An update on genetically encoded lipid biosensors

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ABSTRACT Specific lipid species play central roles in cell biology. Their presence or enrichment in individual membranes can control properties or direct protein localization and/or activity. Therefore, probes to detect and observe these lipids in intact cells are essential tools in the cell biologist’s freezer box. Herein, we discuss genetically encoded lipid biosensors, which can be expressed as fluorescent protein fusions to track lipids in living cells. We provide a state-of-the-art list of the most widely available and reliable biosensors and highlight new probes (circa 2018–2021). Notably, we focus on advances in biosensors for phosphatidylinositol, phosphatidic acid, and PI 3-kinase lipid products.

INTRODUCTION Lipids are fundamental building blocks of cellular life. Their amphipathic nature makes them a keystone of bilayer membranes, as simply and elegantly illustrated by the double-tailed “tadpoles” of so many BioRender cartoons. Yet this deft simplicity belies the diversity of phospholipid, sphingolipid, and sterol species that make up biological membranes. The tightly crafted recipe of these lipids, with their unique shapes and charges, endows key functional properties on membranes: fluidity, curvature, and the capacity to selectively recruit or activate proteins are all regulated by lipids (Meer et al., 2008; Meer and Kroon, 2011; Balla, 2012). For this reason, cell biology demands approaches that can detect and enumerate membrane lipid compositions in their native cellular environment (Stahelin, 2009; Narwal et al., 2018; Dickson and Hille, 2019; Quinville et al., 2021).

This is where the genetically encoded lipid biosensors enter: these are typically lipid-binding domains from effector proteins or pathogen toxins, engineered to incorporate a tag for detection. Most conveniently, this involves fusion to a fluorescent protein for imaging in live cells. In this way, lipid biosensors can give information about the relative abundance, dynamics, and subcellular localization of lipids—in real time and in living cells. On the downside, the biosensors may be subject to biases in their localization, especially when not thoroughly characterized. We previously proposed two main criteria a biosensor should satisfy: 1) Is the biosensor selective for the lipid? This is typically determined in vitro. 2) Is the presence of the lipid both necessary and sufficient to localize the biosensor? This must be determined by modulation of lipids in the native cellular environment, and is often overlooked (Wills et al., 2018). Other caveats that must be considered include limitation to the detection of lipids in the outer plasma membrane or cytosolic membrane, because limits of fluorescence microscopy make interpreting localization in organelle lumens challenging. There are also extreme challenges to calibration, generally preventing quantification in terms of absolute lipid mass or mole fraction, though there have been technical tour-de-force studies that have done so (e.g., Liu et al., 2018). The strengths and weaknesses of lipid biosensors have already been explored in depth by ourselves and others (Balla et al., 2000; Lemmon, 2003; Maekawa and Fairn, 2014; Hammond and Balla, 2015; Wills et al., 2018). Suffice to say here, when it comes to genetically encoded lipid biosensors, a quote from Han Mi-nyeo, a character in the hit Netflix show Squid Game, sums it up: “I’m good at everything, except the things I can’t do.”

There have been many comprehensive reviews detailing currently available lipid biosensors (Stahelin, 2009; Kay et al., 2012; Maekawa and Fairn, 2014; Hammond and Balla, 2015; Narwal et al., 2018; Wills et al., 2018). We refer the reader to these resources for a comprehensive picture. Our goal here is to summarize a few notable

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Abbreviations used: Chol, cholesterol; DAG, diacylglycerol; DGK, diacylglycerol kinase; ER, endoplasmic reticulum; IP3, inositol 1,4,5-trisphosphate; IP4, inositol 1,3,4,5-tetrakisphosphate; NES, nuclear export sequence; PA, phosphatidic acid; PABD, phosphatidic acid binding domain; PASS, phosphatidic acid biosensor with superior sensitivity; PC, phosphatidylcholine; PI, phosphatidylinositol; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PI(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PI3K, phosphatidylinositol kinase; ER, endoplasmic reticulum; IP3, inositol 1,4,5-trisphosphate; IP4, inositol 1,3,4,5-tetrakisphosphate; NES, nuclear export sequence; PA, phosphatidic acid; PABD, phosphatidic acid binding domain; PASS, phosphatidic acid biosensor with superior sensitivity; PC, phosphatidylcholine; PI, phosphatidylinositol; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PI(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PI3K, phosphoinositide 3-kinase; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PI(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PI(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PI3K, phosphoinositide 3-kinase; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PI(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PI(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PI(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PI(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PI(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PI(3,4)P2, phosphatidylinosito
| Lipid          | Biosensor                                | Affinity          | Lipid specific? | Cellular localization of lipid | References                                      |
|---------------|------------------------------------------|-------------------|----------------|-----------------------------|------------------------------------------------|
|               |                                          |                   |                | Lipid dependent? | Lipid sufficient? | References                                      |
| Chol          | D4-PFO + mutants                         | 2–30 mol%         | ✔              | ✔                          | Shimada et al., 2002; Maekawa and Faim, 2015; Liu et al., 2016 |
| SM            | Lysenin                                  | $K_d = 5 \text{nM}$ | ✔              | ✔                          | Yamaji et al., 1998; Kiyokawa et al., 2005; Abe et al., 2012 |
| PA            | NES-PABD-spo20p$_{S^{51-91}}$ (PASS)      | ?                 | ✘              | ✔                          | Zhang et al., 2014                                 |
|               | NES-2xPABD-spo20p$_{S^{51-91}}$           | ?                 | ✘              | ✔                          | Bohdanowicz et al., 2013                          |
| α-Syn-N       |                                          | $K_d = 6.6 \text{μM}$ | ✔              | ✔                          | Yamada et al., 2020                               |
| PS            | C2-lactadherin                           | $K_d \sim 0.5 \text{μM}$ | ✔              | ✔                          | Yeung et al., 2008; Maeda et al., 2013; Vecchio and Stahelin, 2018 |
| DAG           | C1ab-PDK1                                | $K_i (\text{PDBu}) = 0.2 \text{μM}$ | ✔              | ✔                          | Chen et al., 2008; Kim et al., 2011                |
|               | C1ab-PKCε                                | $K_d \sim 10 \text{nM}$ | ✔              | ✔                          | Stahelin et al., 2005; Domart et al., 2012        |
| PI            | BcPI-PLC$^{\text{H2A}}$                  | ?                 | ✔              | ✔                          | Pemberton et al., 2020                           |
|               | BcPI-PLC$^{\text{ANH}}$                  | ?                 | ✔              | ✔                          | Pemberton et al., 2020                           |
| PI4P          | P4M-SidM                                 | $K_d \sim 1 \text{μM}$ or $-18.2 \text{nM FL}$ | ✔              | ✔                          | Brombacher et al., 2009; Schoebel et al., 2010; Hammond et al., 2014 |
|               | P4M-SidMx2                               | $K_d \sim 18.2 \text{nM FL}$ | ✔              | ✔                          | Hammond et al., 2014; Levin et al., 2017         |
|               | P4C-SidC                                 | $K_d \sim 250 \text{nM}$ | ✔              | ✔                          | Dolinsky et al., 2014; Weber et al., 2014; Zewe et al., 2018 |
|               | N-PH-ORP5, N-PH-ORP8                    | $K_d \sim 5 \text{μM for PI}(4,5)P_2$ | ✔              | ✔                          | Chung et al., 2015; Gai et al., 2017; Sohn et al., 2018 |
|               | PH-OSBP, PH-FAPP1                        | $K_d \sim 250 \text{nM}$ | ✔              | ✔                          | Levine and Munro, 2002; Sentztay et al., 2010; Lenoir et al., 2015 |
| PI5P          | 3xPHD (ING2)                             | ?                 | ✘              | ✔                          | Gozani et al., 2003; Pendaries et al., 2006       |
|               | PH-PLCδ1                                 | $K_d \sim 2 \text{μM}$ | ✘              | ✔                          | Garcia et al., 1995; Lemmon et al., 1995; Stauffer et al., 1998; Varnai and Balla, 1998; Hirose et al., 1999; Suh et al., 2006 |
|               | PH-PLCδ4                                 | $K_d > PH-PLCδ1$ | ✘              | ✔                          | Lee et al., 2004; Hammond and Ball, 2015          |

**TABLE 1:** Current genetically encoded lipid biosensors for a variety of selective lipid species.
| Lipid         | Biosensor            | Affinity | Lipid specific? | Cellular localization of lipid | Lipid dependent? | Lipid sufficient? | References                                                                 |
|--------------|----------------------|----------|----------------|-------------------------------|------------------|-------------------|---------------------------------------------------------------------------|
| Tubby<sub>c</sub> | K<sub>d</sub> > PH-PLC81 | X - binds PI(3,4)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> | ✔ | ✔ | ✔ | Quinn et al., 2008; Szentpetery et al., 2008; Halaszovich et al., 2009; Hammond and Balla, 2015 |
| Tubby<sup>332H</sup> | K<sub>d</sub> > Tubby | X - binds PI(3,4)P<sub>2</sub> and PIP<sub>3</sub> | ✔ | ? | ✔ | Quinn et al., 2008 |
| ENTH/ANTH    | K<sub>d</sub> ~ 2 μM | X - binds to PIP<sub>3</sub> | ✔ | ? | ☑ | Ford et al., 2001; Itoh et al., 2001; Yoon et al., 2011 |
| PI3P         | FYVE-Hrsx2            | K<sub>d</sub> ~ 2.5 μM | ✔ | ✔ | ? | Burd and Emr, 1998; Gauilier et al., 1998; Gillooly et al., 2000; Sankaran et al., 2001 |
|              | FYVE-EEA1             | K<sub>d</sub> ~ 45 nM | ✔ | ✔ | ? | Burd and Emr, 1998; Gauilier et al., 1998, 2000 |
|              | PX-p40phox           | K<sub>d</sub> ~ 5 μM | ✔ | ✔ | ? | Bravo et al., 2001; Ellson et al., 2001; Kanai et al., 2001 |
| PI(3,5)P<sub>2</sub>  | ML1-Nx2              | K<sub>d</sub> ~ 5.6 μM | ✔ | ✔ | ✔ | Li et al., 2013; Hammond et al., 2015 |
| PI(3,4)P<sub>2</sub>  | PH-TAPP1-CT          | K<sub>d</sub> ~ 80 nM | ✔ | ✔ | ✔ | Dowler et al., 2000; Thomas et al., 2001; Kimber et al., 2002; Marshall et al., 2002; Manna et al., 2007 |
|              | eTapp1-PH<sup>c</sup> | K<sub>d</sub> ~ 80 nM | ✔ | ✔ | ✔ | Liu et al., 2018 |
|              | TAPP1-cPHx3          | K<sub>d</sub> > 80 nM | ✔ | ✔ | ✔ | Goulden et al., 2019 |
| PIP<sub>3</sub>  | PH-ARNO(2G)-300F<sub>x2</sub> | K<sub>d</sub> ~ 170 nM | X - binds IP<sub>4</sub> | ✔ | ✔ | Goulden et al., 2019 |
|              | eMyox-PHx2<sup>c</sup> | K<sub>d</sub> ~ 33 nM | X - binds IP<sub>4</sub> | ✔ | ✔ | Hokanson et al., 2006; Plantard et al., 2010; Lu et al., 2011; Liu et al., 2018 |
|              | PH-Akt               | K<sub>d</sub> ~ 590 nM | X - binds PI(3,4)P<sub>2</sub> and IP<sub>4</sub> | ✔ | ? | Frech et al., 1997; Watton and Downward, 1999; Manna et al., 2007 |
|              | PH-Btk               | K<sub>d</sub> ~ 80 nM | X - binds IP<sub>4</sub> | ✔ | ? | Fukuda et al., 1996; Salim et al., 1996; Rameh et al., 1997; Kontos et al., 1998; Manna et al., 2007 |
|              | PH-GRP1 (2G), PH-ARNO (2G) | K<sub>d</sub> ~ 170 nM | X - binds IP<sub>4</sub> | ✔ | X - binds Arf/Arl | Klarlund et al., 1997; Venkateswarlu et al., 1998; Gray et al., 1999; Cohen et al., 2007; Hofmann et al., 2007; Li et al., 2007; Manna et al., 2007 |

<sup>a</sup>The accuracy of this probe is disputed.
<sup>b</sup>The <i>K<sub>d</sub></i> value is derived from myosin-c tail and IP<sub>4</sub> headgroup binding.
<sup>c</sup>Requires chemical ligation with a solvatochromic dye for optimal performance.
recent advances and tools available for specific lipids. We also present an updated table (Table 1) showing some of the most widely used and (in our opinion) reliable genetically encoded lipid biosensors.

The phosphoinositide that we all forget about

There have been many iterations of biosensors for phosphoinositides, which are cardinal regulators of membrane function (Dickson and Hille, 2019). These lipids are all phosphorylated derivatives of a single parent lipid, phosphatidylinositol (PI). PI is a major lipid, approximately 10% of cellular phospholipids, with the derivatives being <1% (Vance, 2015), but a biosensor for PI itself had been lacking. As an abundant lipid, its distribution may be assumed to be ubiquitous. Yet, because PI is the key substrate for synthesis of the other phosphoinositides, its availability in given membranes is an important variable best not left to assumption.

Pemberton et al. recently created a PI biosensor utilizing the Bacillus cereus PI-specific phospholipase C (BcPl-PLC). BcPl-PLC was mutated in order to eliminate catalytic activity of the enzyme, yet retain the active site configuration that can accommodate the inositol headgroup (BcPl-PLCH82A). However, in vitro assays showed nonspecific BcPl-PLCH82A binding to PC-containing liposomes. To remove this nonspecific binding, two membrane-penetrating tyrosine residues were mutated to create the BcPl-PLCANH probe. It should be noted, though, that neither the BcPl-PLCH82A nor the BcPl-PLCANH probes are fully specific for PI in vitro, as the BcPl-PLCANH showed enhanced binding to liposomes containing DAG and PI (Pemberton et al., 2020). PI was shown to be necessary for membrane localization of both sensors, because depletion of PI by PI-PLC recruitment or AngII stimulation caused a decrease in membrane localization of the biosensors. The sufficiency of PI for biosensor recruitment was demonstrated when PI levels at the plasma membrane were increased with either pseudoajarin-induced degradation of PI4P and PI(4,5)P2 to PI, or GSK-A1 inhibition of PI4KA-mediated conversion of PI to PI4P (Pemberton et al., 2020). Notably, the BcPl-PLCANH probe showed similar patterns of localization compared with BcPl-PLCH82A within cells. However, the BcPl-PLCANH showed more cytosolic localization than BcPl-PLCH82A, indicating that BcPl-PLCH82A may be a higher affinity probe for PI.

These probes revealed a surprising distribution of PI: an abundance at the endoplasmic reticulum (ER), peroxisomal, Golgi, and mitochondrial cytosolic leaflets, some on the endosomal network, but a notable absence at the plasma membrane at steady state (Pemberton et al., 2020). Satisfyingly, these findings were corroborated by additional approaches, including acute activation of PI-PLC or PI4Ks to generate diacylglycerol or PI4P from PI localized in specific membranes, which could be detected with other biosensors for these lipids (Pemberton et al., 2020; Zewe et al., 2020), and the trafficking of exogenously applied fluorescent PI (Zewe et al., 2020). Taken together, these results support a model where PI within the ER is transferred to the plasma membrane (PM) and then quickly converted into PI4P and PI(4,5)P2 to maintain homeostasis of these crucial PM phosphoinositide species.

The phospholipid backbone: phosphatidic acid

Phosphatidic acid (PA) is a crucial lipid, being both an intermediate in more complex phospholipid biosynthesis, and a second messenger molecule in diacylglycerol kinase (DGK) and phospholipase D (PLD) signaling pathways (Thakur et al., 2019). The most widely used biosensor is the phosphatidic acid biosensor with superior sensitivity (PASS) developed by Zhang et al. (2014). An added nuclear export sequence (NES) to the Spo20 phosphatidic acid-binding domain (PABD) prevented accumulation of PASS within the nucleus. This newly designed probe was able to show clear translation to the PM after stimulation with phorbol-12-myristate-13-acetate, without having to overexpose images. However, the PASS did still retain some slight binding to PI(4,5)P2 and PI3P within liposomes that the original Spo20 biosensor also showed (Zhang et al., 2014). A higher avidity, tandem dimer has also been developed (Bohdanowicz et al., 2013).

The usefulness of these PA biosensors has been recently corroborated by some new, ingenious tools, which have increased confidence in the accuracy of the Spo20-based PA lipid biosensors. An optogenetic bacterial PLD demonstrated that PA production in a variety of organelles is indeed sufficient to recruit PASS (Tei and Baskin, 2020). Additionally, click chemistry was used to label the products of PLD transphosphatidylation reactions as a proxy for PA, PLDs endogenous product. This method showed in real time that active PLDs localize to the PM, ER, and Golgi, with slight localization on endosomes, lysosomes, and the mitochondria (Liang et al., 2019; Tei and Baskin, 2020).

Recent work has gone into characterizing the N-terminus of α-synuclein as a novel PA biosensor (Yamada et al., 2020). Using liposomes, this construct (α-Syn-N) was shown to be selective for PA as compared with other lipids. However, it also showed higher selectivity for 18:1/18:1 PA species, which could limit its use in endogenous systems where many different acyl chains are likely to occur, and the 18:1/18:1 species is rare (Lorent et al., 2020). Within Cos7 cells, the α-Syn-N biosensor was shown to be dependent on PA, as it colocalized with wild-type DGKs and PLD, but not when catalytically dead enzymes or inhibitors were used to prevent PA production. However, it is still not clear that this biosensor will be as sensitive as PASS when PA levels are modulated in a more physiological context (Yamada et al., 2020). Therefore, we still recommend the more robustly characterized Spo20-based PA biosensors.

Class I PI 3-kinase products: both of them

The class I PI 3-kinase pathway is a paramount regulator of growth in metazoa; it is often activated in cancer and other diseases (Fruman et al., 2017). Mechanistically, PI 3-kinase signaling operates through production of the lipid second messenger PIP3 by 3-OH phosphorylation of PI(4,5)P2. PIP3 can then be converted (to varying extents) into an additional signal, PI(3,4)P2, by 5-OH phosphatases (Malek et al., 2017). Both PIP3 and PI(3,4)P2 interact with effector proteins, which may be selective for or both lipids (Hawkins and Stephens, 2016). Therefore, distinguishing these two lipids, and their subcellular localizations, is vital for delineating PI3K signaling at the cellular level.

The most popular biosensor for PIP3 signaling is the lipid-binding pleckstrin homology (PH) domain from its most famous effector, Akt. Although often mistaken for a PIP3-biosensor, this domain actually binds to both PIP3 and PI(3,4)P2 (Manna et al., 2007; Ebner et al., 2017; Liu et al., 2018; Goulden et al., 2019). It is worth noting that the isolated PH domain, from all three isoforms of Akt1–3, actually exhibits a preference for PIP3,4P2, although this preference only holds true for Akt2 in the context of the full-length protein (Liu et al., 2018). Therefore, the Akt PH domain-based biosensors can be fine indicators of PI3K activity, but they report the convolution of PIP3 and PI(3,4)P2 signals.

Our lab has recently published a highly selective and sensitive PI(3,4)P2 biosensor, cPHx3, made of a tandem trimmer of the C-terminal PH (cPH) domain from tandem PH-domain–containing protein 1 (TAPP1) fused to a NES and a fluorescent protein tag (Goulden et al., 2019). The improved sensitivity for PI(3,4)P2, derived from the high avidity of the tandem trimers, was evident when we detected the...
lipid’s synthesis after insulin stimulation, which had not previously been evident with lipid biosensors or many biochemical approaches (Goulden et al., 2019). Through an assortment of orthogonal manipulations in cells, we were also able to demonstrate that PI(3,4)P2 was both necessary and sufficient to drive CHRELP localization in cells.

As an alternative to tandem arrays, Liu and colleagues improved the membrane binding of a single cPH domain by mutating a methionine to a membrane-penetrating tryptophan residue. This would undoubtedly improve the binding of a fluorescent protein conjugate. However, cysteine residues were also removed or inserted to produce a single site for chemical ligation of a solvatochromic dye, generating eTAPP1–cPH (Liu et al., 2018). This solvatochromic dye exhibits a spectral shift when inserted into the hydrophobic bilayer, permitting ratiometric imaging of the probe’s membrane association. When calibrated against known mole fractions of PI(3,4)P2 in liposomes, precise quantification of lipid concentration was realized (Liu et al., 2018). Therefore, precise spatiotemporal detection of PI(3,4)P2 is now possible, which when combined with recent advances in mass spectrometry detection of this lipid (Maquek et al., 2017), will usher in a new era of understanding of this enigmatic lipid’s role in PI3K singling.

It is important to note that single PH domains from TAPP1 had previously been used as highly selective (but less sensitive) PI(3,4)P2 biosensors—but these came in two forms: one corresponding to the isolated cPH domain, and a second that includes the entire C-terminus of the protein. This C-terminal region contains a clathrin-binding domain, which biases the localization of the probe (Goulden et al., 2019). Therefore, it is critical to work with domains restricted to the isolated PH domains.

We also took advantage of the highly PI(P)-selective 2G splice variant of the ARNO (also known as cytohesin-2) PH domain to make a high avidity, tandem dimer probe for this lipid (Cronin et al., 2004). We engineered an I303E mutation into each domain to disrupt a secondary binding site for Arl-family GTPases; this biosensor showed excellent selectivity for PI(P)2 in cells (Goulden et al., 2019). Liu et al. also engineered optimized membrane binding and solvatochromic dye-conjugated derivatives of the PI(P)-selective MyosinX tandem PH domains, eMyoX-PH (Liu et al., 2018). Thus, there are now highly sensitive and selective PI(P)2 biosensors to accompany PI(3,4)P2 biosensors. These are included in Table 1. As noted in the table, a potential caveat to these sensors is their binding to soluble inositol tetrakisphosphate (IP4), the cognate headgroup of PI(P)2. This could potentially limit membrane translocation when PL-mediated IP4 production is triggered in conjunction with PI3K. This is expected to be a more minor caveat for the dimeric probes, where local concentration of the lipid on the membrane will favor high avidity binding to the tandem PH domains.

CONCLUSION
Genetically encoded lipid biosensors continue to be a powerful and convenient tool to study lipid dynamics and function in cell biology. Here, we have focused on a brief refresher of the principles, and highlighted some of the newest biosensors that have appeared in the last 3 years. Given the recent trend from the last 3 years in bio- sensor development, it seems certain that new and improved probes are on the horizon, so we encourage the reader to continue keeping an eye open for the latest developments!

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