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Direct Kinetic Fingerprinting for High-Accuracy Single-Molecule Counting of Diverse Disease Biomarkers

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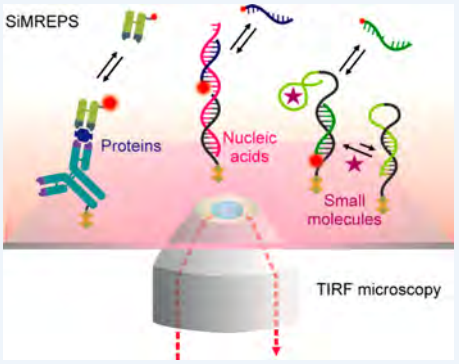
CONSPECTUS: Methods for detecting and quantifying disease biomarkers in biofluids with high specificity and sensitivity play a pivotal role in enabling clinical diagnostics, including point-of-care tests. The most widely used molecular biomarkers include proteins, nucleic acids, hormones, metabolites, and other small molecules. While numerous methods have been developed for analyzing biomarkers, most techniques are challenging to implement for clinical use due to insufficient analytical performance, high cost, and/or other practical shortcomings. For instance, the detection of cell-free nucleic acid (cfNA) biomarkers by digital PCR and next-generation sequencing (NGS) requires time-consuming nucleic acid extraction steps, often introduces enzymatic amplification bias, and can be costly when high specificity is required. While several amplification-free methods for detecting cfNAs have been reported, these techniques generally suffer from low specificity and sensitivity. Meanwhile, the quantification of protein biomarkers is generally performed using immunoassays such as enzyme-linked immunosorbent assay (ELISA); the analytical performance of these methods is often limited by the availability of antibodies with high affinity and specificity as well as the significant nonspecific binding of antibodies to assay surfaces. To address the drawbacks of existing biomarker detection methods and establish a universal diagnostics platform capable of detecting different types of analytes, we have developed an amplification-free approach, named single-molecule recognition through equilibrium Poisson sampling (SiMREPS), for the detection of diverse biomarkers with arbitrarily high specificity and single-molecule sensitivity. SiMREPS utilizes the transient, reversible binding of fluorescent detection probes to immobilized target molecules to generate kinetic fingerprints that are detected by single-molecule fluorescence microscopy. The analysis of these kinetic fingerprints enables nearly perfect discrimination between specific binding to target molecules and any nonspecific binding. Early proof-of-concept studies demonstrated the *in vitro* detection of miRNAs with a limit of detection (LOD) of approximately 1 fM and >500-fold selectivity for single-nucleotide polymorphisms. The SiMREPS approach was subsequently expanded to the detection of rare mutant DNA alleles from biofluids at mutant allele fractions of as low as 1 in 1 million, corresponding to a specificity of >99.99999%. Recently, SiMREPS was generalized to protein quantification using dynamically binding antibody probes, permitting LODs in the low-femtomolar to attomolar range. Finally, SiMREPS has been demonstrated to be suitable for the *in situ* detection of miRNAs in cultured cells, the quantification of small-molecule toxins and drugs, and the monitoring of telomerase activity at the single-molecule level. In this Account, we discuss the principles of SiMREPS for the highly specific and sensitive detection of molecular analytes, including considerations for assay design. We discuss the generality of SiMREPS for the detection of very disparate analytes and provide an overview of data processing methods, including the expansion of the dynamic range using super-resolution analysis and the improvement of performance using deep learning algorithms. Finally, we describe current challenges, opportunities, and future directions for the SiMREPS approach.



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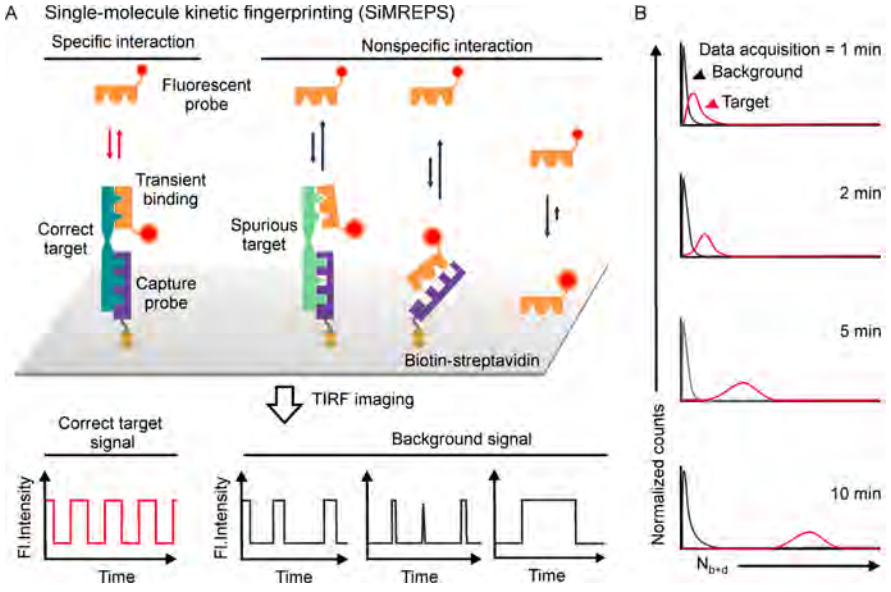

Figure 1. Schematic of the principle of single-molecule kinetic fingerprinting (SiMREPS). (A) SiMREPS uses the transient and reversible binding of low-affinity fluorescent probes to immobilized target molecules to generate distinct kinetic fingerprints that permit high-confidence differentiation for specific binding to correct target and nonspecific background binding. Probe binding and dissociation to single molecules are observed in real time by TIRF microscopy. (B) Predicted distribution of the number of binding and dissociation (N_{b+d}) events as a function of time. With increasing standard acquisition time, a better separation is obtained between specific and nonspecific or background binding.

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1. INTRODUCTION

The detection and quantification of disease biomarkers such as proteins, nucleic acids, hormones, enzymes, peptides, and metabolites at low concentrations in complex biological samples are crucial in a variety of clinical settings, including the early detection of disease,[5] the assessment of the response to therapy,[6] and the prognosis of disease relapse.[7] For instance, prostate-specific antigen (PSA) found at femtomolar levels in human serum has emerged as an important biomarker for prostate cancer recurrence after radical prostatectomy.[8] In addition, cell-free nucleic acids (cfNAs) such as circulating tumor DNA (ctDNA) and microRNA (miRNA) found in biofluids have been increasingly used as biomarkers in so-called liquid biopsies for the early detection of cancers and minimal residual diseases.[9] While the performance of current methods suffices for some clinically important biomarkers, it is challenging to simultaneously achieve high analytical performance with a simple work flow at low cost. Furthermore, with a few exceptions such as single-molecule arrays (Simoa)[10] and single-cell multiomics methods,[11] most techniques do not provide a unified platform for the sensitive quantification of DNA, RNA, protein, and small-molecule biomarkers, necessitating diverse sample handling and measurement methods that complicate analysis.

Recently, our laboratory has developed an approach called single-molecule recognition through equilibrium Poisson sampling (SiMREPS) for the detection and quantification of diverse disease biomarkers with ultrahigh specificity and sensitivity.[1,2,12,13] SiMREPS utilizes the transient and reversible binding of fluorescent detection probes to immobilized target molecules; this repetitive binding is detected at the single-molecule level to generate kinetic fingerprints that permit the differentiation between specific binding (to target molecules) and nonspecific background binding with high confidence. Figure 1A shows a simplified view of the detection of an analyte via SiMREPS along with the resulting distinct single-molecule kinetic fingerprints originating from the specific binding of a fluorescent probe (FP) to the correct target molecule, nonspecific binding to spurious targets, or background binding (i.e., capture probes and assay surfaces). The transient binding and dissociation of probes at equilibrium in a defined observation window can be modeled as a Poisson process wherein the expected number of observed binding events per target molecule becomes more sharply defined (i.e., more deterministic) with longer observation. Thus, with increasing acquisition time, a better separation is obtained between the

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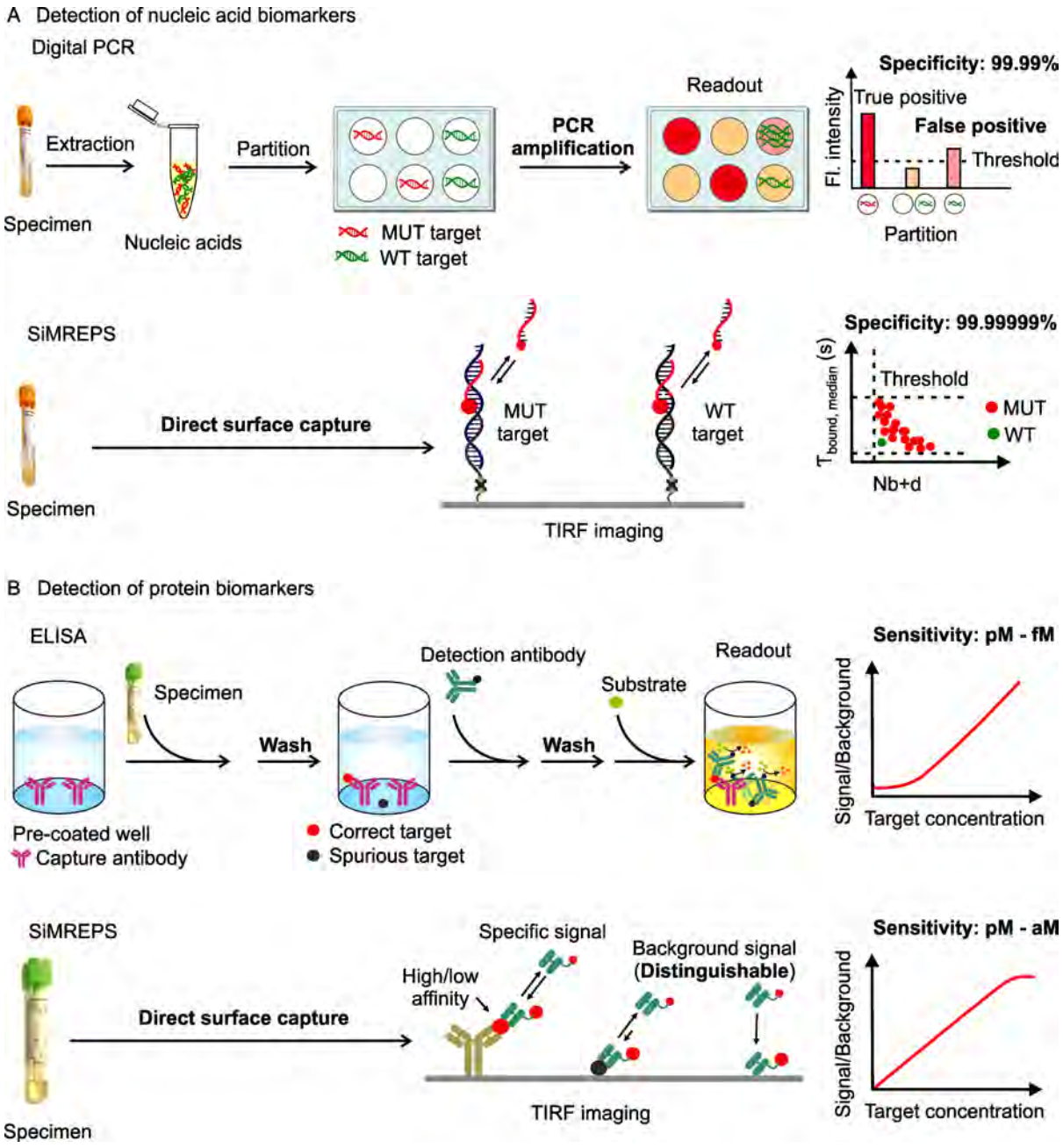

Figure 2. Comparison of conventional and SiMREPS approaches for the detection of nucleic acids and proteins. (A) Comparison between digital PCR and SiMREPS for the detection of mutant (MUT) DNA alleles. Digital PCR is limited by its specificity due to heat-induced chemical modification of nucleobases and amplification bias that can generate false positive signals in wild-type (WT) DNA. SiMREPS is an amplification-free single-molecule kinetic fingerprinting approach that utilizes the transient interaction of a detection probe to achieve arbitrarily high discrimination between closely related nucleic acid sequences. (B) Comparison between ELISA and SiMREPS for the detection of proteins. ELISA utilizes laborious multistep stringent washing protocols and suffers from its lower sensitivity and dynamic range because of high background signals generated by the nonspecific interaction of proteins with the assay surface. SiMREPS uses a direct wash-free protocol for the highly sensitive and specific detection of proteins with a broader dynamic range because of its ability to suppress background signals applying kinetic thresholds.

distribution of the number of binding and dissociation events (N_{b+d}) for specific and nonspecific binding (Figure 1B). To date, SiMREPS has been successfully demonstrated to detect molecular analytes as diverse as miRNAs,[1,14] ctDNAs,[2,15] proteins,[3] and small molecules[16] with high specificity and sensitivity (Section 3 and Table S1). This uncommon ability to detect and accurately count such a broad range of analytes at the single-molecule level suggests great potential for SiMREPS as a generalized platform for biomarker diagnostics.

A wide variety of innovative methods have been developed for the detection of DNA, miRNA, protein, and small molecules with variable analytical performance, either in ensemble or single-molecule assay formats.[13,17−20] The gold-standard methods for detecting nucleic acids include polymerase chain reaction (PCR) and next-generation sequencing (NGS).[19] PCR-based detection methods rely on enzymatic amplification steps in which a small number of target nucleic acid molecules in the sample are exponentially amplified for increasing sensitivity. For instance, digital PCR (dPCR) amplifies and quantifies target

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molecules by partitioning them into individual wells or droplets and allows for absolute target quantification (Figure 2A, top panel). Although dPCR has extremely high sensitivity,[21] PCR-based detection methods suffer from several drawbacks, including the possibility of heat-induced chemical damage,[2,22] amplification bias, inefficient amplification of short nucleic acids (e.g., miRNAs[23]), and interference from PCR inhibitors.[24] Recently, optimized NGS has become popular for the high-throughput sequencing of nucleic acids in a complex mixture, for screening, and for the early detection of cancer.[19,25] However, achieving high sensitivity and specificity with NGS requires high sequencing depth[26] to correct amplification and readout errors, which is time-consuming and often increases cost.[27] In contrast to the above methods, SiMREPS entirely eliminates amplification steps and the errors associated with them, enabling more straightforward sample preparation while achieving very high intrinsic analytical specificity for the detection of a small number of targets (see Table S2 for the advantages and limitations of SiMREPS) through the direct fingerprinting of each molecule (Figure 2A, bottom panel).

To detect protein biomarkers, enzyme-linked immunosorbent assay (ELISA)[28] has long been the preferred technique in clinical research laboratories and hospitals.[29] One of the highest-sensitivity and highest-specificity ELISA formats, sandwich ELISA,[30] utilizes a pair of antibodies to capture and detect protein targets. The specificity of detection is enhanced by the dual recognition by two high-affinity antibodies that bind distinct epitopes of the same antigen. However, the selection and optimization of a pair of high-affinity antibodies for specific protein biomarkers is time-consuming and costly.[31] Moreover, nonspecific binding of the detection antibody to other matrix components or to the assay surface gives rise to significant and variable background signals even in the absence of the antigen, limiting the sensitivity and dynamic range of conventional ELISA (Figure 2B, top panel).

Consequently, conventional ELISA lacks the sensitivity to detect the subpicomolar concentrations of many protein biomarkers in human serum in the early stages of disease.[32] The development of immuno-PCR assays and digital ELISA or single-molecule arrays (Simoa) has enabled the detection of several proteins with LODs in the femtomolar-to-attomolar range; however, these methods require complex workup procedures such as stringent washing steps and enzymatic amplification and still require two compatible high-affinity antibodies per target.[33,34]

Recently, wash-free protein quantification methods have been reported, such as a nanoswitch-linked immunosorbent assay (NLISA),[35] a linker-mediated immunoassay (LMI),[36] and programmable nucleic acid nanoswitches,[37] but these techniques lack the sensitivity of digital ELISA.[38] In contrast, protein SiMREPS enables a one-step, no-wash approach that uses direct kinetic fingerprinting to distinguish the specific signal from nonspecific binding to single molecules and achieves LODs in the femtomolar-to-attomolar concentration range using low-affinity detection probes.[3] Protein SiMREPS achieves a linear dynamic range of about 3.5 orders of magnitude when employing super-resolution analysis,[3] which is larger than that of conventional ELISAs (analog) (Figure 2B, bottom panel) and comparable to that of digital ELISA (Simoa).[38] This wide dynamic range is advantageous given the broad concentration range (attomolar to picomolar) exhibited by protein biomarkers in biofluids.[32]

In this Account, we first introduce the working principles of SiMREPS as well as the most important parameters in obtaining kinetic fingerprints useful for the high-specificity detection of analytes of interest. We then discuss the generality of SiMREPS for the detection and quantification of diverse analytes including nucleic acids, proteins, and small molecules. Next, we provide an overview of standard SiMREPS data analysis as well as recently developed data processing methods that exploit super-resolution localization and deep learning.[4] Finally, we suggest possible future advances of SiMREPS and its application to ever-broader scientific and clinical questions.

2. PRINCIPLES AND METHODS OF SINGLE-MOLECULE KINETIC FINGERPRINTING

2.1. SiMREPS Principles and Assay Design

In 2006, Hochstrasser and colleagues introduced pointillist super-resolution imaging technique PAINT (points accumulation for imaging in nanoscale topology), which relies on the repetitive interrogation of nanoscale structures by transiently binding dye molecules.[39] Subsequently, Jungmann et al.[40] adapted this concept to transiently binding oligonucleotide fluorescent probes, giving rise to a family of methods known as DNA-PAINT.[41] Taking inspiration from these methods, SiMREPS employs the transient binding of FPs not for the imaging of nanoscale features but to generate distinctive temporal patterns (kinetic fingerprints) for the high-confidence detection of single-molecule analytes. Typically, SiMREPS employs TIRF microscopy to suppress background fluorescence from the freely diffusing FPs present in the imaging solution, thus permitting single-molecule detection at or near the surface of a slide or coverslip. The repeated binding of FPs to individual analyte molecules can be modeled as a Poisson process with random arrival times of individual FPs but a well-defined mean number (μ) of binding and dissociation events (N_{b+d}) per target molecule for a given observation time and a standard deviation (σ) proportional to (and theoretically equal to) $\sqrt{N_{b+d}}$.[13,42] As a result, the coefficient of variation (CV = σ/μ) decreases as N_{b+d} increases,[13] implying that any kinetic difference between specific and nonspecific binding, no matter how small, can be resolved with a sufficiently long observation period (Figure 1). At room temperature in 4× PBS (phosphate-buffered saline) buffer and using oligonucleotide FPs 8−10 nucleotides (nt) in length, a 10 min interrogation time is sufficient for discriminating even single-nucleotide variants (SNVs) in RNA or DNA.[1,2]

In principle, any analyte of interest that can be stably bound to a surface and probed repeatedly is a candidate for SiMREPS. The basic requirements of a typical assay include a passivated solid substrate (typically glass or fused silica), a surface-immobilized capture probe (CP), and an FP (Figure 1). The surface is usually functionalized with m-PEG, biotin-PEG, and streptavidin both to provide passivation against excessive nonspecific binding and to immobilize biotinylated CPs. The CP may be, for example, a biotinylated DNA or locked nucleic acid (LNA) strand complementary to part of a target DNA or RNA sequence[1,2] or a biotinylated antibody with strong affinity to a particular epitope of a target protein.[3] The FP may be a fluorescently labeled DNA strand of 8−10 nucleotides in length (in the case of nucleic acid analytes) or a fluorescently labeled detection antibody with a K_D typically in the range of ∼10−600 nM (for proteins). A well-chosen FP has both rapid binding and dissociation kinetics, thus quickly generating kinetic fingerprints

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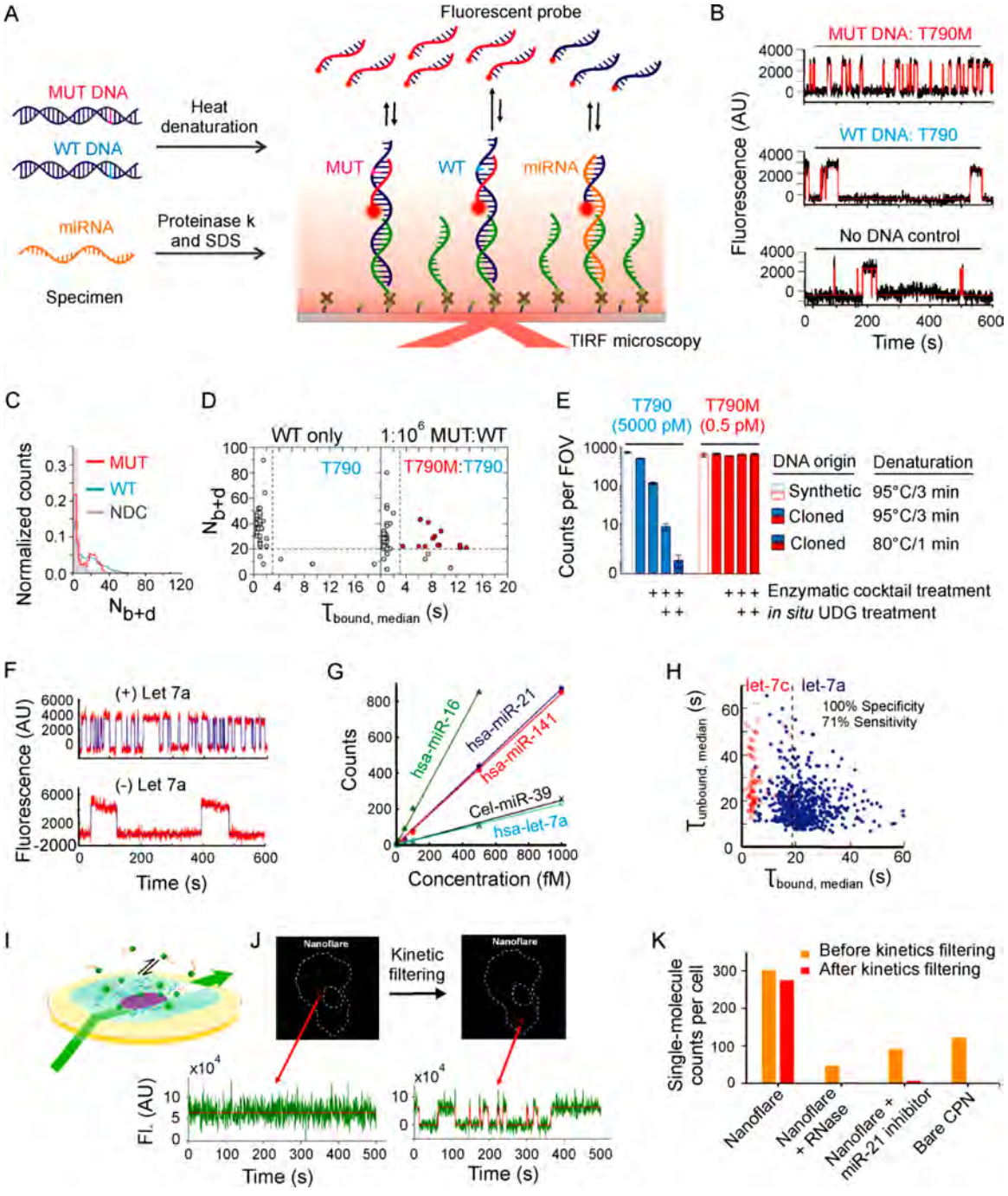

Figure 3. Highly specific and sensitive detection of nucleic acid biomarkers with single-molecule kinetic fingerprinting (SiMREPS). (A) Experimental scheme for SiMREPS assays of DNA and miRNA. (B) Representative single-molecule kinetic traces for MUT DNA (top), WT DNA (middle), and a no-DNA control (bottom) using an FP specific to EGFR mutation T790M (c.2369C>T). (C) Histogram comparing the number of binding and dissociation events (N_{b+d}) observed per single-molecule trace for a no-DNA control (NDC), T790 (WT, 50 nM), and T790M (MUT, 50 fM). (D) Kinetic thresholding based primarily on N_{b+d} and $\tau_{bound,median}$ distinguishes between samples containing WT only and a 1:10^6 mixture of MUT and WT sequences. (E) Varying heat denaturation conditions and enzymatic treatments of T790 (WT, blue) and T790M (MUT, red) demonstrate the impact of spontaneous heat-induced cytosine deamination on specificity. (B–E) Reproduced with permission from ref 2, copyright 2018, American Chemical Society. (F) Representative single-molecule kinetic traces for the *in vitro* detection of miRNA (hsa-let-7a). (G) Standard curves for the *in vitro* detection of five different miRNAs. (H) Dwell time analysis enables high-confidence discrimination between *let-7a* and *let-7c*. (F–H) Reproduced with permission from ref 1, copyright 2015, Springer Nature. (I) Experimental scheme for HILO imaging of single cells using an miR-21-specific nanoflare SiMREPS probe. (J) Time traces illustrating the ability to distinguish single miR-21 molecules from background binding in a single A549 cell. (K) Apparent single-molecule counts from SiMREPS assays of miR-21 under different experimental conditions with and without kinetic filtering. (I–K) Reproduced with permission from ref 14, copyright 2019, American Chemical Society.

with large values of N_{b+d} to achieve sufficient specificity in the shortest possible observation time.

Movies of FPs interacting with all immobilized targets within a microscopic field of view (FOV) in a defined observation time

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window (1−10 min, exposure time 0.1−1 s per frame) are recorded using TIRF and an electron-multiplying CCD (EMCCD) or scientific complementary metal oxide semiconductor (sCMOS) camera and then analyzed using custom MATLAB scripts. (See Section 4 for more details.) Fluorescence intensity versus time traces of single molecules are extracted, and their kinetics are analyzed to distinguish targets from nontargets with high specificity.[1,2,12]

2.2. Assay Chip Preparation

In principle, the SiMREPS concept is compatible with any sample geometry that permits the observation of single FP binding under relatively low oxygen conditions. In practice, the sample chamber design varies depending on the type of microscope used (i.e., prism-type or objective-type TIRF) as well as the desired throughput and sensitivity.[13] Objective-type TIRF permits an open-top chip design and requires only a single substrate functionalized for sample immobilization (i.e., a glass coverslip); sample wells are constructed by cutting pipet tips or 3D printed wells and attaching them to passivated coverslips. In contrast, prism-type TIRF usually requires placing the sample cell between a prism and an objective lens. In this case, closed flow cells sandwiched between a passivated microscope slide (fused silica or glass) and a glass coverslip are preferred. The coverslips or slides are functionalized with an aminosilane followed by a mixture of succinimide esters of biotin-PEG and methoxy-PEG in a certain ratio (e.g., 1:10 or 1:100) and further passivated by disulfosuccinimidyl tartrate to quench the unreacted amine groups. Subsequently, the surface is coated with streptavidin to permit the immobilization of biotinylated CPs. In the case of *in situ* analyte SiMREPS detection within cells (e.g., miRNAs[14]), objective-type TIRF is used together with glass-bottom cell culture dishes. Cellular fixation is performed using treatment with paraformaldehyde or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). The fixed cells are ethanol permeabilized prior to imaging.[43]

2.3. Sample Preparation and Assay Conditions

With no need for enzymatic amplification, SiMREPS assays have shown robust performance in a variety of buffers and minimally treated crude biofluids. Detailed sample preparation and assay protocols are described elsewhere.[12] Briefly, dsDNA samples require a short denaturation (e.g., heating to 80−95°C for 3 min) in the presence of carrier oligonucleotides (e.g., dT_{10}) and cooling to room temperature before surface capturing.[2] For the direct capture of miRNAs from serum or cell extract, samples can be pretreated with SDS and proteinase K.[1] Protein analytes have been directly captured from 1 or 25% serum and can be detected without washing away excess serum or detection probes.[3] Notably, like other techniques utilizing passive surface capture, the sensitivity of SiMREPS is limited by analyte diffusion to the surface and by the capture kinetics (Table S2), typically yielding capture efficiencies of ∼1%.[3] Nevertheless, limits of detection of <10 fM are typical.

The imaging buffers for most SiMREPS assays contained 25−100 nM FP in 1× to 4× PBS buffer. To prolong the usable lifetime (i.e., reduce the photobleaching rate) of fluorophores for more accurate and reproducible kinetic fingerprinting, an oxygen scavenger system comprising 3,4-dihydroxybenzoate, protocatechuate dioxygenase, and Trolox is typically added. In protein-SiMREPS assays, Tween 20 is often added to the imaging buffer to reduce the nonspecific binding of FPs to the imaging surface. To achieve the desired repetitive binding of FPs to targets yielding reproducible kinetic fingerprints distinct from the background, it is important to control the imaging temperature (±2 °C) and the ionic strength.

3. APPLICATION OF SiMREPS TO THE QUANTIFICATION OF DIVERSE BIOMARKERS

3.1. SiMREPS Detection of Nucleic Acids

Cell-free nucleic acids (cfNAs) such as ctDNA, mRNA, and noncoding RNAs (i.e., miRNAs) found in the biofluids of cancer patients have emerged as established or potential biomarkers.[9] Since changes in the levels of ctDNA reflect tumor burden and malignant progression, ctDNAs are increasingly employed as biomarkers in liquid biopsies of cancer. As an example, Cobas *EGFR* Mutation Test v2 for *EGFR* alterations has been approved for use as a companion diagnostic for the selection of therapies in non-small cell lung cancer (NSCLC).[44] Additionally, the expression levels of miRNAs are frequently dysregulated in tumor development, raising the possibility of using circulating miRNAs as biomarkers.[45] For example, miR-21 and miR-125b are deregulated in NSCLC.[45] Several potential cfNA cancer biomarkers have been discussed in recent reviews.[9,45]

The highly specific and sensitive detection of rare mutant DNA alleles in biofluids is challenging because the allelic frequency of ctDNA is often quite low, frequently <1% even in advanced (e.g., Stage IV) cancers.[46] The accurate detection of ctDNA therefore requires high specificity for the mutant allele. To this end, we recently demonstrated the ability of SiMREPS to detect two NSCLC-related *EGFR* mutations—an exon 19 deletion and the T790M (c.2369C>T) point mutation—with extremely high specificity in dsDNA without PCR amplification.[2] Each of the SiMREPS assays used a mutant (MUT)-specific 8 nt oligonucleotide FP to discriminate between specific binding to MUT molecules and nonspecific interactions with spurious or wild-type (WT) nucleic acid sequences (Figure 3). Detailed guidelines for designing SiMREPS FPs have been discussed elsewhere.[1,2,12] Briefly, the maximum theoretical discrimination factor, $Q_{max,therm} = e^{-\Delta\Delta G^0/RT}$,[2,12,47] where $\Delta\Delta G^0$ is the difference in the Gibbs free energy of hybridization of an FP with MUT and of the same FP with the WT DNA target, was calculated for various candidate FPs using web software NUPACK,[48] and the FPs with the largest values of $Q_{max,therm}$ were empirically tested for suitability (i.e., rapid kinetics) in SiMREPS assays.

To permit the surface capture of single-stranded target molecules for the detection of EGFR mutations by SiMREPS, the target dsDNA was subjected to gentle thermal denaturation (at 80 °C to minimize the spontaneous deamination of cytosine to uracil, observed to be suffered by PCR[22]) in the presence of a carrier oligonucleotide (dT_{10}) at high molar excess to substantially reduce reannealing (Figure 3A).

The kinetic fingerprints generated by the transient binding of the optimized MUT-specific FP effectively distinguished among MUT, WT, and no-DNA controls with an acquisition time of 10 min (Figure 3B,C). Both the exon 19 deletion and the T790M mutation were detected at an allelic fraction of as low as 0.0001% (1 MUT molecule in 1 million WT molecules). Notably, the assay for point mutation T790M exhibited an apparent specificity of 99.99999% and an apparent discrimination factor Q_{app} of 1.1×10^7, which is ∼2600 times greater than the maximum thermodynamic discrimination factor estimated from Gibbs free energy calculations by NUPACK (Figure 3D).[2] This achievement attests to the power of kinetic fingerprinting, which, unlike single-measurement thermodynamic discrimina-

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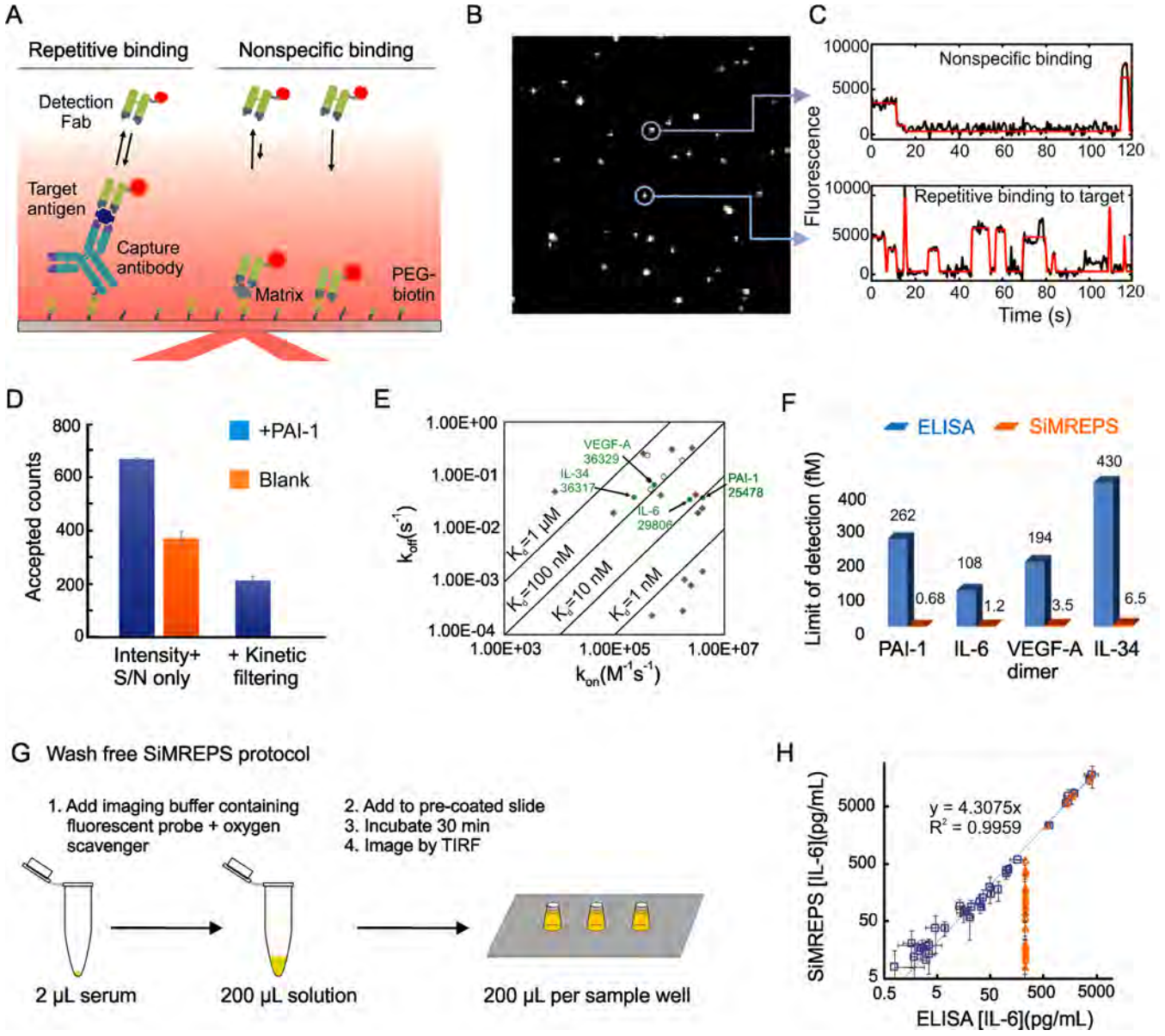

Figure 4. High-confidence detection and counting of single protein molecules by SiMREPS. (A) Experimental scheme for the detection of target protein antigens by SiMREPS. (B) Single movie frame of a representative microscope FOV; the bright puncta represents single FPs bound at or near the coverslip surface. (C) Representative intensity versus time traces showing the distinct kinetic fingerprints of nonspecific binding (top) and repetitive binding to the target antigen (bottom). (D) Impact of kinetic filtering on the number of accepted counts in animal serum samples with and without the spiked-in antigen PAI-1. (E) Scatter plot of binding (k_{on}) and dissociation (k_{off}) rate constants (determined from BLI or SPR measurements) of candidate detection Fabs, with their success or failure as SiMREPS probes at room temperature indicated by color (not suitable, gray diamonds; suitable, green circles; suitable and chosen for final assays, filled green circles). K_d, equilibrium dissociation constant. (F) Bar graph showing the superior sensitivity of SiMREPS (orange bars) compared to ELISA (blue bars) for the same antigens. (G) Wash-free SiMREPS protocol for quantifying IL-6 in serum. (H) Correlation plot of endogenous IL-6 measurements by the wash-free protocol in 34 patient-derived serum samples by SiMREPS (100-fold dilution of all samples) and ELISA with variable dilution factors (4-fold dilution, closed blue squares; 64-fold dilution, open blue squares) or ELISA with 100-fold dilution of all samples (orange triangles).

tion, reaches arbitrarily high specificity by repeated evaluation over an arbitrarily long time window. In particular, the high specificity for C>T mutation T790M arose from minimizing the heat-induced deamination of cytosine to uracil that converts WT *EGFR* to a MUT-like sequence as well as from the enzymatic removal of damaged DNA bases by treatment with UDG (uracil-DNA glycosylase) after denaturation and surface capture (Figure 3E).

For *in vitro* detection of miRNAs by SiMREPS, LNA-modified capture probes 9−11 nt in length were employed to capture miRNA targets from buffer or biofluids pretreated with proteinase K and SDS to protect against RNase activity (Figure 3A). An FP 9 to 10 nt in length generated distinct single-molecule kinetic fingerprints for specific binding to the target miRNA and nonspecific binding (Figure 3F). The generality of this approach was evaluated by detecting four human miRNAs (*hsa-let-7a*, *hsa-miR-21*, *hsa-miR-16*, and *hsa-miR-141*) and one miRNA from *Caenorhabditis elegans* (*cel-miR-39*) with a dynamic range spanning 2 to 3 orders of magnitude and a LOD of approximately 1 fM[1] (Figure 3G). The ability to

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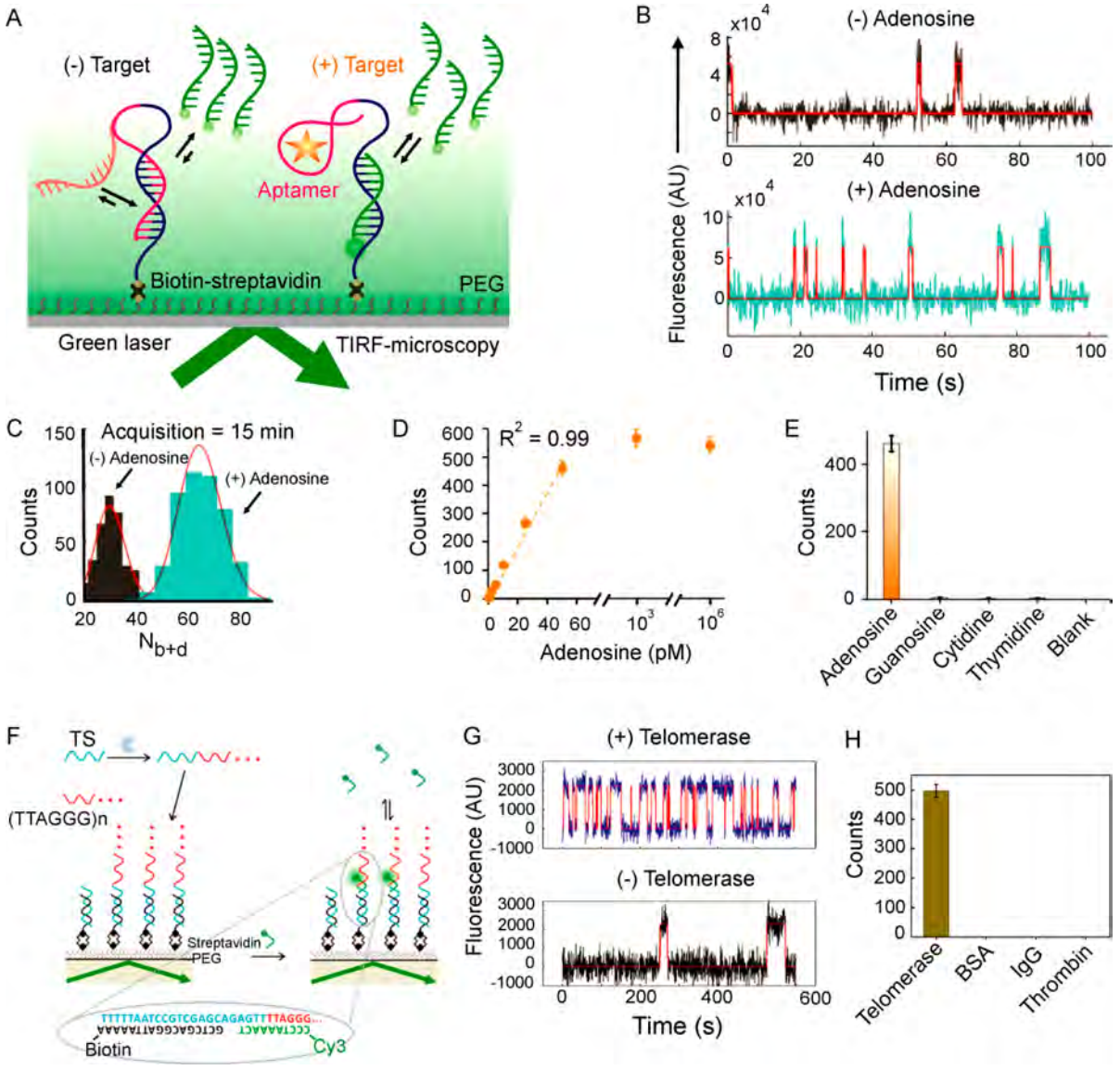

Figure 5. Detection of small molecules and monitoring the enzyme activity using SiMREPS. (A) Experimental scheme showing the use of SiMREPS to probe the state of an aptamer for the high-sensitivity detection of small molecules by TIRF microscopy. (B) Representative intensity versus time traces in the absence and presence of adenosine (50 pM). (C) Histograms of N_{b+d} in the absence (gray) or presence (cyan) of adenosine (50 pM). (D) Standard curve and (E) selectivity of adenosine detection. (A–E) Reproduced with permission from ref 16, copyright 2019, American Chemical Society. (F) Experimental scheme for the detection of telomerase activity at the single-molecule level using SiMREPS. (G) Single-molecule kinetic traces in the (top) presence and (bottom) absence of telomerase activity. (H) The single-molecule assay showed a response in the presence of telomerase but not for other proteins. (F–H) Reproduced with permission from ref 65, copyright 2017, American Chemical Society.

discriminate between single-nucleotide variants was demonstrated by comparing the kinetic fingerprints generated by the FP for *let-7a* in the presence of either *let-7a* or *let-7c* in buffer (Figure 3H); the detection of *let-7* family members was also demonstrated in a cell extract.[1]

Finally, the *in situ* detection of miRNA by SiMREPS within fixed, permeabilized eukaryotic cells was demonstrated by Li et al.[14] using HILO microscopy (Figure 3I). Kinetic fingerprinting enabled strong discrimination between the specific and nonspecific binding of an FP for miR-21 (Figure 3J), permitting the single-molecule counting of miRNAs in single cells *in situ* (Figure 3K). Compared to single-molecule fluorescence *in situ* hybridization (smFISH),[43,49] which typically requires dozens of FPs binding to the same long RNA to achieve discrimination from a spurious FP signal, SiMREPS provides a means of detecting smaller nucleic acids including miRNAs with high accuracy and a low risk of photobleaching using a nanoflare. However, the higher background autofluorescence, potentially high intracellular concentrations of miRNAs, and potential masking by proteins or other binding partners may still pose challenges to the accurate quantification of miRNAs in cells by SiMREPS. The use of expansion microscopy[50] and super-resolution data analysis[2] might solve these problems.

3.2. SiMREPS Detection of Protein Biomarkers

Proteins are involved in many biological processes and are useful biomarkers for differentiating between healthy and diseased states in clinical diagnostics.[51] Mutated or misfolded proteins are associated with multiple diseases, such as Alzheimer's and Parkinson's diseases.[52] Uncontrolled protein expression leads to increased levels of specific proteins in blood that are associated with different types of cancer.[53] Thus, the sensitive and accurate

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quantification of proteins in human biofluids could be critical for the early-stage diagnosis of disease.

For protein detection, the surface-immobilized antigen is allowed to interact transiently and repeatedly with a fluorescent detection probe to generate kinetic fingerprints characteristic of specific binding to the antigen (Figure 4A−D).[3] In contrast to SiMREPS detection of nucleic acids, in which synthetic oligonucleotides can be readily designed for use as FPs, protein-SiMREPS employs a fluorescently labeled detection antibody (typically a monovalent Fab fragment with fast dissociation kinetics) for the FP. Thus, the successful development of a SiMREPS assay for proteins depends on the availability of a detection antibody with suitable kinetics.

Fortuitously, *in vitro* selection methods permit the selection of antibodies with sufficiently rapid dissociation kinetics for use as FPs in SiMREPS. Recombinant monovalent Fab antibodies against a target antigen can be isolated from the HuCAL PLATINUM library, which comprises 45 billion fully human antibody clones that can be screened for antigen binding using phage display and its variants.[54] To facilitate the selection of Fab clones with suitably fast kinetics for SiMREPS, a modified strategy was developed to allow enrichment for clones with high off-rates.[3] ELISA hits from each panning showing binding to antigen were subjected to a high-throughput off-rate screening[55] using Biolayer Interferometry (BLI) and/or Surface Plasmon Resonance (SPR);[55,56] clones with the highest off-rates were sequenced to identify unique Fabs suitable for SiMREPS.

The screening of different *in vitro*-selected Fabs against target antigens IL-6, PAI-1, VEGF-A, and IL-34 by SiMREPS showed that the most useful probes exhibit rate constants of association (k_{on}) in the range of $(0.5–5) \times 10^6$ M^{-1} s^{-1} and rate constants of dissociation (k_{off}) in the range of 0.05−0.5 s^{-1} in PBS at 25−30 °C, corresponding to K_D values of 10−600 nM (Figure 4E),[3] similar to the most useful rate constants for SiMREPS detection of nucleic acids.[15] Encouragingly, 50% of the Fabs that were *in vitro*-selected for high off-rates were found to be suitable as SiMREPS probes. Furthermore, it was found that the kinetics of FP interaction with the antigen could easily be manipulated in SiMREPS measurements by modifying the assay temperature and/or salt concentration.[3]

Since SiMREPS can filter out the signal arising from nonspecific binding based on its kinetic profile (Figure 4C), it removes the background "floor" and achieves higher sensitivity than ELISA and other conventional techniques.[3] For the four antigens tested, SiMREPS achieved LODs in the low-femtomolar to attomolar range, or 55- to 383-fold lower than for commercial ELISA kits for the same antigens (Figure 4F), suggesting that SiMREPS may facilitate the detection of trace protein biomarkers in the earliest stages of disease. Moreover, SiMREPS was shown to be amenable to a wash-free protocol (Figure 4G), meaning that no buffer exchanges are required after the addition of the antigen mixture to the sample well.

In addition to its simpler sample handling requirements, this wash-free SiMREPS assay was more sensitive than ELISA for the measurement of endogenous IL-6 in serum from CAR-T cell therapy patients (Figure 4H). Finally, since SiMREPS requires only one tightly binding antibody (the CP) and since weakly binding FPs are easily found by *in vitro* selection, this approach may prove compatible with antigens for which no high-quality antibody pairs for sandwich ELISA are available.

3.3. SiMREPS Detection of Toxins and Other Small Molecules

In addition to large biomolecules such as proteins and nucleic acids, small molecules such as vitamins, hormones, metabolites, intracellular messengers, and cofactors also play important roles in assessing disease etiology and treatment efficacy.[57] For instance, the concentration of adenosine increases in the plasma of patients with congestive heart failure (CHF),[58] and circulating ATP has emerged as a biomarker of cognitive impairment in HIV.[59]

Aptamers are single-stranded RNA or DNA generated by *in vitro* selection or the systematic evolution of ligands by exponential enrichment (SELEX)[60] and provide a promising approach to the specific detection of small molecules. However, their sensitivity and specificity are often limited by high K_D[61] values as well as the difficulty of completely suppressing a signal in the absence of analyte.

Recently, Weng et al.[16] presented a possible strategy to overcome these challenges, demonstrating the ultrasensitive and specific detection of adenosine biomarkers by combining aptamers with SiMREPS (Figure 5A). In this approach, similar to single-molecule kinetic analysis of the RNA transient structure (SiM-KARTS),[62] the specific binding of an adenosine target with a surface-immobilized hairpin-shaped aptamer induces a conformational change in the aptamer to expose a hairpin stem that transiently and repetitively interacts with FPs (Figure 5A). High-accuracy discrimination of the ligand-bound and ligand-free states was achieved by monitoring the interactions for 15 min under TIRF microscopy, resulting in virtually zero-background measurements of the small-molecule analyte (Figure 5B,C). The LOD for adenosine spiked into chicken meat extract was 0.3 pM (Figure 5D), which is superior to that of recently reported aptasensors.[63] The aptamer-based SiMREPS approach also exhibited high specificity, showing little interference from other small-molecule ligands (Figure 5E).

Aptamer-based SiMREPS (Figure 5A) was further tested by detecting two additional small-molecule toxins such as acetamiprid and PCB-77.[16] The LODs for acetamiprid and PCB-77 were determined to be 0.35 pM and 0.72 pM, respectively,[16] approximately 3 and 70 times lower than for recently reported biosensors.[63,64] SiMREPS thus significantly improves the performance of aptamers in the detection of diverse small-molecule analytes. However, the generality of the SiMREPS approach is limited by the availability of suitable aptamers (Table S2).

3.4. Monitoring of Enzyme Activity with SiMREPS

Given its sensitivity to small chemical differences in single molecules, SiMREPS provides an interesting means to monitor the activity of enzymes. For example, Su et al.[65] employed SiMREPS to monitor the activity of telomerase (Figure 5F), an enzyme that plays a critical role in maintaining chromosomal integrity and is overexpressed in approximately 90% of all malignant tumors.[66] Telomerase activity was monitored *in vitro* by observing the dynamic binding of a short DNA FP with telomerase reaction products (repeated sequence TTAGGG) (Figure 5F), yielding a distinct kinetic signature from background binding (Figure 5G). With this method, the activity of telomerase extracted from as few as 10 cancer cells was detected;[65] in contrast, no such signal was detected in the presence of proteins other than telomerase (Figure 5H).

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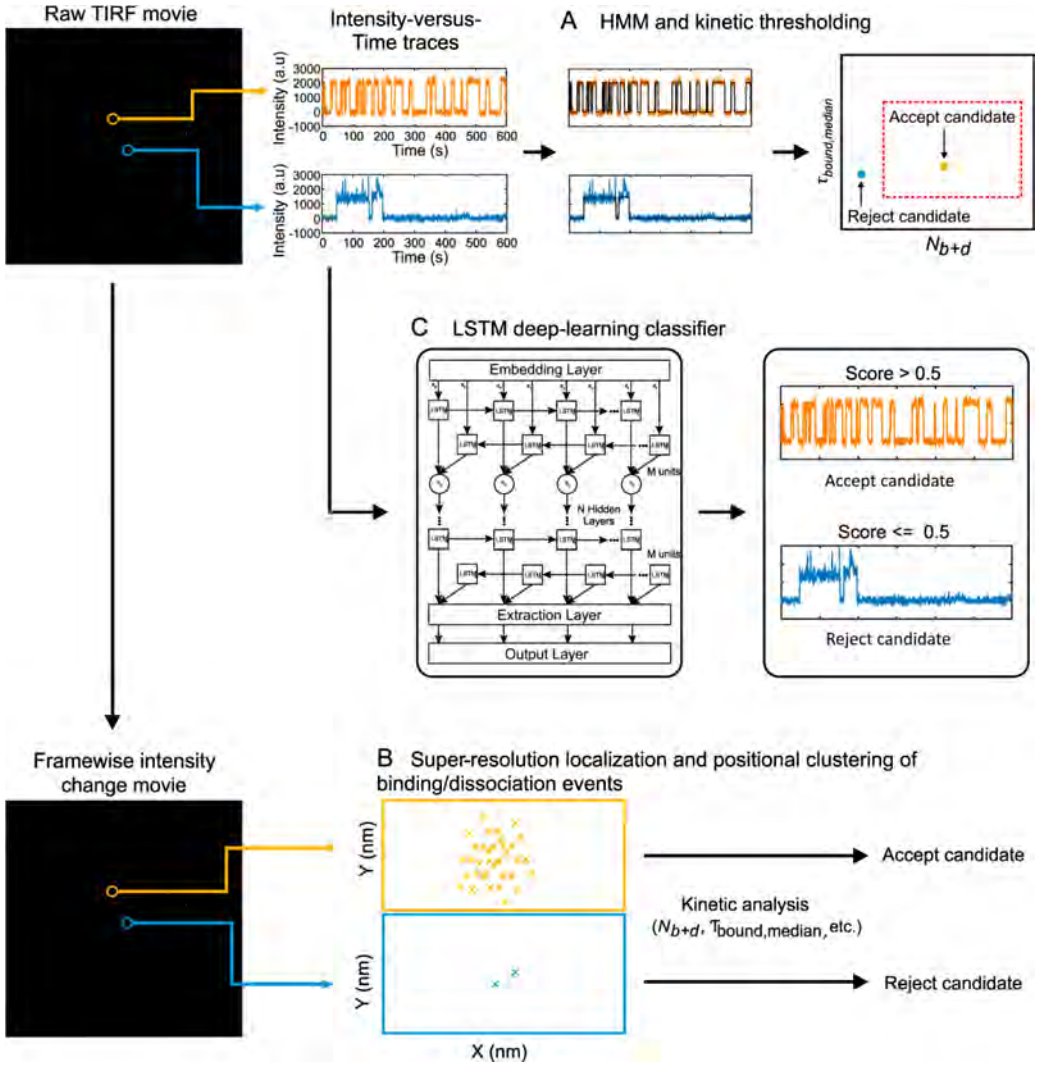

Figure 6. Approaches to SiMREPS data analysis. A TIRF microscopy movie is used to generate single-molecule time traces. (A) These traces are then subjected to HMM and kinetic analysis and accepted or rejected as kinetic fingerprints of analyte molecules. (B) Alternatively, a higher dynamic range can be achieved by performing a frame-by-frame subtraction to yield a framewise intensity change movie, which is then analyzed by super-resolution localization methods to identify clusters of binding events indicative of the presence of analyte molecules. (C) As a third alternative, the single-molecule traces are passed to an LSTM deep-learning classifier that was previously trained using control experiments to score and reject or accept each trace.

4. SIMREPS DATA ANALYSIS AND PROCESSING

4.1. Idealization and Kinetic Analysis of Single-Molecule Intensity Traces

Standard SiMREPS analysis is performed by generating single-molecule intensity versus time traces from TIRF microscopy videos, characterizing the kinetics of FP binding within each trace using hidden Markov modeling (HMM)[67] and either rejecting or accepting each trace as evidence of the presence of a single analyte molecule by enforcing minimum and/or maximum value thresholds for several criteria.[3,12] The typical criteria used to distinguish traces containing specific binding to the analyte from those containing only nonspecific binding include the following:[12]

- signal intensity,
- signal-to-noise ratio,
- number of binding and dissociation events per trace (N_{b+d}),
- median lifetime in the bound ($\tau_{bound,median}$) and unbound ($\tau_{unbound,median}$) states, and
- maximum individual dwell time in the bound and unbound states

Thresholds for the above parameters are usually set empirically by comparing positive (e.g., in the presence of an ~1 pM target) to negative (i.e., matrix-only) control experiments and choosing thresholds that minimize false positives and maximize true positives.

While this standard approach has the advantages of simplicity and transparency, it has two main drawbacks: it is a diffraction-limited analysis method, making it challenging to apply to fields of view with very densely captured analytes (e.g., >1 molecule per 10 μm^2 area), and its output is influenced by the quality of the HMM fitting. To address these limitations, we recently developed super-resolution-[2] and deep learning[4] -based analytic pipelines, respectively.

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4.2. Super-Resolution Analysis

At high concentrations (e.g., >1 pM), multiple analyte molecules may be captured within a single diffraction-limited[68] region. Consequently, the emission of fluorescent probe binding to multiple distinct analyte molecules will overlap, making it difficult or impossible to analyze binding kinetics accurately and placing an upper limit on the dynamic range of SiMREPS measurements performed with standard diffraction-limited analysis.[2]

To overcome this challenge, we developed a super-resolution approach to the analysis of SiMREPS data[2] inspired by microscopy methods.[40] However, unlike conventional super-resolution microscopy, our approach performs subpixel localization using the frame-to-frame changes in fluorescence intensity rather than raw intensity, permitting the analysis of fields of view with very dense probe binding (Figure 6). Hierarchical clustering is used to identify groups of probe binding and dissociation events to the same analyte molecule; these clusters are then subjected to kinetic threshold analysis similar to that performed in HMM analysis.[2] This approach extends the dynamic range by approximately 2 orders of magnitude for both nucleic acid[2] and protein[3] SiMREPS measurements.

4.3. Deep Learning for Fast, Automated, and Accurate Analysis of SiMREPS Data

HMM and super-resolution analysis rely on the fitting of models to naturally noisy raw data, which yield occasional false positives due to, for example, the interpretation of baseline noise or photophysical blinking as binding transitions. As a result, strict thresholds for signal-to-noise ratios and kinetics often must be employed to avoid these errors, which in turn results in the rejection of some true positives and hence lower sensitivity. To overcome these shortcomings, we recently developed a deep learning-based method for SiMREPS data analysis (Figure 6).[4]

Deep recurrent neural network (RNN) methods have been effectively used to learn sequential biological information.[69,70] Long short-term memory (LSTM) is a modification of the RNN architecture to learn long-range dependencies in sequential data,[71] making it suitable for the kinetic analysis of SiMREPS traces. We therefore developed an LSTM-based deep learning approach for the automated classification of SiMREPS traces and found it to yield both higher sensitivity and higher specificity than HMM-based methods in measurements of *EGFR* point mutation T790M.[4] It can be further adapted by transfer learning on new data sets,[4] suggesting an important future role for artificial intelligence approaches in further streamlining SiMREPS-based molecular diagnostics.

5. CONCLUSIONS AND FUTURE DIRECTIONS

SiMREPS is a unique analytical approach that permits the amplification-free detection of single molecules with high sensitivity and specificity using kinetic fingerprinting with transiently binding probes. Because of its lack of analyte-specific chemistry or enzymatic steps, it provides a comprehensive platform for the detection of diverse molecular biomarkers including nucleic acids, proteins, and small molecules. Since its kinetic fingerprinting provides exquisite sensitivity to even minute chemical differences (e.g., single-nucleotide variations or mutations), SiMREPS may offer a future means of detecting epigenetic, epitranscriptomic, and post-translational modifications with single-site and single-molecule sensitivity. Through spatial patterning (e.g., in a microarray or through a water-in-oil droplet emulsion) and/or color encoding, a diverse panel of disease biomarkers could be detected in parallel on a single instrument platform. Similarly, combining *in situ* SiMREPS with expansion microscopy may have potential for single-cell multiomics.[11]

Although standard diffraction-limited analysis methods for SiMREPS already provide very high specificity, the newly developed super-resolution and deep-learning approaches increase the dynamic range and sensitivity of this technique. Future developments, particularly those utlilizing deep learning, may increase the data analysis pipeline efficiency by operating directly on raw movies. Recent publications have shown that attention-based networks[72] and 2D convolutional-based neural networks[73] outperform traditional RNN/LSTM models for processing sequential data. Convolution-based approaches could use raw movie data as input, using fewer hardware resources, eliminating data preprocessing steps, and yielding potentially more accurate classification results.

While the sensitivity of SiMREPS already rivals or surpasses leading techniques for protein and small-molecule detection, its sensitivity for nucleic acids still falls somewhat short of PCR-based amplification approaches. This is not due to an intrinsic sensitivity limit (SiMREPS can detect single molecules) but is attributed to the fact that it is challenging to transport analytes to a surface and image them with ∼100% efficiency. As a result, <0.1% of the analyte molecules present in a sample are detected in a typical measurement. Methods to actively preconcentrate analytes or actively transport them to the imaging surface, as well as optics that permit measurement over a wider field of view, may therefore improve LODs by a factor of 100 or more. Finally, the development of a dedicated, affordable instrument will render the technique accessible to a broad set of scientific and clinical laboratories.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.accounts.0c00621.

Analysis of diverse analytes using single-molecule kinetic fingerprinting; advantages and limitations of SiMREPS for analyzing different biomarkers compared to the existing gold standard technologies (PDF)

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Notes

The authors declare the following competing financial interest(s): The University of Michigan and Bio-Rad Laboratories, Inc. have filed patent applications on technologies described herein, on which A.J.-B., M.T., and N.G.W. are listed as inventors. A.J.-B., M.T., and N.G.W. are co-founders of aLight Sciences, which sponsored part of this work and seeks to commercialize the SiMREPS technology.

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Muneesh Tewari completed his undergraduate studies in biochemistry at Case Western Reserve University and his M.D. and Ph.D. degrees at the University of Michigan in 1997. During his Ph.D., he discovered the apoptosis effector known as Caspase-3. After an internal medicine residency at the University of Michigan, he obtained medical oncology training at the Dana-Farber Cancer Institute and postdoctoral training in systems biology at Harvard Medical School. In 2005, he began his independent career at the Fred Hutchinson Cancer Research Center in Seattle, where his laboratory discovered extracellular microRNAs as a new class of biomarkers. In 2014, he moved back to the University of Michigan to continue his research at the intersection of systems biology, disease biomarkers, and new technology.

Alexander Johnson-Buck received his B.A. from Northern Michigan University. He earned his Ph.D. in chemistry from the University of Michigan, using single-molecule fluorescence microscopy to characterize DNA nanomachines and coinventing SiMREPS. He then performed

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