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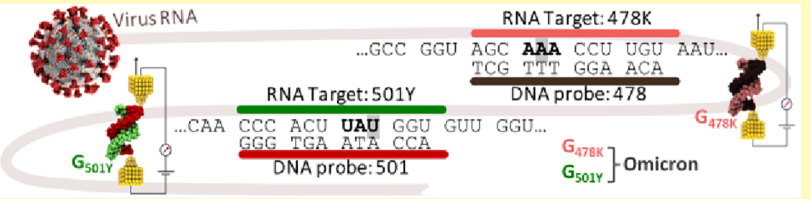
Identifying SARS-CoV-2 Variants Using Single-Molecule Conductance Measurements

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ABSTRACT: The global COVID-19 pandemic has highlighted the need for rapid, reliable, and efficient detection of biological agents and the necessity of tracking changes in genetic material as new SARS-CoV-2 variants emerge. Here, we demonstrate that RNA-based, single-molecule conductance experiments can be used to identify specific variants of SARS-CoV-2. To this end, we (i) select target sequences of interest for specific variants, (ii) utilize single-molecule break junction measurements to obtain conductance histograms for each sequence and its potential mutations, and (iii) employ the XGBoost machine learning classifier to rapidly identify the presence of target molecules in solution with a limited number of conductance traces. This approach allows high-specificity and high-sensitivity detection of RNA target sequences less than 20 base pairs in length by utilizing a complementary DNA probe capable of binding to the specific target. We use this approach to directly detect SARS-CoV-2 variants of concerns B.1.1.7 (Alpha), B.1.351 (Beta), B.1.617.2 (Delta), and B.1.1.529 (Omicron) and further demonstrate that the specific sequence conductance is sensitive to nucleotide mismatches, thus broadening the identification capabilities of the system. Thus, our experimental methodology detects specific SARS-CoV-2 variants, as well as recognizes the emergence of new variants as they arise.

KEYWORDS: *biosensors, molecular electronics, SARS-CoV-2 variant detection, single-molecule break junction, XGBoost machine learning*

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Since the SARS-CoV-2 virus was identified in December 2019, it has spread worldwide, caused nearly seven million fatalities as of October 2023, and has remained a geopolitical, economic, and health issue globally.[1] Much effort during this time has been devoted to developing vaccines to trigger an immune response to the SARS-CoV-2 spike glycoprotein's receptor-binding domain (RBD).[2−8] Unfortunately, as the virus continues to thrive in the human ecosystem new variants of the SARS-CoV-2 virus are continuing to emerge around the globe. Among the emerging variations B.1.1.7 (Alpha), B.1.351 (Beta), B.1.617.2 (Delta), and B.1.1.529 (Omicron) are in the class identified as variants of concern (VOCs).[9−11] Moreover, as modifications to the genome compound in the VOCs, the efficacy of the initial vaccines, which were designed to combat the virus's original genome, has diminished. The quantity, position, and type of mutations on the receptor-binding domain can affect vaccine efficacy over time, and some strains may eventually evade the vaccine induced immunity and trigger another pandemic wave.[12−19] Thus, it is important to monitor circulating variants to track the spread of the disease and implement containment measures in the event of an outbreak. Currently, testing relies heavily on reverse transcription polymerase chain reaction (RT-PCR) and whole-genome sequencing techniques to detect, identify, and track VOCs.[20−23] However, this technique requires significant time and resources for diagnosis and mutation detection. Thus, the COVID-19 pandemic has emphasized the need for fast, sensitive, and cost-effective methods for both diagnostics and variant surveillance, and a new generation of techniques is emerging in an attempt to supplement conventional methods. These include Cas-based assays,[24−27] electrochemical biosensors,[28−30] FET-based biosensors,[31−34] and others.[35−39] Here, we present an electrical biosensor based on single-molecule conductance measurements that allow for sensitive, robust, and cost-effective detection and identification of the VOCs.

The sensing platform is based on the single-molecule break junction (SMBJ) technique (Figure 1g), which has previously been used to demonstrate that (i) the conductance of double-

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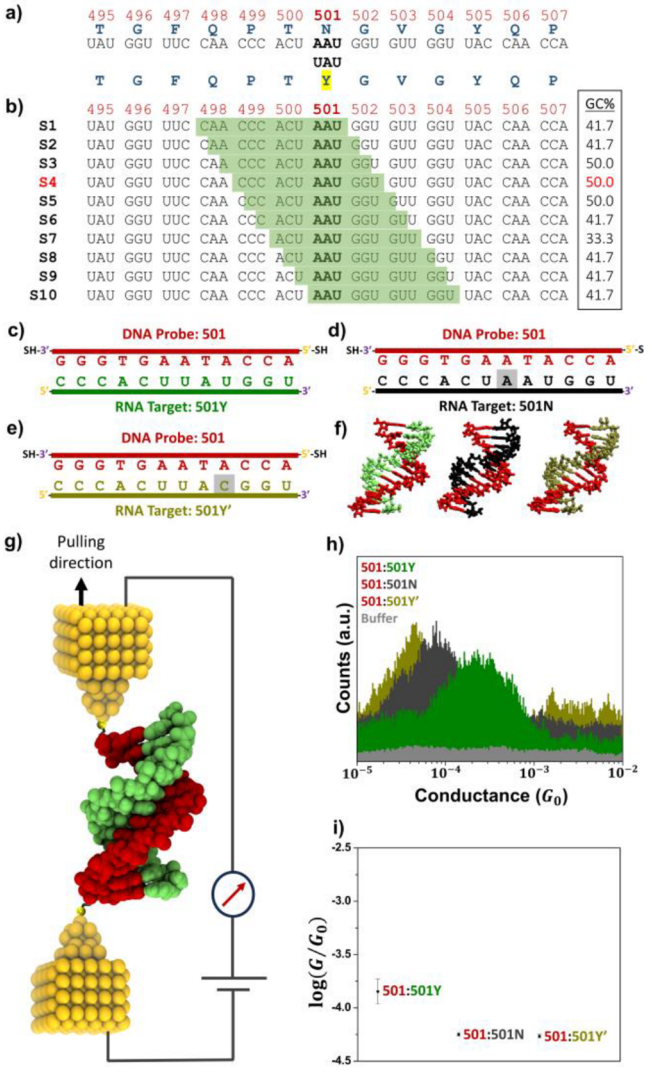


Figure 1. Mutation selection and conductance measurements. (a) Section of the genome including the 501 mutation. (b) 12 base pair sequence windows around each mutation and the corresponding GC content of each; the selected target is shown in red (S4). (c) Designed DNA probe and RNA target for the 501Y mutation, (d) the 501N wild-type RNA target, and (e) the interfering 501Y′ target sequence. (f) 3D molecular representations from MD simulations of 501 DNA probe and 501Y, 501N, and 501Y′ RNA targets, (g) schematic of 501:501Y DNA:RNA hybrid between Au electrodes, (h) conductance histograms for the 501 DNA probe hybridized to 501Y, 501N, and 501Y′ RNA targets, and (i) conductance values for the individual molecules, with error bars indicating standard error of the mean with $N = 4$.

stranded RNA:DNA hybrids can be measured at the single molecule level,[39−44] (ii) the conductance is sensitive to both sequence and base-pair mismatches,[41,42] (iii) it is possible to detect and identify pathogenic bacterial strains using RNA sequences down to attomolar concentrations,[41] and (iv) detection is possible in complex environments.[42] Thus, in light of the emergence of VOCs, here, we examine the utility of single-molecule conductance measurements to identify specific SARS-CoV-2 variants and to explore how this approach can be generalized to identify multiple targets simultaneously.

In the following section, we first discuss the design considerations involved in selecting DNA probes that are compatible with our measurement system and align with our objectives. Specifically, we examine the conductance of RNA:DNA hybrids designed to detect the wild-type (WT) SARS-CoV-2 virus, and the Alpha, Beta, Delta, and Omicron variants, focusing on spike protein mutations N501Y, E484K, and T478K. We demonstrate that these target variants can be accurately identified even in the presence of interfering sequences. Furthermore, we highlight the potential for improvement in target identification by employing a machine learning model, XGBoost, which effectively identifies the presence of these targets within complex solutions containing off-target sequences. Notably, our approach achieves reliable results with a limited number of samples reads (<100), significantly reducing the overall processing time from sample input to obtaining results to mere seconds.

EXPERIMENTAL SECTION

To begin, we use genomic data from GISAID[45] to identify the mutations present in the VOCs. The genomic data indicates that each VOC has a unique set of mutations within the receptor binding domain (RBD) given in Table S1, and by targeting a subset of these mutations, we can unambiguously identify the VOCs of interest. From this analysis, we determine that detecting point mutations at residues 501, 484, and 478 within the spike protein allows us to identify wild-type (WT) SARS-CoV-2, and the Alpha, Beta, Delta, and Omicron VOCs (Table S2), provided the conductance values between perfectly matched and mismatched target sequences are distinguishable.

Thus, the next step is to identify target RNA sequences around each mutation (and thereby the corresponding DNA probe sequence) that would maximize our probability of identifying both the perfectly matched and mismatched sequences. An example of this process is shown in Figure 1a for the mutation at the 501st codon of the spike protein, where a Tyrosine (Y, UAU codon) has mutated into an Asparagine (N, AAU codon). As shown in Figure 1b, we focus on short (12 nucleotides) sequences that are well within the capture and measurement capabilities of the SMBJ approach[41−44,46−48] and then aim to (i) enhance the G:C content, as higher GC content in hybrids of a given length correlates with increased conductivity,[42] (ii) centralize the mutation point within the sequence to ensure structural stability even when a mismatch is present, and (iii) minimize the number of probable confounding sequences within the entire SARS-CoV-2 genome. Based on these criteria, we chose the RNA sequence CCC ACU UAU GGU as our target (Figure 1c) to identify the 501Y mutation, which is present in Alpha, Beta, and Omicron. Next, we chose the complementary DNA sequence as our probe, which we refer to as probe 501. This probe is also able to bind to the RNA sequence CCC ACU **A**AU GGU (501N, Figure 1d), which is present in WT and Delta, as a second target. In addition, we searched for this 12 bp target sequence within the rest of the genome and found no confounding sequences. Finally, since we must also be sensitive to other potential mutations that may occur in this sequence, we chose CCC ACU UA**C** GGU (Figure 1e) as an additional control sequence. In this case, the UAC codon still codes for Tyrosine (Y), so we refer to it as target 501Y′. Thus, the 501 DNA probe is able to bind to RNA targets 501Y, 501Y′, and 501N. A similar approach is utilized for mutations at the 484 and 478 positions, and the amino acid and nucleotide sequences for these are given in Figures S1 and S2, respectively.

Previously, we showed that structural instability in RNA:DNA hybrids can result in unmeasurable conductance values.[41] Therefore, the stability of each selected sequence is a crucial factor in obtaining a reliable measurement. Thus, once the probe and targets are identified for a given point mutation, we examine the structural stability of each hybrid using molecular dynamics (MD) simulations. We perform 50 ns MD simulations, analyze the trajectories in terms of root-mean-square deviation (RMSD), and examine the change in hydrogen bonding over time. MD analyses, whose details are available in Section 2 of the Supporting Information, show that the selected probe-target hybrids are stable over the course of the simulations. In

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mismatched sequences, the RNA can still hybridize with its corresponding DNA probe, although forming a structure that deviates from the perfectly matched case. Additionally, the melting temperatures of the molecules have been calculated and are presented in SI Table S3, indicating that the RNA:DNA hybrids remain stable at room temperature.

Finally, to ensure that the selected targets can be detected and measured, we perform conductance measurements on each using the SMBJ approach (Figure 1g). Here, 1000s of break-junction cycles are repeated, and then conductance traces with identifiable step regimes (SI: Figure S7) are combined to produce a conductance histogram. As can be seen in this case, each target gives a distinct conductance peak, though the 501:501N and the 501:501Y′ hybrids have similar conductance values (Figure 1h,i), a point we will return to below. Histograms displaying the full range of conductance are available in Figure S10 of the SI. The unique conductance values observed can be attributed to the impact of mismatches on the conformation of RNA:DNA hybrids, as documented in the literature.[41] The conductance axes are presented in terms of G_0 where G_0 is the conductance quantum $\frac{2e^2}{h}$ (e is an electron charge, and h is the Planck constant).

RNA target sequences and the DNA probes for 484 and 478 mutations are shown in Figure 2a. The conductance results are shown

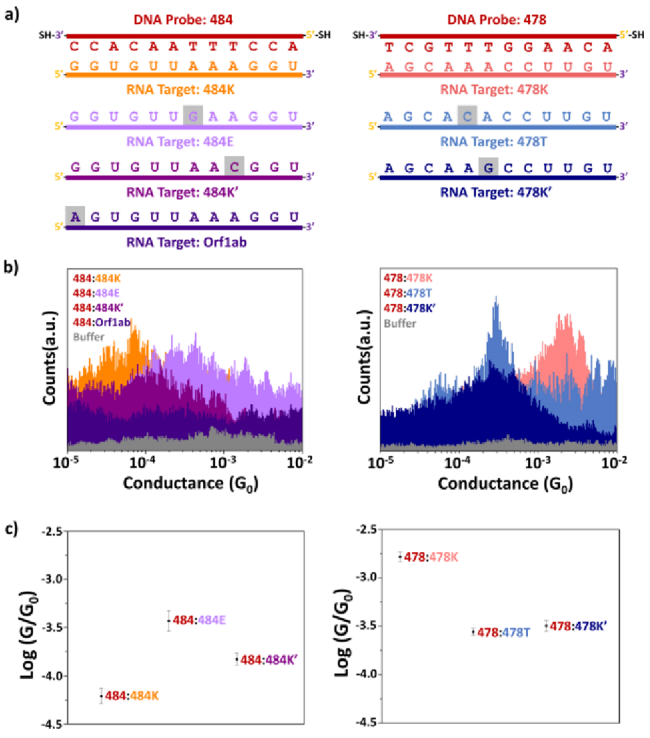


Figure 2. Sequences and conductance measurements for the 484K and 478K mutations. (a) 484 and 478 DNA probes and their complementary and interfering RNA targets, mismatched bases are highlighted. (b) Conductance histograms of the DNA:RNA hybrids, color-coded by corresponding hybrids, and (c) conductance values from a Gaussian fit of $N = 3$ samples with error bars given by the standard error of the mean.

in Figure 2b,c along with the conductance histograms resulting from the SMBJ measurements. Details of the SMBJ technique are provided in Section 3 of the Supporting Information. The 478 probe is designed to detect the 478K mutation, which is present in the Delta and Omicron VOCs, but can also detect the presence of the 478T target (mismatch), which exists in the WT. We also added an additional RNA target to the list, which is named 478K′ to examine possible interference. Similarly, for the 484 DNA probe, we have 484E (mismatch) present in Alpha, Delta, and WT (Table 1), and 484K (perfectly matched) that persists in Beta. In this case, we include 484K′ as a possible mismatched interfering sequence and found a sequence in the SARS-CoV-2 genome that represents a second interference point with a single mismatch to the designed DNA probe (at the end of the sequence) from the Orf1ab protein. As such, we also test for this possibility.

RESULTS AND DISCUSSION

The conductance results from each of these sequences verify that we can distinguish between the perfectly matched complement to our probe sequence and any of the tested mismatches (Figure 1h,i for 501 probe, Figure 2b,c for the 484 and 478 probes). Control experiments utilizing single-stranded DNA probe and RNAs indicate the absence of conductance peaks in the histograms within the designated conductance region (refer to Supporting Information, Figure S6). Figures featuring Gaussian fits have been included in the SI Section 3.5 to improve visual clarity. From these sequences, we can readily identify the perfectly matched cases allowing us to unambiguously detect specific VOCs (Figure 3). For example, if we test a sample with the three probes and find the perfectly matched 501Y but not the perfectly matched 484K or 478K, we would conclude that the sample is COVID positive, and that it is the Alpha VOC. Similarly, (i) Beta can be identified if 501Y and 484K are present, (ii) for Delta, only 478K would be detected, and (iii) for Omicron, both the 501Y and 478K peaks would be present (shown as a representative case in Figure 3). Thus, by focusing only on the perfectly matched sequences that are designed to target specific mutations, we can positively identify specific VOCs and as new VOCs emerge, new probes can be developed to directly target those (Figure S5).

Although we can positively identify the targeted VOCs based on the perfectly matched sequences, the fact that we get well-defined conductance peaks from the mismatched test sequences (501Y′, 484K′, and 478K′) that represent known variations (501N, 484E, and 478T), and the Orf1ab target raises three important questions. Can we positively identify other variants (e.g., WT) based on these results? Can we ensure a COVID positive result even if the new variants arise? And can we positively identify VOCs even if multiple strains are present in a sample (e.g., in wastewater samples)?

The primary issue in answering these questions is that in some cases the conductance values of the mismatches are not significantly different from one another (e.g., 501N and 501Y′, Figure 1h,i). However, if instead of focusing on the dominant peak value alone, we look at the conductance histogram as a "fingerprint" for a specific sequence, we can utilize machine learning techniques to try to distinguish between all possible variations for a given probe sequence. In this approach, we note that while conductance values may be similar, the overall distributions of the conductance histograms are different for each case. Thus, we organize our machine learning algorithm to create histograms from a limited number of traces, and then predict which sample it came from. This is repeated for a number of different traces per histogram. While it is not possible to determine exactly what features of the histogram the algorithm fits to, a generalized way of thinking of this approach is how many traces does it take to create a histogram that can be identified as being similar to one of the "original" complete histograms?

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Table 1. Table Showing How Each of the Variants Can Be Identified Using Results from the XGBoost ML Approach

probe	501		484		478			all	
target	501Y	501N	484K	484E	Orf1ab	478K	478T	501Y′, 484K′, or 478K′	new peak
VOC									
wild-type	0	1	0	1	1	0	1	0	0
Alpha	1	0	0	1	1	0	1	0	0
Beta	1	0	1	0	1	0	1	0	0
Delta	0	1	0	1	1	1	0	0	0
Omicron	1	0	0	0	1	1	0	0	0
new variant	?	?	?	?	1	?	?	?	?
COVID-19 negative	0	0	0	0	0	0	0	0	0

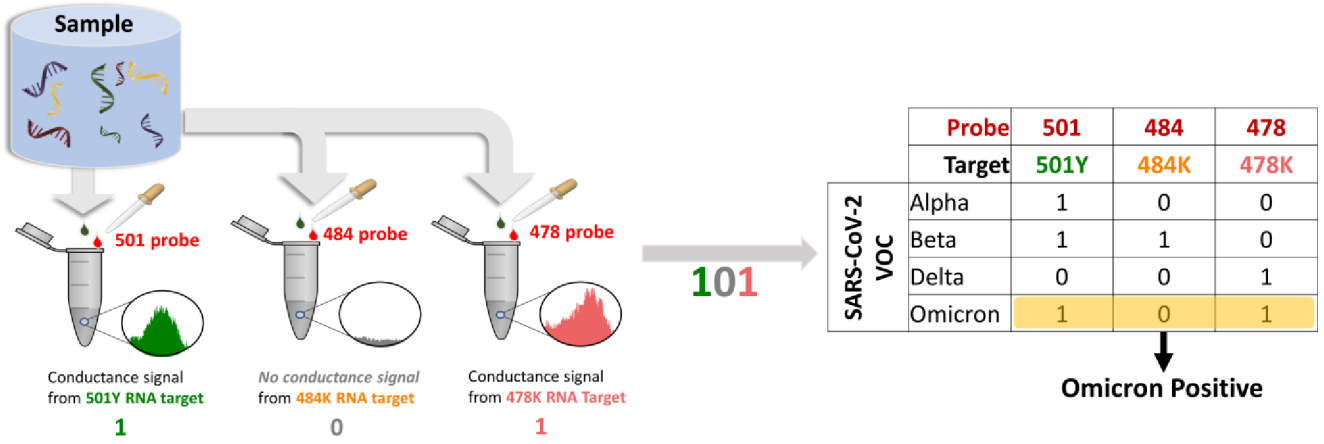


Figure 3. Envisioned process for variant identification using SMBJ conductance values. On the left, a sample is divided into three test samples, one with each of the DNA probes. If the conductance peak corresponding to the perfectly matched sequence for that probe is present, we can use the aggregated data to identify specific VOCs as shown in the table on the right.

Thus, to differentiate between all possible targets including the complementary and mismatched ones, and to automate and speed up the diagnosis process, we apply a machine learning algorithm previously developed by our group based on the XGBoost algorithm.[49−51] This approach decreases the number of conductance measurements required for identification while improving the detection and differentiation accuracy. Here, we design our classifier to detect each of the target sequences independently. To implement the algorithm, we first remove the SMBJ current traces that do not represent a molecular junction from the data sets by implementing an exponential curve fitting test. Therefore, any current traces that have an R^2 value larger than a set threshold (0.95) are discarded. This removes a sufficient number of traces without plateaus to enable the machine learning algorithm to accurately identify sequences. Next, we randomly sample H traces and construct 1000 probability histograms for each data set, with 600 bins over the conductance range from $10^{-7.5}$ to $10^{-1.5}$ G_0. For each data set, we use a train/test split ratio of 70/30, which uses 70% of traces to construct histograms and train the XGBoost[50] classifier, while 30% are used for testing. After training, the classifier can distinguish between each of the sequence targets with an accuracy of >88.7% (484E vs Orf1ab) or >98.5% (the remaining 8 targets) with only H = 100 randomly chosen raw current traces (Figure 4a). Thus, instead of collecting thousands of traces as is typically done in SMBJ measurements to develop a conductance histogram (see Figures 1 and 2), we can positively identify the target present with only 100 sample reads (conductance vs time traces). To determine the minimum number of samples needed, we examine the correlation between the accuracy of prediction for every probe:target hybrid and the number of raw current traces (H), as shown in Figure 4b. Our findings suggest that by independently testing with three different probes and their corresponding sequences using 100 individual current traces each (H = 100), we can accurately verify not just the presence of the targeted VOCs but also identify WT and Orf1ab. The results obtained from the three different probes for the 10 targets considered are summarized in Table 1. The decision matrix produced by the 0s and 1s in this table uniquely determines the target. Note that the Orf1ab producing gene in the target list gives a 0 only if COVID negative. However, positively identifying the new variant type would likely require a new probe, with the VOC detection strategy, which is discussed above.

Finally, to answer the third question above and determine whether SMBJ tests can positively identify specific targets even in the presence of multiple different strains in pooled samples (e.g., wastewater testing), we examine whether a target of interest can be identified in a sample containing both the perfectly matched target and other interfering RNA targets. To test this capability, we add each probe to a solution containing all the target RNA sequences relevant to that probe (Figure 5a). In the 501 and 484 probe cases, because of the dispersion of the conductance histogram for each of the sequences, it is not possible to extract a specific peak for each of the RNA sequences in the sample. However, there is a significant shift in the peak position and overall conductance dispersion when the VOC target is present in the sample and when it is not (Figure 5a). This shift is attributed to an increase in counts in the conductance histogram, particularly around the conductance value position of the matching RNA. For instances like 501

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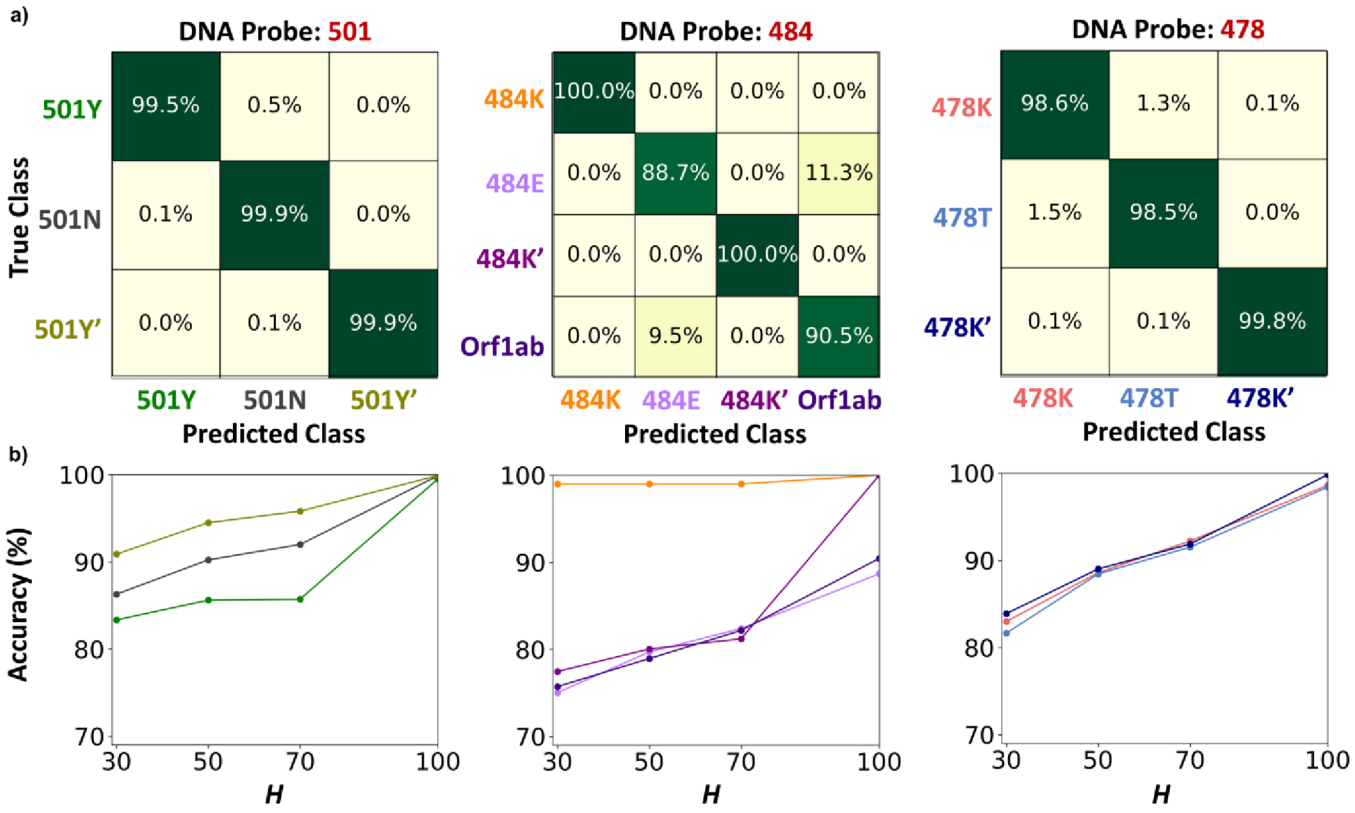


Figure 4. Classifying SARS-CoV-2 mutant sequences using ML. (a) Confusion matrices for the 501, 484, and 478 probes with each of the targets using H = 100 current traces. (b) Classification accuracy of each target (color codes are given above) showing the improvement in accuracy with the number of current traces used in the histograms for the 501, 484, and 478 probes.

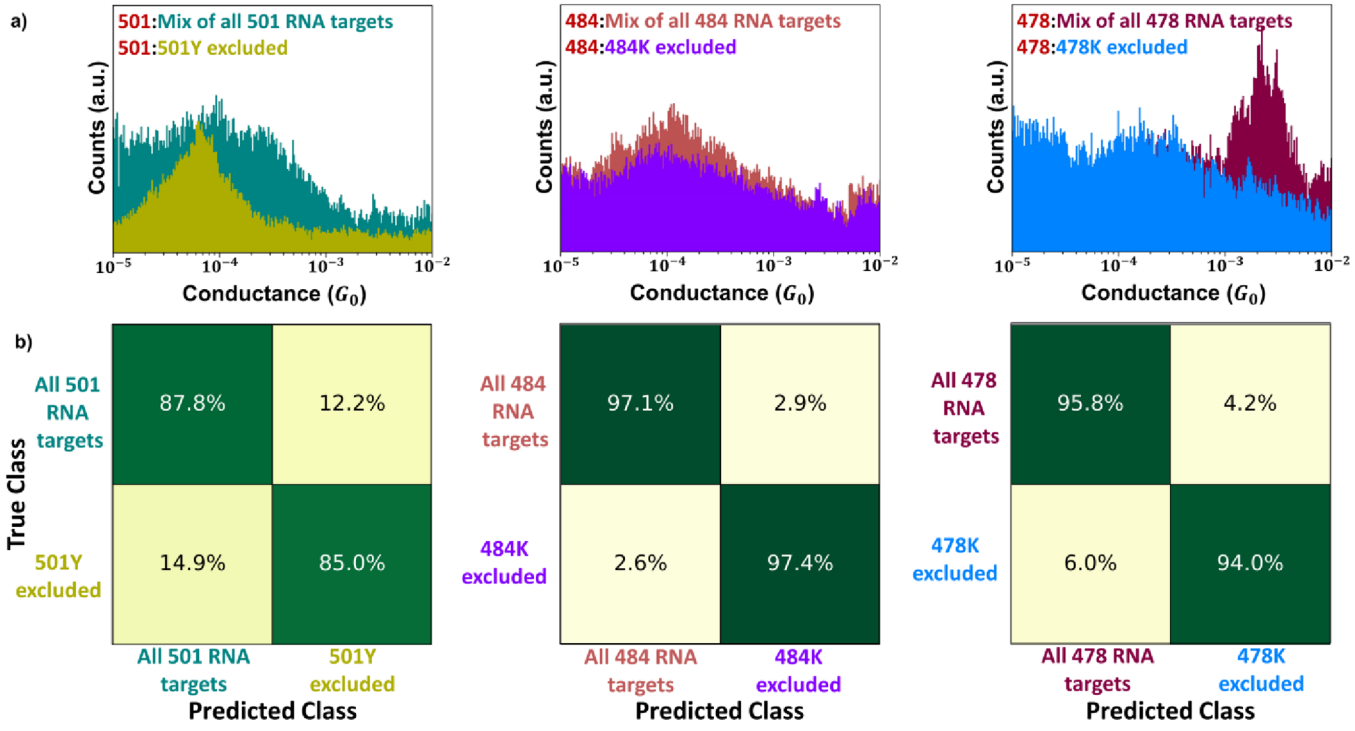


Figure 5. Mutation detection in mixed sample (a) distinct conductance peaks in three probe cases, histograms include conductance of the mix of the corresponding RNA targets of each DNA probe, and one excluding the complementary target RNA molecule from sample. (b) XGBoost classifier confusion matrices based on H = 100 raw current traces illustrating the classifier's ability to detect the variant in mixed samples.

and 484, where the conductance values are closely aligned, the shift is discernible primarily through statistical analysis. Examples of the fits are available in the SI, Section 3.6. A statistical t test analysis indicates p-values of 0.005 (501) and

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0.011 (484) for distinguishing between the cases when the perfectly matched target is present in the mixture and when it is not. In the case of the 478 probe, the peak at 1.65×10^{-3} G_0 appears only in a mixed sample where the perfectly matched sequence is present.

While these *p*-values suggest robust detection of the perfectly matched sequences (and the corresponding VOCs) using conductance measurements with good confidence intervals, it would be preferable to limit the total number of break-junction cycles required to distinguish between cases where the mismatches and targets are both present and cases where only the mismatches are present. Thus, to automate and speed up the diagnostic process and to see if the confidence levels can be improved, we apply the machine learning approach discussed above to distinguish between these cases. Here, we train the classifier using two sets of samples: a mixed sample containing all target sequences for a given probe (both the matched and mismatched sequences) and samples with only the mismatches present. The same 70/30 train/test split ratio is applied to both sets of samples. Using H = 100 current traces, the ML system achieves reasonable accuracy in identifying the presence of different targets in mixed samples. For the case of the 501Y target, the system correctly identifies its presence with 97.8% accuracy. Similarly, it achieves 99.7% accuracy for the 484K target of 484 probe and 99.7% for the 478K target (Figure 5b).

To test the robustness of the classifier, we systematically vary the number of samples examined (Figure S11). This analysis reveals that by increasing the number of samples, the accuracy of the ML system could be improved to the desired threshold. For example, to achieve 98% accuracy, H = 100 histograms are required for the 501Y case, while H = 50 histograms are sufficient for the 484K and 478K cases (Figure S11). This indicates that even in complex samples, we are still able to positively identify specific VOCs that are perfectly matched with their probes, and to positively identify COVID infections regardless of the variant type. Furthermore, these findings demonstrate the potential of the ML approach to be adapted to emerging cases and to improve the accuracy by adjusting the number of current traces examined. By carefully selecting the appropriate number of current traces, the system can achieve high accuracies even in the presence of other targets in a complex environment. Overall, our ML approach offers fast processing capabilities for sample diagnostics with minimal data collection requirements, once sufficient training data has been generated.

CONCLUSIONS

In conclusion, this study presents the successful utilization of the single molecule break junction (SMBJ) technique as an electrical biosensor for the detection and identification of genetic material related to specific SARS-CoV-2 VOCs. By leveraging the specificity of the RNA sequences relative to the VOCs, we designed specific probes targeting key regions of the virus's genome, enabling the detection of specific mutants. The inherent resilience of the hybrids' conductance values to mismatches allows for differentiation between multiple targets in mixed samples, thus expanding the capability to detect a wider range of mutations. This finding highlights the potential of the SMBJ technique to effectively identify and distinguish between various VOCs in complex sample environments. Furthermore, the study demonstrates the application of the XGBoost machine learning technique to enhance the diagnostic process. By utilizing XGBoost, we reduced the number of current traces required from the SMBJ system to allow accurate identification of the VOCs and broaden the detection capabilities. This reduction in data collection not only streamlines the diagnosis process but also paves the way for real-time applications for SMBJ-biosensors, enabling faster and more efficient detection of SARS-CoV-2 VOCs or other pathogens.

This combined experimental and data analysis approach greatly expands detection capabilities and reduces the possibility of both false negatives and false positives, which is important when attempting to track variants globally. The strategy is of potential use in scenarios where quick and accurate identification of variants is crucial, such as in clinical settings. The ability to detect specific sequences and identify or exclude specific variants with a small number of samples also reduces the time and resources needed for analysis, enabling prompt decision-making and potentially early intervention. Thus, this detection system enables the identification of specific VOCs, the ability to track their spread against previous variants, and has the potential to identify the emergence of new VOCs.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensors.3c02734.

Method for choosing the target regions of the genome, MD simulation method and simulation analysis, sample preparation and SMBJ technique, and extra XGBoost Classification data (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Z.A and C.A.G contributed equally.

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Notes

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