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Engineering Protein Switches for Rapid Diagnostic Tests

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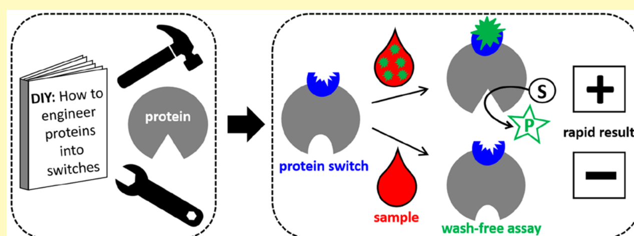
ABSTRACT: Biological signaling pathways are underpinned by protein switches that sense and respond to molecular inputs. Inspired by nature, engineered protein switches have been designed to directly transduce analyte binding into a quantitative signal in a simple, wash-free, homogeneous assay format. As such, they offer great potential to underpin point-of-need diagnostics that are needed across broad sectors to improve access, costs, and speed compared to laboratory assays. Despite this, protein switch assays are not yet in routine diagnostic use, and a number of barriers to uptake must be overcome to realize this potential. Here, we review the opportunities and challenges in engineering protein switches for rapid diagnostic tests. We evaluate how their design, comprising a recognition element, reporter, and switching mechanism, relates to performance and identify areas for improvement to guide further optimization. Recent modular switches that enable new analytes to be targeted without redesign are crucial to ensure robust and efficient development processes. The importance of translational steps toward practical implementation, including integration into a user-friendly device and thorough assay validation, is also discussed.



KEYWORDS: *protein engineering, protein switch, wash-free, homogeneous assay, immunoassay, rapid diagnostics, point-of-care, biosensors*

Point-of-care and in-field tests are needed across broad sectors, in order to improve access, costs, and speed compared to laboratory assays.[1−5] The COVID-19 pandemic highlights the real strength of rapid diagnostics to break transmission and guide timely treatment in infectious disease management,[6−8] and the transformational power of fast on-site results is evident across wide diagnostic applications.[1,3,9] However, the need for quality has been acutely reinforced, and in all sectors robust underpinning technology is required to provide sufficient sensitivity, specificity, and reproducibility in an easy-to-use format with a fast time-to-result.[10,11]

Inspiration can be taken from nature, where signaling is underpinned by protein switches that sense and respond to molecular inputs.[12] Engineered protein switches have been designed for synthetic biology and sensing applications and could be transformational as a rapid alternative to laboratory immunoassays.[13−16] Immunoassays typically utilize separate reagents for analyte recognition and signal generation, necessitating multiple immobilization and wash steps that prohibit direct point-of-need use (Figure 1A).[17] Microfluidic lab-on-a-chip systems can automate the steps, but few, like the Abbott iSTAT point-of-care analyzer, have been commercially successful.[18] Lateral flow devices (LFDs) are the dominant rapid test format in this market, offering a simple, cheap solution by employing the molecular recognition and reporter components on a single test strip (Figure 1B).[19] However, they are often only semiquantitative and insufficiently sensitive for many applications.[19] To overcome such shortcomings, engineered protein switches combine recognition and reporter elements at the molecular level and directly transduce analyte binding to an amplifiable, quantitative signal in a simple, wash-free, homogeneous assay format (Figure 1C).[13,14] They offer great promise to underpin point-of-need tests, compared to current homogeneous assays using chemiluminescent (e.g., AlphaLISA, SPARCL) or TR-FRET technology, which require sophisticated equipment and are better suited to high-throughput settings.[20−22]

Protein switches are well-established tools for lab-based cellular assays and imaging, including widely used commercial examples like protein-fragment complementation assays.[23,24] Despite clear potential, the development of protein switches for *in vitro* diagnostic assays is less mature, and while ever-improving designs are reported,[25−27] they are yet to be established in routine diagnostic use. Here, we review the opportunities and challenges for implementation of this technology and offer prospective solutions by analyzing the relationship between emerging protein switch designs and their performance. Following a comprehensive review of protein

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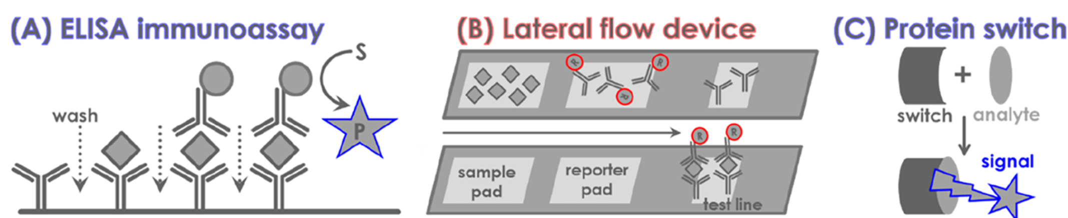

Figure 1. Schematic outline of (A) ELISA immunoassay with separate "capture" and "reporter" reagents, requiring multiple immobilization and wash steps. (B) Lateral flow device immobilizes the "capture" reagent on a test line and free "reporter" reagent on a pad, with binding and washing achieved by flow along the strip. (C) Protein switch combines "capture" and "reporter" elements at the molecular level and directly transduces analyte binding to a signal in a "mix-and-read" assay.

switches in 2015,[14] our focus is on developments from the past 5 years.

DESIGN AND PERFORMANCE

While the conceptual simplicity of protein switch assays offers great potential, devising a design which retains the crucial performance qualities of a laboratory immunoassay remains a significant challenge. Exact specifications are application dependent, but key assay performance characteristics include sensitivity, selectivity, range, reproducibility, functionality in biological matrices, and speed to results. A number of practical considerations including costs, ease of manufacture, shelf life, and ability to multiplex should also be favorable. It must also be understood that despite their limitations the continuing success of ELISAs and LFDs can be attributed to their highly modular format, in which the recognition and reporter components are simply exchanged to develop new assays.[19,28] Recent efforts toward modular protein switch designs are crucial if widespread adoption is to be achieved,[29,30] so development costs and times are competitive with LFDs, while offering improvements in assay performance.

A wealth of protein switch designs have been proposed, each with their own advantages and disadvantages for a particular diagnostic application. Designs can be two-component (proximity switches) or one-component (allosteric switches), but they all transduce analyte binding at a recognition element into activation of a reporter for signal generation (Figure 2).[14] Proximity switches use colocalization of reporter components for activation, whereas allosteric switches utilize a conformational change.[14] Allosteric coupling between the recognition and reporter elements can be achieved by direct domain insertion or modular approaches that limit conformational change to linker regions.[14] The recognition element, reporter, and switching mechanism all impact the performance according to their designs. Understanding these relationships is critical for optimizing protein switches toward the quality standards laid out above but studies analyzing how incremental changes in design parameters affect performance are somewhat lacking in this application driven field. Thus, we will critically review protein switch architectures and evidence our opinion on how designs relate to key performance characteristics, to improve the understanding of structure−function relationships, identify areas for improvement, and guide further optimization.

RECOGNITION ELEMENT

There are broadly four types of recognition element used in protein switches; small molecules, peptides, antibodies, and binding proteins. Their purpose is to bind the target analyte with appropriate selectivity over other molecules in the biological matrix and with sufficient affinity to achieve the desired assay sensitivity and range. It is imperative that these qualities are optimal, to prevent the recognition element limiting performance. Despite this, their affinity (K_d) is often not stated, and only a few studies describe the correlation with protein switch performance.[31,32] Studies into the effect of binding kinetics (k_{on}, k_{off}) may also prove insightful, to guide future designs toward desired properties.

Small molecule recognition elements are limited to known examples and must be conjugated to the sensor, which complicates development and manufacture.[33,34] Peptides are more easily incorporated within the protein switch amino acid sequence, including cleavage sites for proteases[35−37] and linear epitopes for antibody analytes.[25,32,38−40] Linear epitopes are often unavailable, but the scope can be broadened with mimotopes and meditopes, which are disulfide linked cyclic peptides selected by phage display, though still only for antibody analytes.[31,40] So, while small molecules and peptides are useful for certain applications, neither offer the wide analyte scope required for a general, modular protein switch format.

Antibodies are by far the most common recognition element used in ELISAs and LFDs, due to well-established development processes and an enormous existing selection for varied analytes. The ability to easily utilize this vast source of "off-the-shelf" recognition elements within protein switches would improve the scope and uptake of the technology. Whole antibodies have only rarely been used for *in vitro* protein switches,[41] but in the new commercially available Lumit immunoassay, antibodies are linked to each half of a proximity protein switch.[42] Attachment is via chemical modification of the antibodies with synthetic ligands that bind a self-labeling protein tag (HaloTag) on the reporter fragments.[42] This offers the potential to bring the technology into more mainstream research use, as new assays can be developed by simple exchange of commercially available antibodies. However, the combination of HaloTag and the antibody makes the recognition element extremely bulky and unsuitable for switches where geometry and orientation are important. Smaller antibody fragments, including Fab, V_H/V_L, and particularly single domain antibodies like V_HH, simplify the geometric requirements for a functional switching mechanism, and their genetic fusion to protein switches also streamlines manufacture.[29,43−47] The need to know the antibody sequence prohibits use of polyclonal, hybridoma-derived monoclonal and many commercial antibodies, but there are nonetheless robust discovery methods for antibody fragments, so diagnostic development is not restricted if time and budget allow.

Nature does provide alternative binding proteins to antibodies, such as periplasmic binding proteins, but they are limited to known examples and have only been used in protein

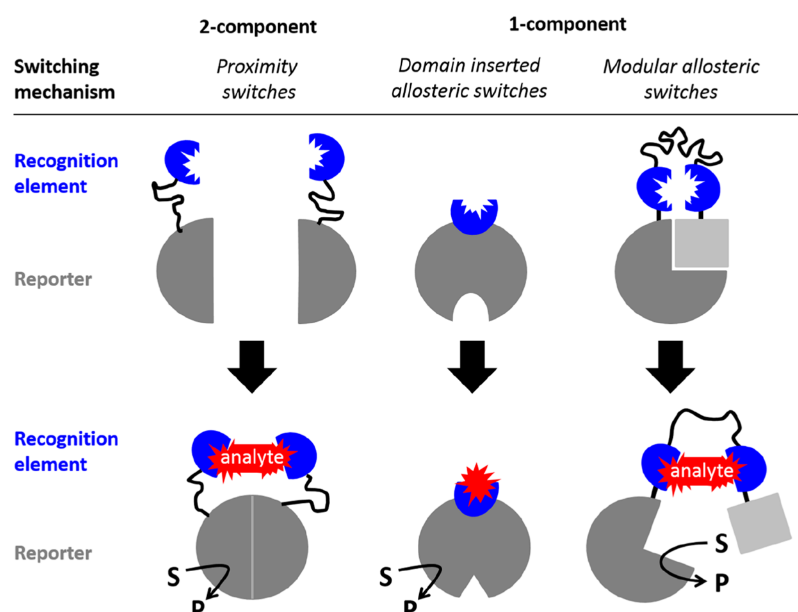

Figure 2. Schematic outline of protein switch designs. (Left) Proximity switches are activated by colocalization of components. (Middle) Domain inserted allosteric switches are activated by conformational change. (Right) Modular allosteric switches are activated by conformational change at linker regions.

switches targeting certain small molecules.[26,29,45,48,49] The scope can be broadened using antibody mimetic non-immunoglobulin binding proteins that consist of a scaffold and variable peptide regions.[50,51] They are selected with high affinity and specificity for new analytes by display methods, so development is quick and straightforward.[50,51] Counter-selection against competing molecules and affinity maturation can be used to further increase specificity and affinity.[52,53] Such proteins are also small, nonglycosylated, and cysteine-free,[50,51] so within protein switches they maintain the geometric benefits of antibody fragments while being easier to recombinantly manufacture. This makes them particularly attractive recognition elements and many, including Affimers, DARPINS, and monobodies, have been successfully utilized in protein switch sensors for varied analytes.[30,44,54,55]

In pursuit of a general, modular protein switch format, it is clearly advantageous to use selectable recognition elements that can be developed to the appropriate affinity and selectivity for wide ranging analytes. However, the current two-step development process to select binders and then optimize their insertion into a protein switch can be laborious. A binder or binding pair selected as optimal using techniques such as ELISA or SPR is not necessarily optimal within the protein switch; for example, that with the highest affinity may not bind the target with the best geometry for switch functionality. The switch may then need to be optimized around it, which complicates development. It would be more efficient if the protein switch had variable binding regions and could be selected from a large library directly based on switch performance. Establishing selection methods may prove difficult, but even an initial display-based screen followed by medium-throughput activity screening of a focused library would enable easier selection of functional switches. Small steps toward this have been made with selection of nonoverlapping binding pairs for proximity switches.[44] Binders which themselves undergo a conformational change have been developed by fusion of selectable binding proteins in clamp-like structures,[56,57] but direct selection of a switching sensor is yet to be achieved.

REPORTER

The purpose of a reporter is to rapidly generate a signal that can be measured by a point-of-need compatible detector, with appropriate sensitivity and range in the biological matrix. Initially, many protein switches had fluorescent protein or dye reporters,[48,55,58] but sensitivity was limited by the lowest measurable fluorescent probe concentration, typically nanomolar, and there were issues with background autofluorescence in biological matrices.[59] They have thus been superseded by enzymatic reporters that enhance sensitivity by signal amplification, as in ELISAs. Despite enormous possibilities, the same set of reporter enzymes encompassing proteases,[37,60] β-lactamase,[30,41,49,54] glucose dehydrogenase,[26,29,35,45,61] and luciferases[27,31−34,36,40,43,44,47,62−64] are repeatedly used in protein switches, perhaps due to their history as reporters in other assays and the ease of recombinant production. Dependent on the enzyme and substrate, there are four key formats: colorimetric, fluorometric, luminescent, and electrochemical.

Colorimetric assays have low sensitivity, limited by the extinction coefficient of the colored product, and poor range due to difficulties in measuring high absorbances.[65] Fluorescence from products of a fluorometric assay can be measured more sensitively and to very high readings compared to absorbance, so the range is greatly improved.[65] Nevertheless, there are issues with background autofluorescence.[65] Protease and β-lactamase (BLA) reporters can use either format depending on the synthetic substrate used.[65] Protease reporters are generally slow with assay time scales on the order of hours, so they are unsuitable for rapid diagnostics.[37,60] BLA is faster with assays taking minutes but is significantly inhibited in serum, which hinders its applicability to many clinical applications.[30]

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Luciferases are bioluminescent enzymes which emit light upon substrate turnover. They are particularly promising reporters due to their brightness and the fact that no incident light source is required.[66] There is no background autofluorescence, so the bright glow is against a dark background, enhancing sensitivity relative to fluorometric methods.[66] The range is also excellent as emitted light can be measured over many orders of magnitude. Even a simple camera can be used for detection, which is compatible with "point-of-need" use.[27,40] The engineered luciferase Nanoluc is the most promising and widely used luciferase within sensor proteins.[25,27,31−34,38,40,43,44,47,62,66] It is small, highly stable, and recombinantly produced,[67] so it works well in a protein switch fusion.[66] It is brighter than other luciferases, which enhances sensitivity, and its "glow" luminescence provides a sustained signal, which simplifies measurement.[67,68] A disadvantage is its low emission wavelength (~460 nm), which is readily absorbed by biological matrices.[66] Although this has been somewhat overcome by the use of thin layers with low path lengths, dilutions can still be required, which reduces sensitivity.[25,27,38,43] Matrix absorption is also reduced at higher wavelengths, and red-shifted Nanoluc variants warrant further investigation in sensor proteins.[36,69−72] Other enzymes with chemiluminescent substrates could expand the scope of luminescent reporters[66] but are yet to be explored in protein switches.

Electrochemical reporters generate an electrical signal which is easier to integrate into a cheap, miniaturized, electronic detection device than optical systems and also overcomes the need for transparent samples.[73] This makes them particularly attractive for point-of-care sensors, as evidenced by the success of the electrochemical glucometer for diabetes management.[74] Reporters that tap into this established technology have been used in protein switches. Trehalase reporters generate glucose so activity can be indirectly measured with a glucometer, although background glucose in the biological matrix complicates measurements.[39,75,76] This is avoided if glucose dehydrogenase (GDH) is itself used as a reporter, with a saturating concentration of glucose substrate. GDH is well developed and offers advantages in respect to its ease of manufacture, high stability, turnover rate, and functionality in biological matrices.[26,29,35,45,61] Nevertheless, detection of glucose oxidation by GDH protein switches has largely been done colorimetrically, and sensitivity is very low when measured electrochemically with a glucometer.[26,29,35,45,61] Standard glucometers are set up to measure turnover by fully active GDH, and adaptations are needed to improve sensitivity for protein-switch measurements. Redox enzyme reporters like GDH oxidize or reduce substrates, and this electron transfer must be interfaced with the electrode for measurement as an amperometric signal.[77] In a standard glucometer, this is done inefficiently by diffusion of redox mediators, but sensitivity is improved by enzyme attachment to the electrode for direct electron transfer.[77] This also reduces interfering reactions, and work is underway to interface GDH protein switches in this way.[35,78] Nevertheless, enzyme attachment in the correct orientation for electron transfer can be a significant challenge.[79]

Aside from GDH, there are a plethora of other redox enzymes that could be explored as reporters, should their operating potential be in a window free from background matrix signals. Further to this, enzymes that change the redox activity of their substrate (e.g., alkaline phosphatase with p-NPP and β-galactosidase with PAPG) have been used for electrochemical ELISAs and should be explored, as the product can be accumulated and potentially redox-cycled to enhance sensitivity.[80−82] Overall, electrochemical reporters are an underexplored tool within protein switches and further work to study the sensitivity, range, and other crucial performance characteristics of such systems would be insightful. The lack of development to date may be due to the added complication of interfacing the assay with an electrode, so future work should be directed here.

SWITCHING MECHANISM

The purpose of the switching mechanism is to transduce analyte binding at the recognition element into a signal change at the reporter. The mechanism should minimize background activity and maximize recovered activity with the analyte, to ensure maximal signal change between the "off" and "on" states (response dynamic range). It should also be functional for wide-ranging analytes if a general, modular format is to be achieved. Mechanisms are based on either two-component proximity switches or one-component allosteric switches, with domain inserted or modular designs (Figure 2).[14]

Proximity Switches. In proximity switches, analyte binding induces colocalization of sensor components, which results in a change in response (Figure 2). The most common approach is split-enzyme complementation, in which an enzyme is split into two inactive fragments, usually directly but sometimes via mutations at a dimerization interface.[39,41,44−47,75,83] Each fragment is attached to recognition elements that bind nonoverlapping epitopes on the analyte, such that binding colocalizes the fragments and induces reconstitution of the active enzyme.[39,41,44−47,75,83] This approach is insensitive to the exact geometry of the analyte and binding domains, so long as linkers between the binder and fragment are sufficient. Once colocalized, the high effective concentration and residual affinity between fragments should drive reconstitution, regardless of exact orientations. A split-GDH showed the potential generality of such systems, with the same design able to detect small molecules, proteins, and proteases by simple exchange of recognition elements.[45] Nevertheless, split-enzyme fragments can be unstable and difficult to produce *in vitro*.[45,83,84] Reconstituted activity can be a fraction of the wild-type enzyme, and high residual affinity between fragments causes background complementation, which reduces the sensitivity and dynamic range of the reporter response.[45−47,83] A big problem with two-component systems is that they are concentration driven, so dependent on the absolute and relative component concentrations.[26,59] To prevent background complementation, fragment concentrations must be well below the residual binding K_d, but this limits the analyte concentration range that can be measured. Also, as recognition elements are on two separate components, there are no avidity effects to increase affinity, so each binder must have sufficient affinity to achieve the required sensitivity. A final consideration is that at high analyte concentrations, a "Hook" effect can be observed, where instead of two recognition elements binding one analyte to colocalize sensor components, the analyte binds to each separately and reduces the response. This can be overcome by control of the component concentration or use of co-operative binders.

Protein engineering to overcome split-enzyme deficiencies is a significant task but worth the time investment because once a particular system is optimized it can form a modular format

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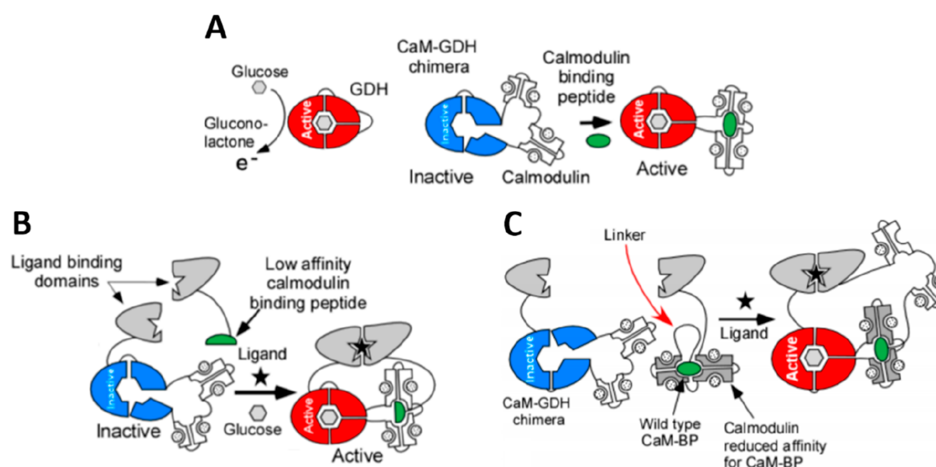

Figure 3. Allosteric GDH switches designed by Alexndrov and co-workers.[26] (A) Core design. (B) Higher order proximity switch architecture. (C) "Caged" system. Reprinted with permission from ref 26. Copyright 2020 American Chemical Society.

that is repeatedly used. Engineering of the NanoLuc reporter into the NanoBit split-enzyme system is an excellent example.[24] Circularly permutated NanoLuc variants with cleavable linkers were screened to identify a suitable dissection site, yielding a small and large fragment with a proximity constraint for activity.[24] The larger polypeptide fragment underwent two rounds of mutagenesis for stabilization and to increase reconstituted activity to 37% of NanoLuc itself.[24] The smaller peptide fragment was separately mutated to reduce residual affinity to K_d = 190 μM and diminish background complementation, even at micromolar fragment concentrations.[24] The NanoBit system was linked to antibodies to form the basis of the recent Lumit cell-lysate immunoassay.[42] To utilize NanoBit for diagnostic assays, both Dixon et al. and Ohmuro-Matsuyama et al. further split the system, to give a polypeptide and two peptide fragments.[44,47] The two small peptide fragments could be produced more easily in fusion with recognition elements and the polypeptide could be held at a separately high concentration in the assay.[44,47] The sensitivity (pM), response dynamic range (up to 100 000%), and analyte linear range (>3 orders of magnitude) of these systems are superior to other *in vitro* split-enzyme assays.[39,41,44−47,75,83] These qualities are a testament to the engineering effort that enabled low background complementation and high luminescent activity recovery. Nevertheless, tests were in a maximum 5% serum matrix, and use as a general format for wide ranging analytes is yet to be shown.

Domain Inserted Allosteric Switches. Allosteric switches based on domain insertion involve fusion of the recognition and reporter domains such that they are allosterically coupled, and binding at the recognition element is transduced into a change in activity of the reporter (Figure 2). Ascertaining a tolerant insertion site for optimal allostery between the domains is challenging.[14] Recently, this has been done by rational design, as in the case of calmodulin insertion into GDH,[61] and by screening of random insertions of circularly permutated BLA into periplasmic binding proteins.[49] Upon analyte binding, both calmodulin and periplasmic binding proteins undergo a conformational change, which is conferred into a change in conformation and activity of the inserted reporter domain.[49,61] However, such structure-switching binding proteins are limited, and generating allostery is more challenging with common recognition elements that undergo minimal conformational change. When BLA was randomly inserted into DARPIN and monobody binding proteins, allosteric switches could be selected.[54] However, binding affinity was reduced in the fusion, and micromolar analyte concentrations were required to drive a switching effect.[54] Further, only one of four attempts to target new analytes by exchange of binding proteins was successful, highlighting the lack of generality to the approach.[54] A solution may lie in the development of selectable structure-switching binding proteins, such that allosteric coupling to the reporter is more reliable. Affinity clamps are selectable bidomain proteins, which enclose around peptides and have been used to generate peptide-responsive allosteric switches.[29,56,85] Building on this, Léger et al. fused selectable monomers into bidomain proteins that open up to bind protein analytes.[57] However, it remains to be seen if they can target varied analytes and be allosterically coupled to reporter enzymes.

An alternative approach is to establish an allosteric switch and then integrate it into a higher order architecture to target varied analytes. Alexandrov and co-workers used structure-guided design to insert calmodulin into GDH (CaM-GDH) at a site where cofactor interacting residues are displaced (Figure 3A).[29] Binding of calmodulin binding peptide (CaM-BP) induces a contraction that is allosterically coupled to GDH and restores cofactor interactions, recovering 50% of wild-type activity. CaM-GDH and CaM-BP were then fused to recognition elements, so analyte binding drives colocalization for activity recovery (Figure 3B). A range of small molecules and proteins was successfully targeted by exchanging recognition elements (binding proteins, V_HH), so integration of the proximity mechanism gives a modular format independent of exact binding orientations. However, low affinity CaM-BP was used to minimize background activation and the truncated peptide did not induce the same structure change in CaM, reducing activity recovery. Concentrations of the two-component sensor also had to be lower than the residual CaM-BP binding K_d, which limited the analyte range that could be measured (∼1−10 nM for rapamycin). To overcome this, native CaM-BP was "caged" with low affinity CaM, giving a kinetic and thermodynamic barrier to background activation (Figure 3C).[26] At micromolar component concentrations, the "uncaged" sensor was fully self-activated, whereas the "caged" sensor had low background activation and so was analyte responsive.[26] Rapamycin was measurable over ∼10−1000 nM, with a response dynamic

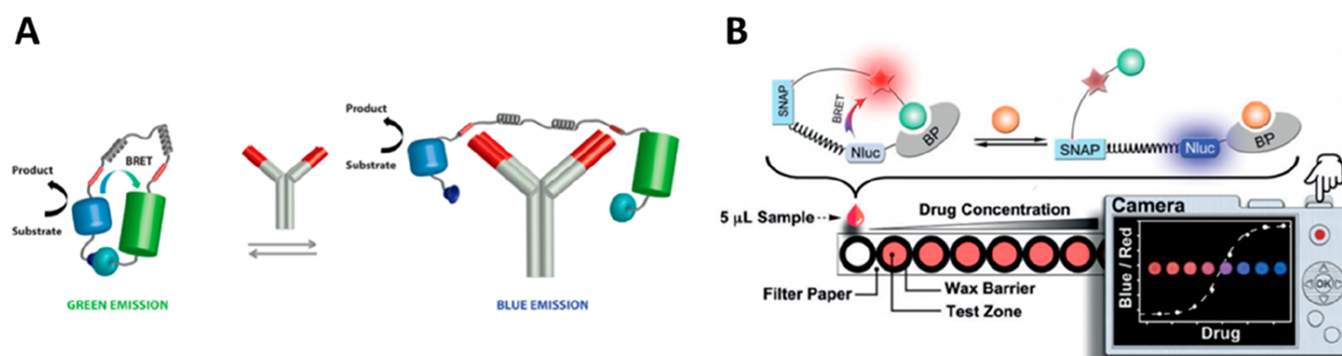

Figure 4. BRET-based modular allosteric switches. (A) LUMABS developed by Merkx and co-workers.[32] Reprinted with permission from ref 32. Copyright 2016 American Chemical Society. (B) LUCIDs developed by Johnsson and co-workers.[43] Reprinted with permission from ref 43. Copyright 2017 Wiley.

range of ∼1300%, but this was dependent on the two-component concentrations.[26] Progress toward optimizing the CaM-BP/CaM "cage" system was made and may lead to further improvements in sensor performance.[26] Fluorescent protein and NanoLuc based switches could be similarly constructed but unlike the "uncaged" sensors performance in biological matrices was not verified.[26,29]

Modular Allosteric Switches. Modular allosteric switches comprise structurally distinct recognition and reporter domains, with conformational change limited to linker regions and induced by mutually exclusive binding interactions (Figure 2).[14] They are generally based on modulation of an enzyme−inhibitor interaction or bioluminescent resonant energy transfer (BRET).[27,30−32,34,40,43,62,85,86] Unlike domain-inserted switches, the structurally distinct recognition element should maintain its affinity and need not be structure-switching, so common ones can be used interchangeably. The intact reporter domain should also retain its full activity and stability, unlike split and domain-inserted reporters that can be structurally compromised and suffer weaker activity recovery. One-component modular allosteric switches are less concentration dependent than two-component systems but binding geometry can be more critical to ensure disruption of the competing interaction.

Autoinhibited enzyme switches comprise an enzyme tethered to an inhibitor via a linker containing recognition elements.[14] Analyte binding induces a linker conformational change, which disrupts the enzyme−inhibitor interaction and restores activity.[14] In a recent example, BLA was tethered to its inhibitor protein (BLIP) via a flexible linker containing two Affimer binding proteins.[30] This gives an avidity effect, and with anti-hCRP Affimers of K_d = 12 nM, hCRP was measurable over 30 pM to 30 nM.[30] As in a previous switch, the BLA-BLIP interaction was weakened to enable disruption, but this increases background activity and is a limiting factor to sensitivity.[30,86] Nevertheless, 83% of wild-type BLA activity was regained upon activation, and the response dynamic range was up to 1200%.[30] Previous autoinhibited switches targeting peptides and antibodies have been reliant on the exact geometries of those analytes.[85,86] The use of selectable Affimer binding proteins and an optimized flexible linker enabled a range of proteins to be targeted (antibody, hexameric protein, virus).[30] However, these large multimeric proteins can induce significant linker conformational change, and it remains to be seen if monomeric proteins or small molecules can be targeted without significant linker redesign for each analyte. Furthermore, only a 1% serum matrix could be used,[30] and better performing electrochemical and luminescent reporters are yet to be explored, perhaps due to the difficulty in generating appropriate inhibition domains.

BRET sensors are based on a modulation of distance and thus resonant energy transfer between a luciferase and fluorophore.[66] Merkx and co-workers introduced LUMABS (LUMinescent AntiBody Sensor), in which blue light emitting NanoLuc and linked mNeonGreen fluorescent protein are held close together by helper domains (Figure 4A).[32] Antibody binding to two recognition elements (epitopes, cyclic peptides, or small molecules) extends the linker between them and disrupts the helper domain interaction.[31−33] The distance between NanoLuc and mNeonGreen increases, which decreases BRET and the ratio of green/blue emitted light.[31−33] Such ratiometric measurements are independent of sensor concentration and matrix effects on absolute signal intensity, and so are reproducible.[31−33] The maximal response dynamic range was ∼300%, limited by how close the domains are able to get in the "closed" state and the large background NanoLuc signal at mNeonGreen's emission wavelength.[33] The recognition elements need sufficient affinity to overcome the helper domain interaction, but use of two gives an avidity effect, and with HIVp17 epitopes of K_d = 42 nM, the anti-HIVp17 antibody was measurable over 10−1000 pM.[32] The sensors were also functional in undiluted plasma but with light intensity and the sensitivity reduced 10-fold.[32] LUMABS are restricted to antibody analytes as they depend on the Y-shaped presentation of two antigen binding domains at a sufficient distance to induce a measurable change in BRET.[31−33] Small molecules have been targeted but in a competitive format, functional only at low millimolar analyte concentrations.[33] An alternative NB-LUMABS sensor is based on intramolecular split NanoLuc complementation and BRET to a directly attached red fluorescent dye.[40] In the "closed" state, NanoLuc and the fluorophore are closer, and thus BRET is more efficient than when using bulky fluorescent protein and helper domains.[40] The background NanoLuc signal is also lower at the red fluorophore emission wavelength, which improves the response dynamic range (∼500%) and sensitivity (low pM).[40] Fluorophores with large differences between their excitation and emission wavelengths may be useful, so excitation overlaps well with NanoLuc emission for efficient BRET, but emission is at red-shifted wavelengths with a low background from NanoLuc.

Johnsson and co-workers developed LUCIDs (LUCiferase-based Indicators of Drugs), which comprise a receptor protein, NanoLuc and SNAP-tag labeled with a red-emitting fluorophore and synthetic ligand analogue of the target drug (Figure 4B).[43,87] An intramolecular interaction between the ligand analogue and receptor protein is disrupted by target drug binding, which increases the distance between NanoLuc and the fluorophore.[43,87] The reduction in BRET efficiency is reproducibly measured as a ratiometric change in red/blue emitted light.[43,87] Antibody fragments (Fabs) have a common 3D structure, so they can target new drugs without an optimization of sensor geometry for efficient BRET.[43] However, the synthetic ligand/Fab interaction must be weakened, so that the free drug can compete and be measured over a clinically relevant range.[43] This requires mutation and synthetic ligand optimization for each new sensor, based on structural knowledge that may not always be available.[43] It does enable sensor tuning, and for example, methotrexate is measurable over 53 nM to 5 μM or 1−130 μM using appropriately tuned sensors.[43] The response dynamic range is up to 2155%, due to the close proximity of NanoLuc and fluorophore in the "closed" state and their distance in the "open" state, enforced by a long rigid proline linker (Pro30).[43] Importantly, LUCIDs are also functional in 50% serum and 10% whole blood.[43] Similar sensors have been optimized to transition from an "open" to "closed" state upon binding NADPH or NAD+, which enables indirect measurement of many associated metabolites and enzymes via production of these cofactors.[27,62] Another similar sensor has an additional ligand that binds protein, driving a transition from the "closed" to "open" state by steric hindrance.[34] Streptavidin and dihydrofolate reductase have been targeted, but it remains to be seen if this is a generally applicable strategy.[34] Resonant energy transfer has an inverse sixth-power distance dependence, so designing sensor formats that are independent of target geometry is challenging and an important area for research.

■ ASSAY VALIDATION

New protein switch designs are often only analyzed in terms of a few qualities and are rarely rigorously assessed against all the appropriate performance criteria. More thorough and comparable analysis between sensors would make the quality and potential of new designs much clearer, and better highlight areas for improvement. Assay speed is absolutely critical in the context of the test application; for example, in many countries, doctors' consultations last 10−15 min, so any test used at this point in the care pathway must be significantly faster. Total assay time is perhaps the only universally reported quality, and most protein switches operate on multiminute time scales, which compares unfavorably with blood glucose monitors that give a response in seconds. In terms of sensitivity, the limit of detection (LOD) is often not explicitly calculated, and where it is, different methods are used. The often used $\text{mean}_{\text{blank}} + 3\sigma_{\text{blank}}$ method only accounts for variability in blank measurements, and we feel that stricter definitions which also account for variability in test measurements are more reliable.[88] Target selectivity is sometimes studied; for example, specificity for a certain IgG over others has been tested for various antibody sensors,[25,30,31,38] but such assessments could be more widely adopted. Reproducibility is extremely important if an assay is to ultimately be used for real-world analysis, but some studies do not even include repeat measurements. The standard deviation of three repeats is often reported, but large scale analysis of intra- and interassay coefficients of variation is rare.[27] Batch-to-batch reproducibility is also crucial to ensure consistency between lots, but this is also only rarely investigated.[25,30,38] The analyte range over which a protein switch assay is functional is usually reported, but rigorous calculations of the upper and lower limits of quantification alongside the accuracy and precision of measurements in between are omitted. Performance in biological matrices should always be thoroughly studied but is often lacking. Detection of an analyte spiked in a certain percentage of matrix is often shown, but data on the exact effect of this matrix compared to buffer are frequently absent. Studies are usually limited to assessment of the analytical accuracy with spiked samples and a comparison of measurements between the protein switch and a "gold standard" assay.[27,29,30,62,76] Evaluation of the diagnostic accuracy in patient or other real-world samples is infrequently completed but is absolutely critical to understand the utility of the assay. In terms of practical considerations, although shelf life is frequently studied,[25,27,29,30,38,43,45,62] other factors including costs and ease of manufacture are largely ignored, despite being vital for real-world uptake.

While comprehensive analysis of all these parameters may not always be appropriate, we do feel that more consistent reporting standards would aid comparison between protein switches and guide further improvements. Furthermore, translation of protein switch assays into real-world use is reliant on extensive validation, and it will not be adopted until this is done. For well-developed protein switches, the next important step is thorough analytical validation of assay performance to regulatory standards, for example, the U.S. FDA criteria for bioanalytical method validation.[89] Only then will such assays show their potential to be used in routine diagnostic use. For clinical diagnostics, further evaluations are then needed to determine how the test works in patient samples (the diagnostic accuracy) and in a clinical setting to improve patient care (the clinical utility). Co-design of devices, with the end-user involved at an earlier stage of the design process, may be a means to improve the rate at which assays are pulled through to these evaluations.

■ DEVICES

While the discussed protein switches offer promise, the assays still require liquid handling steps (e.g., pipetting) and often use laboratory detection devices (e.g., plate readers), prohibiting direct use at the point-of-care or in-field. Most research is focused on switch design, but methods to fully integrate such assays into a user-friendly point-of-need device are urgently required if practical implementation of the technology is to be achieved. Device integration can also improve some aspects of assay performance, including speed, sample volume requirements, functionality in biological matrices, and ability to multiplex. Existing technology for point-of-care immunoassays, including chip- and paper-based microfluidic devices, can be tapped into, and integration with no-wash protein switch assays should, in fact, be easier.[90−92] A fully device-integrated electrochemical protein switch assay has yet to be achieved, largely due to the described complexities of interfacing the assay and electrode, while preventing fouling by the biological matrix. Electrochemical protein switches have been attached to screen-printed gold electrodes via covalent linkers and carbon fiber electrodes via graphene nanosheets.[35,78] Other interfacing

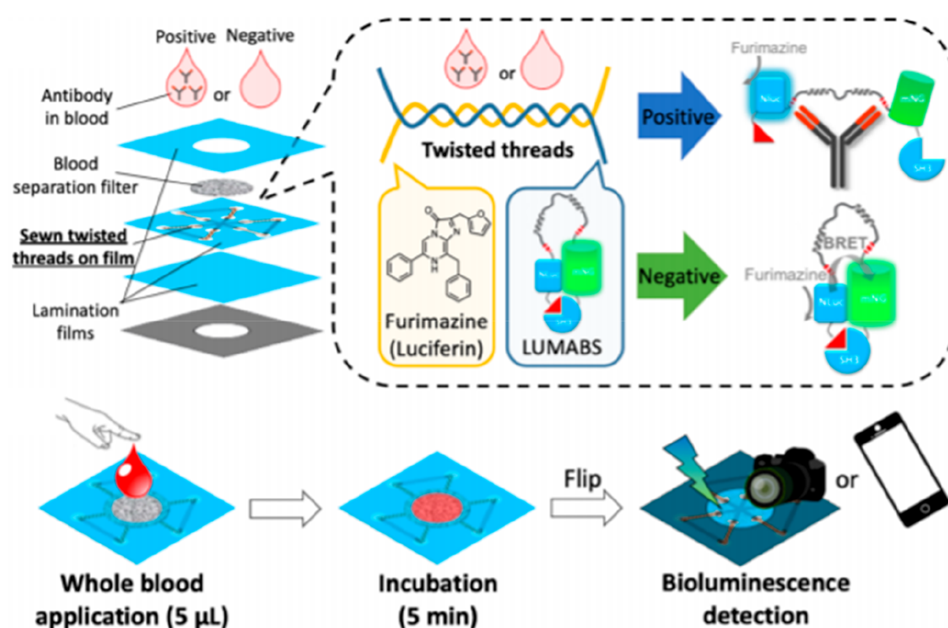

Figure 5. Antibody detection in whole blood using LUMABS on a μTAD developed by Citterio and co-workers.[25] Reprinted with permission from ref 25. Copyright 2020 American Chemical Society.

strategies from biosensors,[93−95] or even complementary technology such as biofuel cells,[96] should be explored. Once interfacing is optimized, devices encompassing liquid handling and detection can be developed from existing electrochemical point-of-care technology, such as the glucometer.[97,98] Device integration is further developed for BRET sensors, and recent microfluidic approaches have shown great potential.[25,36,38]

Weihs et al. have integrated a BRET-switch assay into a microfluidic glass chip comprising a chaotic mixer for the sensor, substrate and sample mixing, an incubation channel, and a narrow chamber for optical detection.[36] This minimizes luminescent signal absorption for measurements in a 10% serum matrix, and the total channel volume of 23.6 μL allows for low sample volumes.[36] The chip is used in a device containing syringe pumps for sample flow, a heat-block for temperature control, and microphotomultiplier tubes for signal detection.[36] While this does enable a fast (5 min), low cost assay, some liquid handling is still required, and the 6.5 kg device affords poor portability.[36] Tenda et al. describe a smaller, fully integrated microfluidic paper-based analytical device (μPAD) for BRET-switch assays.[38] Whole blood is processed by a plasma separation membrane, which flows by capillary action into paper layers impregnated with the substrate and then the LUMAB sensor.[38] The bioluminescent signal is simply detected by a camera.[38] This design has recently been improved by using a 3D-printed attachment to a smart-phone camera alongside a microfluidic thread-based analytical device (μTAD; Figure 5).[25] The substrate and sensor protein are instead impregnated on intertwined threads, which reduces the dead volume, reducing assay times and sample volume requirements.[25] In fact, in just 5 min, 5−15 μL of whole blood can be tested without sample volume dependency, making it compatible with a finger prick of blood.[25] The design enabled up to 6-plex detection on different zones of the device,[25] which is an important development as no protein switch in itself offers multiplex detection. Clinical diagnoses often require laboratory analysis of panels of analytes, and efficient uptake of point-of-care alternatives relies on incorporation of all of the desired analysis.[99] The multiplex device was also stable for a month, could be reproducibly fabricated and is cheap to produce for low-resource settings.[25] Crucially, it was compatible with an alternative NB-LUMABS sensor, so it has the potential to be used with other BRET protein switches without device redesign.[25] A plethora of other microfluidic technologies could be explored, to find optimal solutions to drive the much needed translation of protein switch assays into practical use. This is arguably now the most pressing area of research, as no assay will ever actually be used at the point of need, unless fully integrated into an easy-to-use device.

■ OPPORTUNITIES

Should the described challenges be overcome, the opportunities for protein switch technology in quantitative point-of-need diagnostics is vast. Protein switches have thus far been largely developed for healthcare applications, where there is great potential to improve disease prediction, speed up diagnosis, and monitor drug dosing, to give better patient outcomes. There are wide opportunities for protein switch antibody tests: those developed for infectious disease serology could be widened to autoimmune, allergy, and broader immunology applications,[25,32,38,40,59] and those for therapeutic drug monitoring can guide dosing in the ever-growing biologics market.[25,30,31,40] Small molecule tests have also been developed for drug monitoring,[26,43] as well as metabolic[27,62] and disease biomarker assays.[33,100] Tests for a range of proteins have also been reported, and the scope expands as new biomarkers are discovered.[29,30,41,44] Human health applications dominate the literature, but the significant opportunities for quantitative on-site tests should be explored in broader sectors. Some protein switch assays have been developed for food standards,[63] agricultural disease monitoring,[30] and veterinary immunology applications,[75,76] but there are much wider prospects in these settings and others,

including environmental monitoring, illicit drug detection, and sports medicine.[1]

■ CONCLUSION

Engineered protein switches directly transduce analyte binding into a detectable signal in a simple, wash-free, homogeneous assay format. They comprise a recognition element, reporter, and switching mechanism, which all impact on performance according to their designs. In terms of recognition elements, antibody fragments, and selectable nonimmunoglobulin binding proteins offer the widest target scope. Luminescent reporters afford high sensitivity and range, while the potential of electrochemical reporters for simple integration into electronic devices is underexplored. Domain inserted allosteric switches are challenging to develop for new targets and have been largely superseded by more modular approaches. Two-component proximity switches are largely independent of target binding geometry but can suffer concentration dependent background activation. One-component modular allosteric switches are less concentration dependent, but exact binding geometries are more important. While such comparisons between approaches can be made, academic studies making systematic changes to a certain design aspect while keeping others constant would give a much clearer perspective on design-performance relationships and guide optimization of future designs. Crucially, protein switch signaling in nature operates down to the millisecond time scale, and more studies should evaluate the rate limiting steps of synthetic protein switch responses to guide assay times down from the current multiminute time frames.[26] The dynamic range of protein switch assays is highly variable and is another key area where the design-performance relationship must be better understood. Recent efforts toward modular switches that can target new analytes without redesign are crucial to ensure a fast and robust development process. In the future, high-throughput, computational, and de novo design approaches may guide more efficient design and development.[101,102]

Protein switches could underpin quantitative point-of-need diagnostics needed across wide applications to improve access, costs, and speed compared to laboratory assays. Yet despite this potential, they are yet to be established in routine diagnostic use. Practical implementation can only be achieved through integration into user-friendly devices and thorough validation of the assay. It is particularly crucial to compare the protein switch to a "gold standard" laboratory assay in its ability to measure analytes in clinical or real-world samples. While design of novel protein switches with improved performance characteristics is of course important to drive progress, it is crucial to remember that they cannot be practically implemented until the same efforts are guided toward translational research.

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Notes

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■ VOCABULARY

Allostery, the process by which proteins translate the binding of an effector molecule at one site to another functional site, thereby regulating activity; Bioluminescence, a specific form of chemiluminescence observed in living organisms, in which light is emitted upon turnover of a luciferin substrate by a luciferase enzyme. The reaction involves an excited state intermediate, which decays to a ground state via emission of light energy; Electrochemical, chemical reactions that involve the transfer of electrons and can be measured as an electrical signal; Immunoassay, a biochemical test that detects or quantifies an analyte in solution, using an antibody or antigen binding reagent; Microfluidics, the manipulation and control of fluids that are constrained to sub-millimeter channels

■ REFERENCES

(1) Cummins, B. M.; Ligler, F. S.; Walker, G. M. Point-of-care diagnostics for niche applications. *Biotechnol. Adv.* **2016**, *34* (3), 161−176.

(2) Gubala, V.; Harris, L. F.; Ricco, A. J.; Tan, M. X.; Williams, D. E. Point of care diagnostics: status and future. *Anal. Chem.* **2012**, *84* (2), 487−515.

(3) Cassedy, A.; Mullins, E.; O'Kennedy, R. Sowing seeds for the future: The need for on-site plant diagnostics. *Biotechnol. Adv.* **2020**, *39*, 107358.

(4) Bell, R.; Harr, K.; Rishniw, M.; Pion, P. Survey of point-of-care instrumentation, analysis, and quality assurance in veterinary practice. *Vet. Clin. Pathol.* **2014**, *43* (2), 185−192.

(5) Nayak, S.; Blumenfeld, N. R.; Laksanasopin, T.; Sia, S. K. Point-of-care diagnostics: recent developments in a connected age. *Anal. Chem.* **2017**, *89* (1), 102−123.

(6) Chen, H.; Liu, K.; Li, Z.; Wang, P. Point of care testing for infectious diseases. *Clin. Chim. Acta* **2019**, *493*, 138−147.

(7) Ravi, N.; Cortade, D. L.; Ng, E.; Wang, S. X. Diagnostics for SARS-CoV-2 detection: A comprehensive review of the FDA-EUA COVID-19 testing landscape. *Biosens. Bioelectron.* **2020**, *165*, 112454.

(8) Kozel, T. R.; Burnham-Marusich, A. R. Point-of-care testing for infectious diseases: past, present, and future. *J. Clin. Microbiol.* **2017**, *55* (8), 2313−2320.

(9) Jani, I. V.; Peter, T. F. How point-of-care testing could drive innovation in global health. *N. Engl. J. Med.* **2013**, *368* (24), 2319.

(10) Döhla, M.; Boesecke, C.; Schulte, B.; Diegmann, C.; Sib, E.; Richter, E.; Eschbach-Bludau, M.; Aldabbagh, S.; Marx, B.; Eis-Hübinger, A.-M.; Schmithausen, R. M.; Streeck, H. Rapid point-of-care testing for SARS-CoV-2 in a community screening setting shows low sensitivity. *Public Health* **2020**, *182*, 170−172.

(11) Vashist, S. K.; Luppa, P. B.; Yeo, L. Y.; Ozcan, A.; Luong, J. H. Emerging technologies for next-generation point-of-care testing. *Trends Biotechnol.* **2015**, *33* (11), 692−705.

(12) Wodak, S. J.; Paci, E.; Dokholyan, N. V.; Berezovsky, I. N.; Horovitz, A.; Li, J.; Hilser, V. J.; Bahar, I.; Karanicolas, J.; Stock, G.; Hamm, P.; Stote, R. H.; Eberhardt, J.; Chebaro, Y.; Dejaegere, A.; Cecchini, M.; Changeux, J.-P.; Bolhuis, P. G.; Vreede, J.; Faccioli, P.; Orioli, S.; Ravasio, R.; Yan, L.; Brito, C.; Wyart, M.; Gkeka, P.; Rivalta, I.; Palermo, G.; McCammon, J. A.; Panecka-Hofman, J.; Wade, R. C.; Di Pizio, A.; Niv, M. Y.; Nussinov, R.; Tsai, C.-J.; Jang,

H.; Padhorny, D.; Kozakov, D.; McLeish, T. Allostery in its many disguises: from theory to applications. *Structure* **2019**, *27* (4), 566−578.
(13) Vallée-Bélisle, A.; Plaxco, K. W. Structure-switching biosensors: inspired by Nature. *Curr. Opin. Struct. Biol.* **2010**, *20* (4), 518−526.
(14) Stein, V.; Alexandrov, K. Synthetic protein switches: design principles and applications. *Trends Biotechnol.* **2015**, *33* (2), 101−110.
(15) Stein, V. *Synthetic Protein Switches: Methods and Protocols*; Springer: New York, 2017.
(16) Greenwald, E. C.; Mehta, S.; Zhang, J. Genetically encoded fluorescent biosensors illuminate the spatiotemporal regulation of signaling networks. *Chem. Rev.* **2018**, *118* (24), 11707−11794.
(17) Vashist, S. K.; Luong, J. H. *Handbook of Immunoassay Technologies: Approaches, Performances, and Applications*; Academic Press, 2018.
(18) Jung, W.; Han, J.; Choi, J.-W.; Ahn, C. H. Point-of-care testing (POCT) diagnostic systems using microfluidic lab-on-a-chip technologies. *Microelectron. Eng.* **2015**, *132*, 46−57.
(19) Koczula, K. M.; Gallotta, A. Lateral flow assays. *Essays Biochem.* **2016**, *60* (1), 111−120.
(20) Beaudet, L.; Rodriguez-Suarez, R.; Venne, M.-H.; Caron, M.; Bédard, J.; Brechler, V.; Parent, S.; Bielefeld-Sévigny, M. AlphaLISA immunoassays: the no-wash alternative to ELISAs for research and drug discovery. *Nat. Methods* **2008**, *5* (12), No. an8.
(21) Lautenschlager, C.; Hurt, S.; Titus, D.; Parent, S. A comparison of AlphaLISA and TR-FRET homogeneous immunoassays in serum-containing samples. *Perkin Elmer Application Note*; PerkinElmer, Inc.: Waltham, MA, 2009. Available from http://www.perkinelmer.co.jp/Portals/0/resource/tech/tech_ls/protocol_collection/APP_ALPHALISA_VS_TR-FRET.pdf.
(22) Sandetskaya, N.; Isserstedt-John, N.; Kölsch, A.; Schattschneider, S.; Kuhlmeier, D. An integrated homogeneous SPARCL immunoassay for rapid biomarker detection on a chip. *Anal. Methods* **2019**, *11* (19), 2542−2550.
(23) Michnick, S. W.; Ear, P. H.; Manderson, E. N.; Remy, I.; Stefan, E. Universal strategies in research and drug discovery based on protein-fragment complementation assays. *Nat. Rev. Drug Discovery* **2007**, *6* (7), 569−582.
(24) Dixon, A. S.; Schwinn, M. K.; Hall, M. P.; Zimmerman, K.; Otto, P.; Lubben, T. H.; Butler, B. L.; Binkowski, B. F.; Machleidt, T.; Kirkland, T. A.; Wood, M. G.; Eggers, C. T.; Encell, L. P.; Wood, K. V. NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *ACS Chem. Biol.* **2016**, *11* (2), 400−408.
(25) Tomimuro, K.; Tenda, K.; Ni, Y.; Hiruta, Y.; Merkx, M.; Citterio, D. Thread-based bioluminescent sensor for detecting multiple antibodies in a single drop of whole blood. *ACS Sens.* **2020**, *5* (6), 1786−1794.
(26) Edwardraja, S.; Guo, Z.; Whitfield, J.; Lantadilla, I. R.; Johnston, W. A.; Walden, P.; Vickers, C. E.; Alexandrov, K. Caged activators of artificial allosteric protein biosensors. *ACS Synth. Biol.* **2020**, *9* (6), 1306−1314.
(27) Yu, Q.; Xue, L.; Hiblot, J.; Griss, R.; Fabritz, S.; Roux, C.; Binz, P.-A.; Haas, D.; Okun, J. G.; Johnsson, K. Semisynthetic sensor proteins enable metabolic assays at the point of care. *Science* **2018**, *361* (6407), 1122−1126.
(28) Lequin, R. M. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). *Clin. Chem.* **2005**, *51* (12), 2415−2418.
(29) Guo, Z.; Johnston, W. A.; Whitfield, J.; Walden, P.; Cui, Z.; Wijker, E.; Edwardraja, S.; Retamal Lantadilla, I.; Ely, F.; Vickers, C.; Ungerer, J. P. J.; Alexandrov, K. Generalizable protein biosensors based on synthetic switch modules. *J. Am. Chem. Soc.* **2019**, *141* (20), 8128−8135.
(30) Adamson, H.; Ajayi, M. O.; Campbell, E.; Brachi, E.; Tiede, C.; Tang, A. A.; Adams, T. L.; Ford, R.; Davidson, A.; Johnson, M.; McPherson, M. J.; Tomlinson, D. C.; Jeuken, L. J. C. Affimer-enzyme-inhibitor switch sensor for rapid wash-free assays of multimeric proteins. *ACS Sens.* **2019**, *4* (11), 3014−3022.
(31) van Rosmalen, M.; Ni, Y.; Vervoort, D. F.; Arts, R.; Ludwig, S. K.; Merkx, M. Dual-color bioluminescent sensor proteins for therapeutic drug monitoring of antitumor antibodies. *Anal. Chem.* **2018**, *90* (5), 3592−3599.
(32) Arts, R.; Den Hartog, I.; Zijlema, S. E.; Thijssen, V.; van der Beelen, S. H.; Merkx, M. Detection of antibodies in blood plasma using bioluminescent sensor proteins and a smartphone. *Anal. Chem.* **2016**, *88* (8), 4525−4532.
(33) Arts, R.; Ludwig, S. K.; van Gerven, B. C.; Estirado, E. M.; Milroy, L.-G.; Merkx, M. Semisynthetic bioluminescent sensor proteins for direct detection of antibodies and small molecules in solution. *ACS Sens.* **2017**, *2* (11), 1730−1736.
(34) Schena, A.; Griss, R.; Johnsson, K. Modulating protein activity using tethered ligands with mutually exclusive binding sites. *Nat. Commun.* **2015**, *6* (1), 7830.
(35) Bollella, P.; Edwardraja, S.; Guo, Z.; Kirill Alexandrov; Katz, E. Control of Allosteric Protein Electrochemical Switches with Biomolecular and Electronic Signals. *J. Phys. Chem. Lett.* **2020**, *11* (14), 5549−5554.
(36) Weihs, F.; Gel, M.; Wang, J.; Anderson, A.; Trowell, S.; Dacres, H. Development and characterisation of a compact device for rapid real-time-on-chip detection of thrombin activity in human serum using bioluminescence resonance energy transfer (BRET). *Biosens. Bioelectron.* **2020**, *158*, 112162.
(37) Yang, D.; Park, H. J.; Yoo, T. H. A colorimetric protease activity assay method using engineered procaspase-3 enzymes. *Anal. Methods* **2016**, *8* (33), 6270−6276.
(38) Tenda, K.; van Gerven, B.; Arts, R.; Hiruta, Y.; Merkx, M.; Citterio, D. Paper-based antibody detection devices using bioluminescent BRET-switching sensor proteins. *Angew. Chem., Int. Ed.* **2018**, *57* (47), 15369−15373.
(39) Drikic, M.; De Buck, J. Split trehalase as a versatile reporter for a wide range of biological analytes. *Biotechnol. Bioeng.* **2018**, *115* (5), 1128−1136.
(40) Ni, Y.; Arts, R.; Merkx, M. Ratiometric bioluminescent sensor proteins based on intramolecular split luciferase complementation. *ACS Sens.* **2019**, *4* (1), 20−25.
(41) Yu, F.; Alesand, V.; Nygren, P. Å. Site-specific photoconjugation of beta-lactamase fragments to monoclonal antibodies enables sensitive analyte detection via split-enzyme complementation. *Biotechnol. J.* **2018**, *13* (7), 1700688.
(42) Hwang, B. B.; Engel, L.; Goueli, S. A.; Zegzouti, H. A homogeneous bioluminescent immunoassay to probe cellular signaling pathway regulation. *Commun. Biol.* **2020**, *3* (1), 8.
(43) Xue, L.; Yu, Q.; Griss, R.; Schena, A.; Johnsson, K. Bioluminescent antibodies for point-of-care diagnostics. *Angew. Chem.* **2017**, *129* (25), 7218−7222.
(44) Dixon, A. S.; Kim, S. J.; Baumgartner, B. K.; Krippner, S.; Owen, S. C. A Tri-part Protein Complementation System Using Antibody-Small Peptide Fusions Enables Homogeneous Immunoassays. *Sci. Rep.* **2017**, *7* (1), 8186.
(45) Guo, Z.; Murphy, L.; Stein, V.; Johnston, W. A.; Alcala-Perez, S.; Alexandrov, K. Engineered PQQ-glucose dehydrogenase as a universal biosensor platform. *J. Am. Chem. Soc.* **2016**, *138* (32), 10108−10111.
(46) Su, J.; Beh, C.; Ohmuro-Matsuyama, Y.; Kitaguchi, T.; Hoon, S.; Ueda, H. Creation of stable and strictly regulated enzyme switch for signal-on immunodetection of various small antigens. *J. Biosci. Bioeng.* **2019**, *128* (6), 677−682.
(47) Ohmuro-Matsuyama, Y.; Ueda, H. Homogeneous noncompetitive luminescent immunodetection of small molecules by ternary protein fragment complementation. *Anal. Chem.* **2018**, *90* (5), 3001−3004.
(48) Xue, L.; Prifti, E.; Johnsson, K. A general strategy for the semisynthesis of ratiometric fluorescent sensor proteins with increased dynamic range. *J. Am. Chem. Soc.* **2016**, *138* (16), 5258−5261.

(49) Tullman, J.; Nicholes, N.; Dumont, M. R.; Ribeiro, L. F.; Ostermeier, M. Enzymatic protein switches built from paralogous input domains. *Biotechnol. Bioeng.* **2016**, *113* (4), 852−858.
(50) Löfblom, J.; Frejd, F. Y.; Ståhl, S. Non-immunoglobulin based protein scaffolds. *Curr. Opin. Biotechnol.* **2011**, *22* (6), 843−848.
(51) Škrlec, K.; Štrukelj, B.; Berlec, A. Non-immunoglobulin scaffolds: a focus on their targets. *Trends Biotechnol.* **2015**, *33* (7), 408−418.
(52) Orlova, A.; Magnusson, M.; Eriksson, T. L.J.; Nilsson, M.; Larsson, B.; Hoiden-Guthenberg, I.; Widstrom, C.; Carlsson, J.; Tolmachev, V.; Stahl, S.; Nilsson, F. Y. Tumor imaging using a picomolar affinity HER2 binding affibody molecule. *Cancer Res.* **2006**, *66* (8), 4339−4348.
(53) Adamson, H.; Nicholl, A.; Tiede, C.; Tang, A. A.; Davidson, A.; Curd, H.; Wignall, A.; Ford, R.; Nuttall, J.; McPherson, M. J.; Johnson, M.; Tomlinson, D. C. Affimers as anti-idiotypic affinity reagents for pharmacokinetic analysis of biotherapeutics. *BioTechniques* **2019**, *67* (6), 261−269.
(54) Nicholes, N.; Date, A.; Beaujean, P.; Hauk, P.; Kanwar, M.; Ostermeier, M. Modular protein switches derived from antibody mimetic proteins. *Protein Eng., Des. Sel.* **2016**, *29* (2), 77−85.
(55) Carlin, K. B.; Cruz-Teran, C. A.; Kumar, J. P.; Gomes, C.; Rao, B. M. Combinatorial pairwise assembly efficiently generates high affinity binders and enables a "mix-and-read" detection scheme. *ACS Synth. Biol.* **2016**, *5* (12), 1348−1354.
(56) Huang, J.; Koide, S. Rational conversion of affinity reagents into label-free sensors for peptide motifs by designed allostery. *ACS Chem. Biol.* **2010**, *5* (3), 273−277.
(57) Léger, C.; Di Meo, T.; Aumont-Nicaise, M.; Velours, C.; Durand, D.; Li de la Sierra-Gallay, I.; van Tilbeurgh, H.; Hildebrandt, N.; Desmadril, M.; Urvoas, A.; Valerio-Lepiniec, M.; Minard, P. Ligand-induced conformational switch in an artificial bidomain protein scaffold. *Sci. Rep.* **2019**, *9* (1), 1178.
(58) Golynskiy, M. V.; Rurup, W. F.; Merkx, M. Antibody detection by using a FRET-based protein conformational switch. *ChemBioChem* **2010**, *11* (16), 2264−2267.
(59) Banala, S.; Arts, R.; Aper, S. J.; Merkx, M. No washing, less waiting: engineering biomolecular reporters for single-step antibody detection in solution. *Org. Biomol. Chem.* **2013**, *11* (44), 7642−7649.
(60) Stein, V.; Nabi, M.; Alexandrov, K. Ultrasensitive scaffold-dependent protease sensors with large dynamic range. *ACS Synth. Biol.* **2017**, *6* (7), 1337−1342.
(61) Guo, Z.; Johnston, W. A.; Stein, V.; Kalimuthu, P.; Perez-Alcala, S.; Bernhardt, P. V.; Alexandrov, K. Engineering PQQ-glucose dehydrogenase into an allosteric electrochemical Ca2+ sensor. *Chem. Commun.* **2016**, *52* (3), 485−488.
(62) Yu, Q.; Pourmandi, N.; Xue, L.; Gondrand, C.; Fabritz, S.; Bardy, D.; Patiny, L.; Katsyuba, E.; Auwerx, J.; Johnsson, K. A biosensor for measuring NAD+ levels at the point of care. *Nat. Metab.* **2019**, *1* (12), 1219−1225.
(63) Caron, K.; Trowell, S. C. Highly sensitive and selective biosensor for a disaccharide based on an AraC-like transcriptional regulator transduced with bioluminescence resonance energy transfer. *Anal. Chem.* **2018**, *90* (21), 12986−12993.
(64) Smirnova, D. V.; Samsonova, J. V.; Ugarova, N. N. The bioluminescence resonance energy transfer from firefly luciferase to a synthetic dye and its application for the rapid homogeneous immunoassay of progesterone. *Photochem. Photobiol.* **2016**, *92* (1), 158−165.
(65) Gibbs, J.; Kennebunk, M. Selecting the detection system—colorimetric, fluorescent, luminescent methods. *Corning Life Sciences ELISA Technical Bulletin*; Corning Incorporated Life Sciences: Kennebunk, ME, 2001. Available from https://www.dutscher.com/data/pdf_guides/en/CLS-DD-AN-458.pdf.
(66) Yeh, H.-W.; Ai, H.-W. Development and applications of bioluminescent and chemiluminescent reporters and biosensors. *Annu. Rev. Anal. Chem.* **2019**, *12*, 129−150.
(67) Hall, M. P.; Unch, J.; Binkowski, B. F.; Valley, M. P.; Butler, B. L.; Wood, M. G.; Otto, P.; Zimmerman, K.; Vidugiris, G.; Machleidt, T.; Robers, M. B.; Benink, H. A.; Eggers, C. T.; Slater, M. R.; Meisenheimer, P. L.; Klaubert, D. H.; Fan, F.; Encell, L. P.; Wood, K. V. Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. *ACS Chem. Biol.* **2012**, *7* (11), 1848−1857.
(68) England, C. G.; Ehlerding, E. B.; Cai, W. NanoLuc: a small luciferase is brightening up the field of bioluminescence. *Bioconjugate Chem.* **2016**, *27* (5), 1175−1187.
(69) Shakhmin, A.; Hall, M. P.; Machleidt, T.; Walker, J. R.; Wood, K. V.; Kirkland, T. A. Coelenterazine analogues emit red-shifted bioluminescence with NanoLuc. *Org. Biomol. Chem.* **2017**, *15* (40), 8559−8567.
(70) Yeh, H.-W.; Karmach, O.; Ji, A.; Carter, D.; Martins-Green, M. M.; Ai, H.-w. Red-shifted luciferase-luciferin pairs for enhanced bioluminescence imaging. *Nat. Methods* **2017**, *14* (10), 971.
(71) Yeh, H.-W.; Xiong, Y.; Wu, T.; Chen, M.; Ji, A.; Li, X.; Ai, H.-w. ATP-independent bioluminescent reporter variants to improve in vivo imaging. *ACS Chem. Biol.* **2019**, *14* (5), 959−965.
(72) Su, Y.; Walker, J. R.; Park, Y.; Smith, T. P.; Liu, L. X.; Hall, M. P.; Labanieh, L.; Hurst, R.; Wang, D. C.; Encell, L. P.; Kim, N.; Zhang, F.; Kay, M. A.; Casey, K. M.; Majzner, R. G.; Cochran, J. R.; Mackall, C. L.; Kirkland, T. A.; Lin, M. Z. Novel NanoLuc substrates enable bright two-population bioluminescence imaging in animals. *Nat. Methods* **2020**, *17* (8), 852−860.
(73) Labib, M.; Sargent, E. H.; Kelley, S. O. Electrochemical methods for the analysis of clinically relevant biomolecules. *Chem. Rev.* **2016**, *116* (16), 9001−9090.
(74) Turner, A. P. Biosensors: sense and sensibility. *Chem. Soc. Rev.* **2013**, *42* (8), 3184−3196.
(75) Drikic, M.; Olsen, S.; De Buck, J. Detecting total immunoglobulins in diverse animal species with a novel split enzymatic assay. *BMC Vet. Res.* **2019**, *15* (1), 374.
(76) Drikic, M.; Windeyer, C.; Olsen, S.; Fu, Y.; Doepel, L.; De Buck, J. Determining the IgG concentrations in bovine colostrum and calf sera with a novel enzymatic assay. *J. Anim. Sci. Biotechnol.* **2018**, *9* (1), 69.
(77) Monteiro, T.; Almeida, M. G. Electrochemical enzyme biosensors revisited: Old solutions for new problems. *Crit. Rev. Anal. Chem.* **2019**, *49* (1), 44−66.
(78) Koushanpour, A.; Gamella, M.; Guo, Z.; Honarvarfard, E.; Poghossian, A.; Schöning, M. J.; Alexandrov, K.; Katz, E. Ca2+-switchable glucose dehydrogenase associated with electrochemical/electronic interfaces: applications to signal-controlled power production and biomolecular release. *J. Phys. Chem. B* **2017**, *121* (51), 11465−11471.
(79) Yates, N. D.; Fascione, M. A.; Parkin, A. Methodologies for "wiring" redox proteins/enzymes to electrode surfaces. *Chem. - Eur. J.* **2018**, *24* (47), 12164−12182.
(80) Goggins, S.; Naz, C.; Marsh, B. J.; Frost, C. G. Ratiometric electrochemical detection of alkaline phosphatase. *Chem. Commun.* **2015**, *51* (3), 561−564.
(81) Spring, S. A.; Goggins, S.; Frost, C. G. Ratiometric electrochemical detection of β-galactosidase. *Org. Biomol. Chem.* **2017**, *15* (34), 7122−7126.
(82) Akanda, M. R.; Choe, Y.-L.; Yang, H. Outer-sphere to inner-sphere" redox cycling for ultrasensitive immunosensors. *Anal. Chem.* **2012**, *84* (2), 1049−1055.
(83) Su, J.; Dong, J.; Kitaguchi, T.; Ohmuro-Matsuyama, Y.; Ueda, H. Noncompetitive homogeneous immunodetection of small molecules based on beta-glucuronidase complementation. *Analyst* **2018**, *143* (9), 2096−2101.
(84) Stains, C. I.; Furman, J. L.; Porter, J. R.; Rajagopal, S.; Li, Y.; Wyatt, R. T.; Ghosh, I. A general approach for receptor and antibody-targeted detection of native proteins utilizing split-luciferase reassembly. *ACS Chem. Biol.* **2010**, *5* (10), 943−952.
(85) Stein, V.; Alexandrov, K. Protease-based synthetic sensing and signal amplification. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111* (45), 15934−15939.

(86) Banala, S.; Aper, S. J.; Schalk, W.; Merkx, M. Switchable reporter enzymes based on mutually exclusive domain interactions allow antibody detection directly in solution. *ACS Chem. Biol.* **2013**, *8* (10), 2127−2132.
(87) Griss, R.; Schena, A.; Reymond, L.; Patiny, L.; Werner, D.; Tinberg, C. E.; Baker, D.; Johnsson, K. Bioluminescent sensor proteins for point-of-care therapeutic drug monitoring. *Nat. Chem. Biol.* **2014**, *10* (7), 598−603.
(88) Armbruster, D. A.; Pry, T. Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev.* **2008**, *29*, S49−S52.
(89) Meesters, R.; Voswinkel, S. Bioanalytical method development and validation: from the USFDA 2001 to the USFDA 2018 guidance for industry. *J. Appl. Bioanal.* **2018**, *4* (3), 67−73.
(90) Mou, L.; Jiang, X. Materials for microfluidic immunoassays: a review. *Adv. Healthcare Mater.* **2017**, *6* (15), 1601403.
(91) Li, F.; You, M.; Li, S.; Hu, J.; Liu, C.; Gong, Y.; Yang, H.; Xu, F. Paper-based point-of-care immunoassays: Recent advances and emerging trends. *Biotechnol. Adv.* **2020**, *39*, 107442.
(92) Christodouleas, D. C.; Kaur, B.; Chorti, P. From point-of-care testing to eHealth diagnostic devices (eDiagnostics). *ACS Cent. Sci.* **2018**, *4* (12), 1600−1616.
(93) Ronkainen, N. J.; Halsall, H. B.; Heineman, W. R. Electrochemical biosensors. *Chem. Soc. Rev.* **2010**, *39* (5), 1747−1763.
(94) Wan, Y.; Su, Y.; Zhu, X.; Liu, G.; Fan, C. Development of electrochemical immunosensors towards point of care diagnostics. *Biosens. Bioelectron.* **2013**, *47*, 1−11.
(95) Dai, Y.; Liu, C. C. Recent Advances on Electrochemical Biosensing Strategies toward Universal Point-of-Care Systems. *Angew. Chem.* **2019**, *131* (36), 12483−12496.
(96) Rasmussen, M.; Abdellaoui, S.; Minteer, S. D. Enzymatic biofuel cells: 30 years of critical advancements. *Biosens. Bioelectron.* **2016**, *76*, 91−102.
(97) Sun, A. C.; Hall, D. A. Point-of-care smartphone-based electrochemical biosensing. *Electroanalysis* **2019**, *31* (1), 2−16.
(98) da Silva, E. T.; Souto, D. E.; Barragan, J. T.; de F. Giarola, J.; de Moraes, A. C.; Kubota, L. T. Electrochemical biosensors in point-of-care devices: recent advances and future trends. *ChemElectroChem* **2017**, *4* (4), 778−794.
(99) Lingervelder, D.; Koffijberg, H.; Kusters, R.; Ijzerman, M. J. Point-of-care testing in primary care: a systematic review on implementation aspects addressed in test evaluations. *Int. J. Clin. Pract.* **2019**, *73* (10), e13392.
(100) Teanphonkrang, S.; Ernst, A.; Janke, S.; Chaiyen, P.; Sucharitakul, J.; Suginta, W.; Khunkaewla, P.; Schuhmann, W.; Schulte, A.; Ruff, A. Amperometric detection of the urinary disease biomarker p-HPA by allosteric modulation of a redox polymer-embedded bacterial reductase. *ACS Sens.* **2019**, *4* (5), 1270−1278.
(101) Langan, R. A.; Boyken, S. E.; Ng, A. H.; Samson, J. A.; Dods, G.; Westbrook, A. M.; Nguyen, T. H.; Lajoie, M. J.; Chen, Z.; Berger, S.; Mulligan, V. K.; Dueber, J. E.; Novak, W. R. P.; El-Samad, H.; Baker, D. De novo design of bioactive protein switches. *Nature* **2019**, *572* (7768), 205−210.
(102) Quijano-Rubio, A.; Yeh, H.-W.; Park, J.; Lee, H.; Langan, R. A.; Boyken, S. E.; Lajoie, M. J.; Cao, L.; Chow, C. M.; Miranda, M. C. De novo design of modular and tunable allosteric biosensors. *bioRxiv* **2020**, DOI: 10.1101/2020.07.18.206946.