road, Taipei 112, Taiwan, ROC. E-mail address: dmyang@vghtpe.gov.tw (D.-M. Yang).

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

Journal of Chinese Medical Association. (2021) 84: 745-753.

Received May 27, 2021; accepted June 18, 2021.

doi: 10.1097/JCMA.0000000000000574.

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Table 1**Selected events of Pb poisoning with significant symptoms**

Years	Area	Source	Symptoms	Ref
1994	Michoacán, Mexico	Ceramic folk-art	Renal, reproductive, neuromuscular dysfunctions, behavior alterations in children, etc.	6
2004	Guangdong, China	Electronic waste	Skin damage, headaches, vertigo, nausea, chronic gastritis, gastric ulcers, etc.	7
2008	Shaanxi, China	Metal smelting	Abdominal pain, developmental delay, irritability, etc.	8
2010	Zamfara, Nigeria	Mining	Seizures, hearing problems, irritability, etc.	9
2012	Hunan, China	Chemical plant	Developmental delay, memory loss and abdominal pain in children, etc.	10
2013	Kabwe, Zambia	Lead-zinc mine	Central nervous system damages, etc.	11
2020	Taichung, Taiwan	Traditional Chinese medicine	Abdominal pain, insomnia, etc.	12

Table 2**Regulations which limit the contents of Pb within blood of human or detected in the water, or foods**

Standard/unit conversion	ppb (µg/L)	µg/dL	nM
Blood lead level (BLL) for adult	100	10	500
BLL for children	50/25	5/2	250/100
WHO 2017 Pb in tap water	10	1	50
CNS 8088: Pb from faucet Taiwan	7	0.7	35
Food containing Pb	300	30	1500
Mushroom containing Pb	3000	300	15 000

BLL = blood lead level.

officially suggested not to exceed 10 µg/dL in adults and should be <5 µg/dL in children (Table 2).^{15,16} More recently, Pb content in urine or serum was also used in toxicity diagnosis alternatively. However, the standard values for safe permissible levels of urinary/serum Pb levels are yet to be determined. Followingly, the observation of possible entry routes for Pb such as drinking water and intake of foods was made. The permissible concentration of Pb in tap water, foods, and mushroom (dry weight) are

set at 7 ppb (0.7 µg/dL, Taiwan CNS 8088) or 10 ppb (1 µg/dL, WHO 2017), 300 µg/Kg (30 µg/dL), and 3000 µg/Kg (300 µg/dL), respectively (Table 2).¹⁷

Many issues need to be overcome in the examination of Pb concentration from blood (BLL) or from other tested targets (water or foods—the ingestion sources). For example, Pb reagent preparation requires the use of strong acid and base, which need to be handled with care to avoid the risk of occupational disaster. In addition, it requires professional training for personnel to operate the precision instruments (eg, atomic absorption spectroscopy or inductively coupled plasma mass spectrometry). Of course, gaining Pb-content data using the whole complicated procedure is time-consuming. Finally, such tests can be carried out only in limited places, either in hospitals (blood drawing) or special companies equipped with atomic absorption spectroscopy or inductively coupled plasma mass spectrometry, and needs specialists for operating the equipment (Fig. 1).

Through long-term observation, scientists gradually proposed that no safe BLL exists, if safety is defined as the level not harmful to human life.¹⁸ In fact, chronic exposure to even lowest BLL (as low as 2 µg/dL) in children has been confirmed to possibly lead to various kinds of neurodevelopmental impairments,

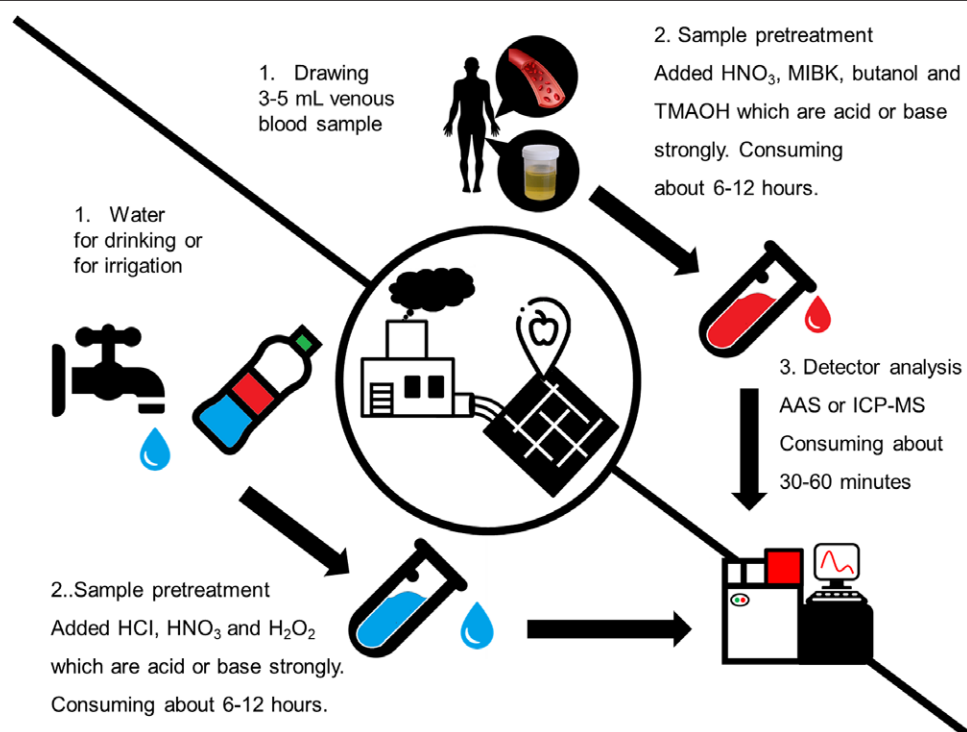
**Fig. 1** Measurement procedure of Pb content extracted from environment or human body.

Table 3**Selected historical events for low BLL induced defects (<5 or even 2 µg/dL)**

Years	Affect	Ref
2000	A small increasing in the number of red blood cells and in girls with reducing mean corpuscular volume and mean corpuscular hemoglobin.	20
2006	Irregular menstruation, Increasing the risk for infertility.	21
2007	Correlated to simple reaction time that reflects attention ($p = 0.05$), and digit span ($p = 0.08$).	22
2012	A higher semen lead concentration was correlated with lower sperm count.	23
2014	Decreasing birthweight and increasing the odds of preterm birth among boys.	24
2017	An increasing risk of dental caries of the deciduous teeth	25
2017	Correlated positively with red cell distribution width; and negatively with child size, age, body mass index, hemoglobin, platelet distribution width, gamma-glutamyl transferase (γ -GT) and IQ.	26

BLL = blood lead level.

ranging from permanent cognitive damages to numerous neurodegenerative diseases, without specific behavioral alterations or clear significant symptoms.¹⁹ Furthermore, low-level Pb exposure was also confirmed to be a risk factor that contributes to cardiovascular disease and increases the overall mortality rate, once entering and staying in human body (Table 3).²⁷ Recent studies from Taiwan also reported on the association of urinary Pb with cardiovascular disorder (by measuring the thickness of carotid intima-media) and with metabolic syndrome in young generations.^{28,29} Thus, the toxicological mechanisms at very low

contents of Pb exposure need to be urgently explored, especially in young populations. In addition to BLL, knowing Pb contents within the living body is another challenge for understanding more about the toxicology of the heavy metal Pb.

3. BIOSENSORS TO EXPLORE THE SECRET OF LIFE

The fluorescent biosensors (FBs) in various forms (i.e. either chemical indicators or genetically-encoded [GE] fluorescent protein [FP] biosensors [GEFBs, Fig. 2]) that are compatible with a spectral/signal recorder or a fluorescent microscope can be used for the real-time detection of specific targets whether extracted from environments or tested inside living body.³¹ By applying such FBs, the content dynamics of a targeted molecule within or even outside the living body can be directly detected and shown at the aspects of time and space. The functions of the probed interests can be further understood through the help of these GEFBs.

The concept for probing interested targets by GEFBs is adapting the sensing key as a specific receptor within the FP domain, either inside single FP biosensors for the conformational changes (Fig. 2A, B)³¹ or between the two FP pairs for the reaction of fluorescence resonance energy transfer (FRET, Fig. 2C, D).³⁰ In single FP biosensors, the fluorescence intensity (FI) of single FP increases to turn “on”, or decreases to turn “off” after receptor-target recognition-binding, when sensor-target exists (Fig. 2B). In FRET-based biosensors, such receptor-target binding within FRET pairs generate FRET signals (Fig. 2D). In both ways, the sensing work can be accomplished.

“Cameleon”³⁰ is the first GFP biosensor borne in 1997 by Prof. Roger Tsien, who won the 2008 Nobel Prize for Chemistry. This biosensor monitors intracellular calcium (Ca) ions through

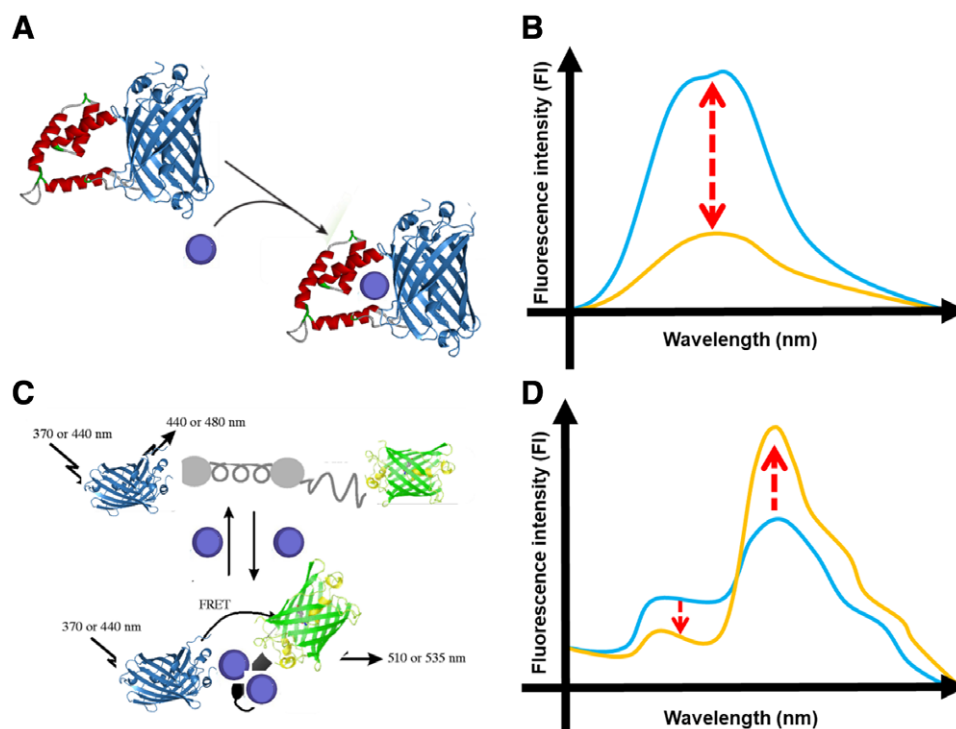


Fig. 2 Design of genetically-encoded fluorescent biosensors (GEFBs). A, Single fluorescent protein biosensor will proceed conformational changes after target-sensor binding. B, The fluorescent emission spectra of such a single-FP biosensor will either in increase (on, ECFP Blue) or decrease (off, EYFP Yellow) mode. C, Fluorescent energy resonance transfer (FRET)-based biosensor uses two FRET FP pair proteins, either EBFP with EGFP or ECFP with EYFP. D, Conformational changes happen when target-sensor binding exists. The fluorescence intensity (FI) of EGFP (or EYFP) increases, and then EBFP (or ECFP) decreases. C, Graph was adapted from previous report.³⁰

acquiring event signals of FRET between 2 FRET pair FPs.³⁰ Such brilliant concept has been proved to be workable and allowed measuring the dynamics of intracellular targeted signals inside living cells in a time-lapse manner, and alternatively allowed amplifying chemical indicators, which needs an additional preloading procedure. Following Cameleon, more than 50 kinds of FRET-based or similar biosensors were developed continuously (Table 4). These tools can help scientists to observe certain conditions of living cells such as the oncogenic processes of tumors⁶⁸ and even to detect severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).⁶⁷ To build up metal ion FBs or GEFBs (eg, lead [Pb],⁶⁹ cadmium [Cd],⁷⁰ silver [Ag],⁷¹ copper [Cu],⁷² or zinc [Zn],^{63,73} etc), more criteria like molecular selectivity are required. Thus, developing GEFBs for detecting metal ions is relatively hard and therefore is less to be seen.

4. CHEMICAL PB BIOSENSOR

Idealistically, we live in a relatively healthy place if there is no violation against the law, no unscrupulous adding toxic stuffs into the food, water or in the air. However, the real world is somehow we need to be precocious about resources with more abnormal ingredients around our environment on purpose. We should take more caution to survey convenient and precise methods to verify the contents of toxicants such as the heavy metal Pb we intake incidentally. Taking advantage of drinking or even breathing through any kind of perception technology

is available from specialized hospitals or companies (Fig. 1). For precisely understanding the environmental (outside human body) and poisoned (inside human body, BLL, or *in vivo*) status of heavy metal Pb and monitoring their lethal contents will be direct and efficient on the aspect of source/absorbance control.^{74,75} The sensor methods and required regulations for the so-called accepted amounts of Pb in certain targets should be solid and confirmed, as mentioned in Table 2.¹⁵⁻¹⁷

To deal with the Pb issue, we had previously applied a kind of chemical indicator indo-1 (originally for Ca) as a novel Pb sensor.⁷⁶ The crucial point of indo-1 being able to sense Pb is that Pb can specifically quench the fluorescent intensity (FI) of indo-1 at spectral measurement around 450-470 nm (Fig. 3A). FI of indo-1 is Ca-insensitive at 440-450 nm (Fig. 3B).⁷⁷ With this chemical indicator, we provide evidence that Orai1 with STIM-1 as a kind of store-operated calcium channels (SOCs) plays a dominant role in cytosolic Pb entry (Fig. 3C-E).^{76,78} It seems to be relatively convenient using indo-1 as an alternative method to measure the existence of Pb, although this chemical probe has many drawbacks. The first drawback is that the photo-instability of indo-1 causes the photo-toxicity and even photo-activation of the reagents within the tested cells. Second, due to the weak FI of indo-1, the cell-loading procedure takes more time, with an extra problem in difficulty distinguishing the reduced FI signals from the illumination-induced photo-bleaching and the Pb-dependent photo-quenching. Third, none of the chemical probes can be trapped into specific subcellular

Table 4
Examples of FRET-based biosensors

Application	Examples	Mechanism	Sensory key(s)	FRET pair	Ref
Protein binding interaction	Multimerization of IL-17RA	Inter	IL-17RA with itself	CFP YFP	32
	GPCR subunit association	Inter	Gα with Gβγ	CFP YFP	33
	Transcriptional factor Erg and Jun interaction	Inter	Erg with Jun	CFP YFP	34
Protein conformational change	Sensing membrane potential	Intra S	Potassium channel voltage sensing domain	ECFP EYFP	35
	Activation and signaling of rac and cdc42	Intra M or Intra S	Cdc42 or rac with GTPase binding domains	CFP YFP	36, 37
				ECFP EYFP	
Protease activity	Caspases	Cleavage	Caspase proteolytic substrate	CFP YFP	38-42
				Cerulean Venus	
	Calpain	Cleavage	Calpain proteolytic substrate	ECFP EYFP	43
Kinase/phosphatase activity	Factor Xa	Cleavage	Factor Xa proteolytic substrate	BFP5 RSGFP4	44
	MLCK and MLCP	Intra S	RMLC (regulatory myosin light chain)	ECFP Citrine	45
	Kinetics and potencies of 12 known PKC ligands	Intra S	PKCδ	ECFP EYFP	46
	Detection of PKC activities	Intra S	Truncated pleckstrin containing PH and DEP domains	ECFP EYFP	47
	Phosphorylation by insulin receptor	Intra M	Phosphorylation recognition domain and its binding substrate	CFP YFP	48
	Activities of EGFR, Src and Ab1	Intra M	SH2 with phosphorylation substrates for EGFR, Src and Ab1	CFP YFP	49
	Activation of Src	Intra M	SH2 with phosphorylation substrates for Src	CFP YFP	50
Metabolic molecules	Glucose	Intra S	Glucose binding protein	ECFP EYFP	51-53
	Maltose	Intra S	Periplasmic binding proteins	ECFP EYFP	54
	Glutamine	Intra S	Glutamate/aspartate binding protein ybeJ	ECFP Venus	55
Signalling molecules	cAMP	Inter	PKA with cAMP-dependent binding substrate	CFP YFP	56
	IP3	Intra S	InsP3 receptors	CFP YFP	57, 58
	cGMP	Intra S	GKI and PDE	CFP YFP	59
	Estrogen receptor ligand	Intra S	Estrogen receptor ligand binding domain	CFP YFP	60
	Ca ²⁺ in ER	Intra S	apoK1-er	CFP YFP	61
	Ca ²⁺	Intra M	CaM M13	CFP YFP	30
				BFP GFP	
Other molecules	Zn ²⁺	Intra M	Atox1 WD4	CFP YFP	62, 63
	ATP	Intra M	ε subunit of the bacterial FoF1-ATP synthase.	CFP Venus	64
	Specific RNA sequence	Intra S	HIV-1 Rev protein	ECFP EYFP	65
	SARS CoV-2 Spike protein	Intra M	hACE2	Cy3 Cy5	66
	SARS CoV-2	Cleavage	3-chymotrypsin-like cysteine protease (3CL ^{pro}) substrate	ECFP Venus	67

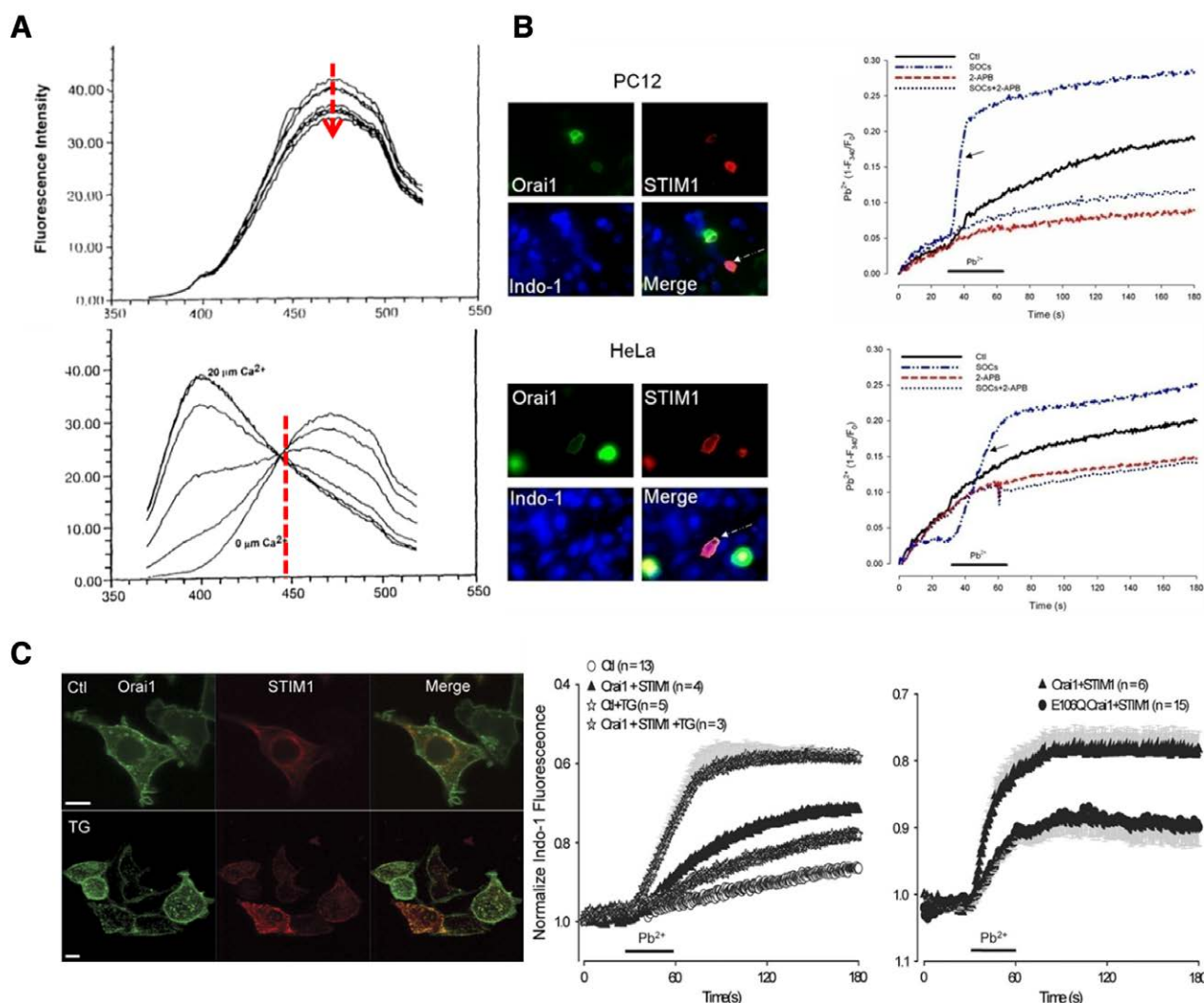


Fig. 3 The use of chemical fluorescent sensor indo-1 to detect intracellular entry of Pb from extracellular environment. A, The fluorescent emission spectra of indo-1 at different concentrations of Pb (upper) or those of Ca (lower). Pb can quench the fluorescent signals of Indo-1 at around 450 to 470nm (red arrows shown in upper part), and this wavelength area is almost Ca-insensitive (red dash line shown in the lower part). These figures are originally from Legare et al⁷⁷ and permission has been obtained to use the same here. B, Functional role of store-operated Ca channel (SOC, composed by a membrane channel Orai1 shown with green fluorescence, and an ER membrane protein STIM1 shown with red fluorescence) for the intracellular entry of Pb probed by indo-1 (shown with blue fluorescence) using different types of cells (upper: PC12; lower: HeLa). Right: The time-lapse recordings of indo-1 at different conditions—for example, control (Ctrl), activated SOC, SOC blocker 2-APB, activated SOC with SOC blocker. The data are originally from Chang et al,⁷⁶ and permission has been obtained to use the same here. C, Further evidence on the role of SOC through overexpression of Orai1 and STIM1. Left: Confocal images of Orai1 (green) and STIM1 (red) is shown in the localization of them. Right: The time-lapse recordings of indo-1 at different conditions. The data are originally from Chiu et al⁷⁸, and permission has been obtained to use the same here.

compartment to sense target molecule at present. The fourth key point is the cost. Such kinds of chemical probes generally cost high, and they require relatively a large amount for dye loading and the following sensing processes.

5. FRET-BASED PB BIOSENSORS

Since we did not have much experience on constructing GEFBs previously, it was indeed a great challenging task to develop Pb GEFBs. Thanks to Prof. Roger Tsien for giving us personal encouragement and suggestions in early 2008 before he gained the Nobel Prize. In 2012, we made the first version of FRET-based Pb biosensor Met-lead 1.59, so that the in-cell content monitoring of Pb can finally be done alternatively.⁶⁹ PbrR (a novel Pb binding protein) was selected as the Pb-sensing key within Met-leads. PbrR⁷⁹ was originally found from a special

bacteria *Cupriavidus metallidurans* (CH34),⁸⁰ which helps the organism to survive longer in the waste water of factories. The major functional domain of PbrR was cloned and re-ligated into the backbone of YC3.6 (replacing the Ca sensing motif: calmodulin and M13)⁸¹ to form Met-leads (molecular structure proposed in Fig. 4A). Finally, the performance of fluorescent spectral Met-lead (Fig. 4B) provides a direct evidence to demonstrate the FRET signal manipulation (functional Pb sensing) when Pb exists.

Discussing about sensor ability of the first version of Met-lead 1.59 (ie, the dynamic range [DR] and the sensitivity [limit of detection, LOD]), the DR is less than 2-fold (emission ratio from 3.3 to 5.7; Fig. 4C), and the practical LOD of Met-lead 1.59 is 100 nM (~2 $\mu\text{g}/\text{dL}$) or 500 nM (~10 $\mu\text{g}/\text{dL}$) with or without ionophore (ionomycin), respectively (Fig. 4D).⁶⁹ The sensing ability of Met-lead 1.59 was obviously not fully

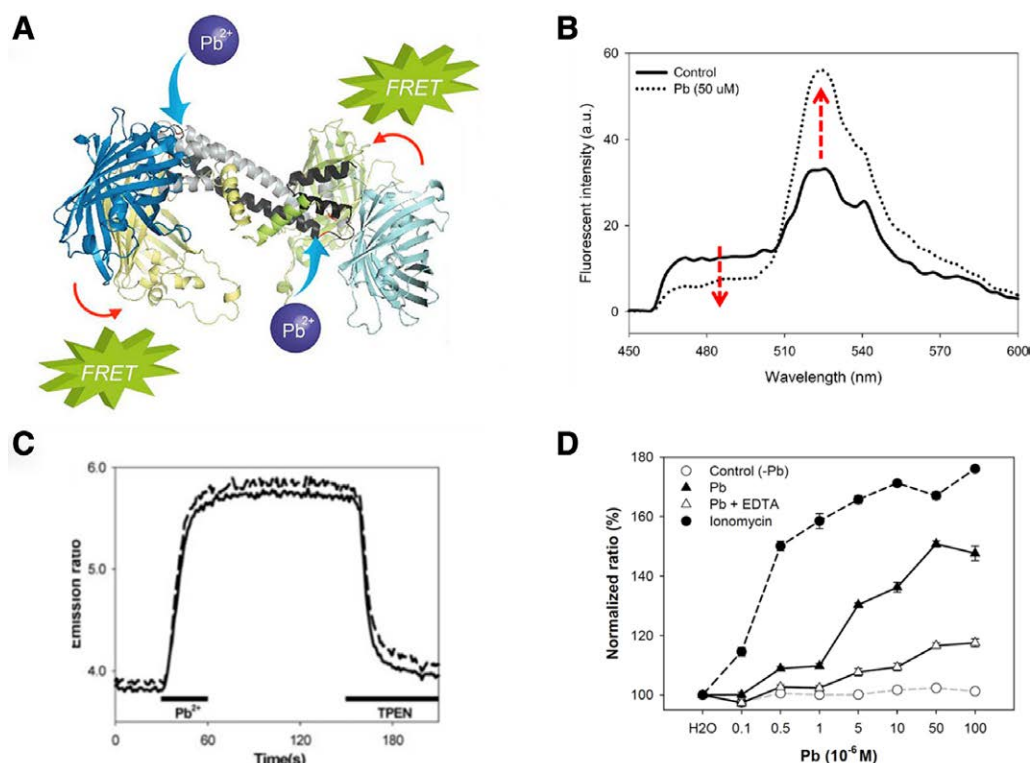


Fig. 4 Design, spectra, and performance of FRET-based Pb biosensor Met-lead. A, FRET design of Met-lead. B, The spectra of Met-lead shown FRET event could happen when Pb exists. C and D, Time-lapse record of Met-lead displays the emission ratio increase when Pb exists with (C) or without ionomycin (D). The data shown in (A and B) and (C and D) are originally from Yang et al.⁸² and Chiu and Yang⁶⁹, and permission has been obtained to use the same here.

qualified for further real applications (compared with the regulation required in Table 2), although it was a very good start for the development of FRET-based Pb biosensor. For

the specificity of Met-lead 1.59, the ionic selectivity of Met-lead 1.59 has been tested on various ions (eg, Ca, Mg, Mn, Fe, Cu, and Zn). The only interfered ions are Cu and Zn.⁶⁹ In addition to the FRET-based Pb GEFBs, we also developed a FRET-based cadmium (Cd) biosensor by applying CadR as the sensing key.⁷⁰

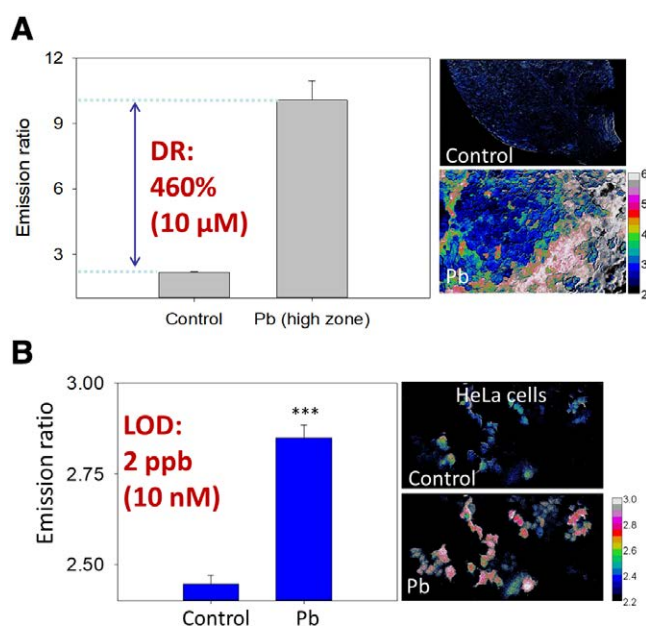


Fig. 5 Sensing ability of optimized FRET-based Pb biosensor Met-lead 1.44 M1. A, The dynamic range (DR) of FRET-ratio changes is up to 460% (within cotyledons of *Arabidopsis* seedlings). B, The limit of detection (LOD) is 10 nM (2.0 ppb). The data are originally from Yang et al.⁸² and permission has been obtained to use the same here.

6. OPTIMIZATION OF FRET-BASED PB BIOSENSORS

As described above, the low level of Pb is indeed quietly threatening human health without apparent signs of considerable dangers. Due to the relatively low DR (less than 2-fold) and sensitivity/LOD (only fare for adult BLL level: ~10 μg/dL) is not well-verified for children's limit 5 μg/dL. Met-lead 1.59, as the first version of FRET-based biosensor, was not a good for further Pb biosensing.^{69,82} Therefore, we tried to improve the sensing ability of Met-leads through different ways. First, utilizing the original structure of PbrR with six α-domains⁸³ to let us consider the adjustment of PbrR in lengths (different number of α-domains) may change the space distance between the two FRET pair FPs to modify the sensing level of Met-leads upgraded.^{69,84} Second, dimerization of PbrR via constructing a three-cysteine Pb-binding socket is required to send MerR-like protein family.⁸³ As the multiple-meric property could cause functional instability of Met-leads, it could be possible to break in a such multimer by inserting a repeat sequence (linker) within the middle position of PbrR. Actually, the sensing ability of Met-leads will be improved alternatively afterward.⁸⁴

So far, Met-lead 1.44 M1 is the optimized version with the best DR (almost 5-electronic fold, Fig. 5A) and LOD (10 nM, 2 ppb; Fig. 5B).⁸⁴ The dramatically expanded DR of Met-leads led us to explore the basic Pb toxicological researches involving

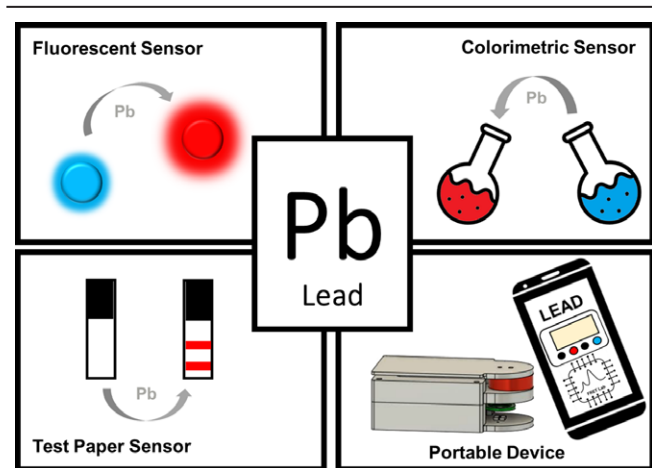


Fig. 6 The integration of various Pb detection methods into a portable device.

in vivo biosensing (eg, on live species such as *Drosophila* and *Arabidopsis* (Fig. 5A), respectively).⁷⁸ Newly-developed Met-lead 1.44 M1 with a high sensitivity (Fig. 5B) is five times lower than WHO-permitted level for tap water (10 ppb, Table 2) and 50 times lower than the BLL for children, 5 µg/dL (50 ppb, Table 2). Thus, Met-lead 1.44 M1 has met many important potential practical needs: the Pb detections from environment (*in vitro*, drinking or irrigation water) or body fluid (in-cells, serum or urine), and others (*in vivo*, whole animal or plant), which has been widely well-noticed in researches.

7. FUTURE PERSPECTIVES

Scientists have tried to combine 3C electronics such as smart phone to construct easy-to-use biosensors.^{84–94} Such portable devices would gradually become popular because of having a new advanced camera. Through visible light information or fluorescent signals, the mobile-tools can achieve good sensing abilities either using cuvettes or plates/microfluidic chamber to support target sources. Thus, it would be a great task to combine smart-phone with Met-leads to construct a new portable FRET-based sensing device in the future (Fig. 6). The new easy-to-handle device containing a biosensor-chip like Met-leads will allow us to imply real-time, and to precisely measure the contents of Pb everywhere, such as in tap or irrigation water, human bloods/serums or urines, etc. (Figs. 1 and 6). Meanwhile, the single FP-based Pb biosensors (Fig. 2A, B) can be more conveniently applied than FRET-based biosensors (because FRET-based biosensors occupy two fluorescent channels,⁹⁵ but single FP-based biosensors needs only one), and can be formulated as per the guidance of molecular simulation in the future (for examples of animated Met-lead, visit <https://reurl.cc/ygb4ny>).⁷⁸

ACKNOWLEDGMENTS

This work was supported by Ministry of Science and Technology of Taiwan (MOST 108-2745-8-075-001-, MOST 105-2320-B-075-002, NSC-97-2320-B-075-005-MY3, NSC-100-2320-B-075-004, NSC-102-2320-B-075-002) and Taipei Veterans General Hospitals (V98C1-052, V99C1-002, V100C1-032, V101C1-072, V102C-163, VGHUST102-G7-1-2).

We thank Professors Daniel van der Lelie for providing the bacteria CH34; Takeharu Nagai for sharing the FRET backbone of YC 3.6; Chia-Lin Wu for gifts of fly strains *Cha-gal4*, *R13F02-gal4*, and *TH-gal4*.

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