Methods and Applications in Fluorescence

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Single-molecule chemistry. Part I: monitoring oxidation of G in oligonucleotides using CY3 fluorescence

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Abstract

Single-molecule hybridisation of CY3 dye labelled short oligonucleotides to surface immobilised probes was investigated in zero-mode waveguide nanostructures using a modified DNA sequencer. At longer measuring times, we observed changes of the initial hybridisation fluorescence pulse pattern which we attribute to products created by chemical reactions at the nucleobases. The origin is a charge separated state created by a photoinduced electron transfer from nucleobases to the dye followed by secondary reactions with oxygen and water, respectively. The positive charge can migrate through the separated state created by a photoinduced electron transfer from nucleobases to the dye followed by secondary reactions with oxygen and water, respectively. The positive charge can migrate through the hybrid resulting in base modifications at distant sites. Static fluorescence spectra were recorded in order to determine the properties of CY3 stacking to different base pairs, and compared to pulse intensities. A characteristic pulse pattern change was assigned to the oxidation of G to 8-oxoG besides the formation of a number of secondary products that are not yet identified. Further, we present a method to visualise the degree of chemical reactions to gain an overview of ongoing processes. Our study demonstrates that CY3 is able to oxidise nucleobases in ds DNA, and also in ss overhangs. An important finding is the correlation between nucleobase oxidation potential and fluorescence quenching which explains the intensity changes observed in single molecule measurements. The analysis of fluorescence traces provides the opportunity to track complete and coherent reaction sequences enabling to follow the fate of a single molecule over a long period of time, and to observe chemical reactions in real-time. This opens up the opportunity to analyse reaction pathways, to detect new products and short-lived intermediates, and to investigate rare events due to the large number of single molecules observed in parallel.

1. Introduction

Astraphloxin was first synthesized and patented by König at the University of Dresden in the early 1920ies. Later, the dye was re-invented by Waggoner during his search for labelling dyes for biomolecules, and named CY3 [1, 2]. Derivatives of CY3 and the pentamethine CY5 soon became the standard dyes for the emerging microarray technology from the late 1990s, mainly used in two-colour hybridisation (‘red-green’) microarray experiments to measure gene expression of various biological organisms, and many other applications.

The reason for the neglect of the dye for decades is its low fluorescence quantum yield [3, 4]. In the presence of nucleosides, the fluorescence efficiency of CY3 and sulfonated derivatives is increased by interactions that reduce the efficiency of trans-cis isomerisation as the main deactivation pathway of the first excited singlet state [3, 5–10]. Blocking the isomerisation by conjugation with nucleosides and oligonucleotides, proteins, chemical derivatisation, in a solvent of high viscosity, or in a frozen solvent, resulted in an increase of fluorescence lifetime and quantum yield [4, 11–16]. In single-stranded (ss) oligonucleotides, CY3 fluorescence was found to depend on the labelling position, the nature of the linker and the linking nucleotide, and sequence context. When attached to the 3’ or 5’ end, in double-stranded (ds) DNA the dye
interacts by stacking to the terminal base pair. This interaction increases the stability of the hybrid, particularly for short oligonucleotides which is comparable to the effect of an additional base pair [8, 17–20]. Molecular dynamics calculations revealed that the position of CY3 is rather flexible especially when linked to the 3′ end [21, 22].

Interestingly, although isomerisation efficiency was expected to be reduced, stacking of CY3 to a base pair results in a decrease of fluorescence efficiency. It was found that the dye exists in a dynamic equilibrium with a non-stacked conformation [5]. Later it was demonstrated that CY3 blinks on a timescale of μs which makes the dye interesting for single-molecule applications [18, 23]. Whereas the photophysics of DNA-CY3 conjugates is well investigated, the determination of electrochemical properties is impaired by intramolecular proton and charge transfer (CT) processes making it difficult to reliably measure redox potentials [24, 25]. It was shown experimentally and by calculations that base pairing and stacking have a tremendous influence on redox potentials [26, 27]. In consecutive G rich sequences, each additional GG step lowers the oxidation potential by approximately 0.1 eV [28].

All these studies demonstrate that CY3 cannot be regarded as an inert fluorescence label. Interactions with nucleobases have to be taken into account since they inevitably change the behaviour of the system under study. In order to eliminate the influence of dye photophysics in single-molecule experiments, additives were used to reduce the oxygen concentration and to deactivate the dye triplet state as the origin of bimolecular chemical reactions in the excited state [29–31]. For example, in single-molecule real-time (SMRT) sequencing photoinduced oxidation damages the polymerase resulting in a reduced enzyme lifetime and consequently in much shorter read-lengths. This can be avoided by adding an oxygen scavenging system and a triplet quencher [29, 32]. Nevertheless, we were able to show that single-molecule hybridisation of CY3 labelled oligonucleotides can be studied without such additives. In a feasibility study for the use of a DNA sequencer to measure single-molecule hybridisation kinetics we demonstrated that results obtained under these conditions are well comparable with surface plasmon resonance (SPR) data [18]. However, measurements were compromised by two effects. First, the dissociation rate constant was found to be reduced by rebinding due to the presence of a large number of molecules at the chip surface. Second, a prominent second component in single-molecule kinetic data indicated the existence of additional processes. Here we show that this is caused by chemical reactions leading to modified nucleobases.

Nucleobase oxidation was extensively investigated over the last 25 years for its relevance in cellular processes leading to the damage of genomic DNA, which is associated with a number of diseases and ageing [33]. For this reason, a profound understanding of the damaging reactions in detail is of utmost importance. Guanine was identified to be the main target of oxidation by reactive radical species and high energy irradiation [34–36]. Starting in the 1990ies, various electron acceptors covalently linked to oligonucleotides were used in model studies to disclose the electron transfer (ET) properties of ds DNA [37–41]. One important outcome of these early studies was the conclusion that after charge separation (CS) ds DNA is able to transfer charges at high rates over long distances to G, G dimers or trimers where irreversible chemical reactions with water and oxygen take place, resulting in base modifications that are potentially lethal to the cell [35, 42–49].

We introduce a new approach of investigating oxidation of G in oligonucleotides by measuring single molecule hybridisation. We show that G can be oxidised by a photoinduced electron transfer (PET) to CY3 which functions as an electron acceptor. Originating from the charge separated state, a large number of products were found including the formation of 8-oG as a prominent reaction product, followed by subsequent reactions that we assign to the formation of higher oxidation products. Results of single-molecule experiments were substantiated by static fluorescence and SPR measurements. A more detailed analysis of fluorescence traces will be presented in a forthcoming publication, along with the kinetic data.

2. Materials and methods

Measurements were performed in HBS buffer (150 mM sodium chloride, 10 mM HEPES, 3 mM EDTA, 0.05% v/v Tween-20, Teknova, Holister, USA) at 20 °C. Ultra pure and filtered water (‘MilliQ’, Millipore, Billerica, USA) was used for preparation of solutions. Streptavidin and DTT were from Sigma/ Merck (Buchs, Switzerland). D2O was from Acros Organics (Geel, Belgium). PCD, PCA and TSQ, and SMRT cells were received from Pacific Biosciences (Menlo Park, USA). HPLC purified oligonucleotides were from Microsynth (Balgach, Switzerland). The sequences were designed for non-self-complementarity and absence of secondary structures. Probe/analyte pairs were designed such that estimated melting temperature was about 24 °C in order to obtain sufficient hybrid stability. Sequence data are given in table 1.

2.1. Oligonucleotides

The molecular structure of CY3 is shown in figure 1.

2.2. Single-molecule real-time measurements

Single-molecule measurements were performed using a modified RSH DNA sequencer (Pacific Biosciences, Menlo Park, CA). The operating principle was previously described in detail [18, 32, 50]. Biotinylated
oligonucleotides were immobilised on the SMRTcell chip surface which was coated in a first step with streptavidin (0.05 mg ml$^{-1}$ in HBS, 5 min). 45 μl of analyte solution were added, typically at 20–300 nM. Dye fluorescence was excited at 532 nm and monitored in parallel in 150/250 ZMWs over a period of typically 5–25 min at 20 °C which can be extended to 6h. During measurements, the second laser emitting at 642 nm was switched off. Measurements were performed in the absence of dye protecting agents. For control measurements, PCD/PCA and TSQ were added following a protocol provided by Paciﬁc Biosciences. For comparison with aqueous buffer, the stock solution of 20xHBS buffer was diluted (1 + 19) with D$_2$O.

### 2.3. Data evaluation of single-molecule measurements

Data evaluation was described in a previous publication [18]. Pulse width (PW) and interpulse duration (IPD) histograms were evaluated with an in-house developed software using a 1 + 1 and a sum of two exponentials kinetic model, respectively. For time interval analysis, kinetic data for measurements over 25 min were calculated for 100 s time intervals.

### 2.4. Fluorescence measurements

Fluorescence spectra were recorded using a Tecan Safire2 plate reader (Männedorf, Switzerland) with sample volumes of 100 μl in black Greiner 96 well plates. The reader has a spectral resolution of 1 nm. The hybrids of 200 nM CY3-26 and 1 μM of TL derivatives in HBS were excited at 510 nm. Monochromator slits were set to 10 nm/10 nm for excitation and emission, respectively. Spectra were recorded between 530 and 750 nm. For sample preparation, solutions were mixed, heated for 2 min at 80 °C, and allowed to cool slowly in the thermomixer. As a non-matching control, ON34 was used. Mean intensities and the error calculated from three measurements were plotted.

### 3. Results

#### 3.1. Single-molecule hybridisation measurements

Near-field fluorescence microscopy measurements were performed in the absence of a triplet quencher and oxygen scavenger as described previously [18]. Hybridisation in a ZMW nanostructure manifests in pulses characterised by PW, frequency (IPD), and

| Oligonucleotide | Sequence                                                                                           |
|-----------------|----------------------------------------------------------------------------------------------------|
| Bio34           | 5’Biotin-TTTTTGGAAACTGTATTGGCACTGAGTAGACTCC                                                      |
| Bio34G3         | 5’Biotin-TTTTTGGAAACTGTATTGGCACTGAGTAGACTCC                                                      |
| Bio34ap         | 5’Biotin-TTTTTGGAAACTGTATTGGCACTGAGTAGACTCC                                                      |
| CY3-7           | 5’CY3-CAGTGCC                                                                                   |
| CY3-8           | 5’CY3-CAGTGCCA                                                                                   |
| CY3-7T          | 5’CY3-TCAGTGCC                                                                                   |
| BioAT15         | 5’Biotin-ATAATAAATAATAAT                                                                       |
| BioAT15G        | 5’Biotin-ATAATAAATAATAAG                                                                       |
| CY3-TA12        | 5’CY3-ATTATTATTATT                                                                               |
| CY3-TA12C       | 5’CY3-CTATTATTATT                                                                               |
| CY3-26          | 5’CGATCAAGTACAGATCGCGTGCGGG                                                                     |
| TLG             | 5’TCCGGAGGTATGTGTATCGATCGATCGACGTGCAAC                                                         |
| TLoG            | 5’TCCGGAGGTATGTGTATCGATCGATCGACGTGCAAC                                                         |
| TLap            | 5’TCCGGAGGTATGTGTATCGATCGATCGACGTGCAAC                                                         |
| TTL             | 5’TCCGGAGGTATGTGTATCGATCGATCGACGTGCAAC                                                         |
| TLA             | 5’TCCGGAGGTATGTGTATCGATCGATCGACGTGCAAC                                                         |
| TLC             | 5’TCCGGAGGTATGTGTATCGATCGATCGACGTGCAAC                                                         |
| TLU             | 5’TCCGGAGGTATGTGTATCGATCGATCGACGTGCAAC                                                         |
| TLI             | 5’TCCGGAGGTATGTGTATCGATCGATCGACGTGCAAC                                                         |
| ON34            | TTTTTGGAAACTGTATTGGCACTGAGTAGACTCC                                                             |

**Figure 1.** Molecular structure of CY3. The dye is attached to the 5’ phosphate of the oligonucleotide via a C3 aliphatic linker.
fluorescence intensity which defines a specific pattern. PW correlates with the lifetime of the hybrid whereas IPD is determined by the rate of the association step and analyte concentration from which rate constants of dissociation and association, respectively, can be calculated. Classically, these variables can be determined by SPR and static fluorescence spectroscopy. Ideally, for hybridisation of oligonucleotides an undisturbed train of pulses could be expected as shown in figure 3(a). Depending on sequence, hybridisation conditions, the presence of additives, and laser intensity, diverse deviations from a simple pulse pattern were found as well as sequences of multiple pattern changes. As a consequence of the aforementioned effects, calculated rate constants were found to depend on measurement time.

Measurements were evaluated in two ways. In the first analysis, carried out for quantification purpose, IPD and PW were determined from all ZMWs having a single molecule, and rate constants, relative weights and the number of events were calculated and plotted for time intervals of 100 s, a procedure we call time interval analysis. Typically, events from 10000–50000 single-molecules were collected giving rise to up to 200000 events in a time interval. For comparison with SPR data, rate constants calculated for the first time interval were used which were least affected by light induced processes. In the second analysis, visual inspection of at least 500 fluorescence traces was carried out to identify deviations from the original pulse pattern. It should to be noted that the few examples given in this publication represent a selection from millions of fluorescence traces showing similar features. A more detailed analysis of fluorescence traces will be presented in a subsequent publication.

3.1.1. Hybridisation to Bio34 and derivatives
Since the dye used in the experiments is an obvious component involved in the processes leading to the changes in the fluorescence traces, the effect of the stacking base pair was investigated using the oligonucleotide Bio34 as a model binding partner. In Bio34/CY3-7 and Bio34G3/CY3-7, the dye was stacking to G and to a G trimer, respectively, and in Bio34/CY3-7T to a T:A base pair. In Bio34ap/CY3-8, having an abasic site, the dye was thought to stack only to the C to which the dye was linked. Here, an 8nt analyte was needed to detect binding since the affinity of CY3-7 was too low. For illustration, hybrids are depicted in figure 2.

Analysis of fluorescence traces for hybridisation of CY3-7 to Bio34 reveals interesting details. Continuous trains of pulses (figure 3(a)) were found in only 9% of fluorescence traces for which we analysed 500 traces from ZMWs containing a single molecule. Often, deviations from perfect trains of pulses were observed. In half of all trace files (51%), a change from the initially long to short pulses was found (figures 3(b)–(d)), and 42% terminate (figure 3(e)). A remarkable feature was a pulse pattern characterised by an intensity decrease by about 50%, which after about one minute changes to a pattern comprising of short pulses (figure 3(f)).

Time interval analysis, calculated for 100 s time intervals, reveals strongly differing rate constants and relative weights of their components, accompanied by a large decrease of the number of events in aqueous and D2O based HBS buffer (figures 4(a), (b)). The analysis reflects the fact that in the majority of fluorescence traces changes of the initial pulse pattern have occurred. Addition of the redox reagent DT T results in comparable rate constants but fewer terminations (figure 4(c)). The presence of an enzymatic oxygen scavenging system (PCD/PCA) and a triplet quencher (TSQ) strongly stabilises the system (figure 4(d)).

For Bio34G3/CY3-7, fluorescence traces show very similar features as for Bio34/CY3-7 (data not shown). Pulse pattern changes occur much faster as demonstrated in figure 5.

Bio34/CY3-7T behaves similar to Bio34/CY3-7. Differences mainly relate to the extent of specific features found in the fluorescence traces. Continuous pulse pattern were more frequent (22%) and found to feature intensity changes (supplement figure 1 is available online at stacks.iop.org/MAF/8/035010/mmedia), in some cases accompanied by apparent temporal changes of PW and IPD (data not shown). The most frequent pattern changes include the formation of shorter pulses (47%) and termination (29%). Time interval analysis plots are shown in figure 6.

Substituting G of the stacking base pair by an abasic site as in Bio34ap/CY3-8 leads to fluorescence traces mainly unaffected by pattern changes. Here, 70%
Figure 3. Single-molecule fluorescence traces for hybridisation of 100 nM CY3-7 to Bio34. Reaction products are outlined. Reduction of PW indicates a loss of hybrid stability whereas intensity changes are caused by a different degree of fluorescence quenching due to changes in the stacking base pair. (a) Undisturbed train of pulses. (b), (c) Formation of low affinity products due to a reaction in the stacking C:G base pair revealed by changes of PW, IPD, and pulse intensity. (d) Formation of a low affinity product presumably in the distal 5′G dimer of the hybrid. (e) Termination due to formation of a low affinity product characterised by a loss of affinity. (f) Formation of 8-oG (arrow) followed by a secondary reaction creating a low affinity product.
of traces show a regular pulse pattern (data not shown). In particular, very few intensity changes were observed (<5%). Changes of the initial pulse pattern to shorter pulses (10%) and terminations (5%) were much less frequent than for systems in which the dye was stacking to a base pair. The small degree of changes in fluorescence traces is reflected by time interval analysis (figure 7).

3.1.2. Hybridisation to BioAT15 and BioAT15G
Hybrids of AT oligonucleotides are shown in figure 8.
In order to simplify our model system, we investigated BioAT15G/CY3-TA11C with the dye stacking to the lone G in the sequence. As a control, the parent compound BioAT15 was used which is lacking G. Hybridisation of BioAT15/CY3-TA12 mainly produced fluorescence traces showing essentially continuous pulse patterns (75%). Interestingly, there are no large, irreversible intensity changes which were found to be a characteristic feature in systems where the dye was stacking to a C:G base pair. In 8% of the traces, the pulse pattern changes to shorter pulses, and 6% terminate. Time interval analysis shows very similar association and dissociation rate constants, respectively, low contribution of the additional fast
components, and a constant number of events over a period of 25 min (figure 9). For all time intervals, a single association rate constant was found whereas dissociation always contained a second component. The situation is substantially different when a single G is present in the probe sequence. Due to a higher affinity, more frequent and longer pulses were observed for BioAT15G/CY3-TA12C in good agreement with SPR data. Here, only 15% of fluorescence traces show an undisturbed pulse pattern (data not shown). In 50% of the traces, the initial pulse pattern changes to shorter pulses, and 33% terminate.

Figure 5. Time interval analysis of Bio34G3/CY3-7 measured in HBS buffer.

Figure 6. Time interval analysis of Bio34/CY3-7T measured in HBS buffer.
Noticeable are traces with characteristic intensity changes, often associated with sequential pattern changes (supplement figure 2). In agreement with this, time interval analysis indicates large differences of the association rate constant, relative weights of the components, and a strong decrease of the number of events calculated for individual time intervals (figure 10). As in the case of Bio34/CY3-7, addition of DTT results in a strong increase of the number of continuous trains of pulses (70%) at the expense of transitions to shorter pulses (8%) and terminations (8%). Kinetic data of single-molecule hybridisation are summarised in table 2.

3.2. Static fluorescence measurements

Static fluorescence spectra of CY3-26 hybridised with TL derivatives were recorded to investigate the properties of CY3 stacking to different base pairs. Fluorescence intensities were expected to correlate with pulse intensities in single-molecule fluorescence traces. Non-hybridised CY3-26 was used as control, which was also measured in the presence of a non-matching oligonucleotide, ON34. The shapes of hybrid spectra were very similar to CY3-26 showing neither a significant spectral shift nor broadening of the fluorescence band on hybridisation [20].

Figure 11 illustrates the influence of mismatch base pairs, base modifications, and an abasic site on CY3 fluorescence in different sequence contexts. In all cases, the intensity of hybrids was lower than that of the ss oligonucleotide. The most prominent feature observed was a two-fold intensity reduction of TLoG compared with TLG. This characteristic difference can be used to identify the formation of 8-oG in single-molecule fluorescence traces.

4. Discussion

Hybridisation studied by SPR revealed that immobilised oligonucleotides were stable over a period of many hours [18]. We therefore concluded to attribute changes observed in single-molecule fluorescence traces to processes originating from electronic excitation of the dye giving rise to the additional components in the kinetics of single-molecule hybridisation. In particular, the dye initiates chemical reactions that change the nature of the probe molecule under study.

Due to the close proximity of the electronic systems in the hybrid, the stacking dye in Bio34/CY3-7 forms a donor–acceptor system in which a photo-induced electron transfer can proceed [20]. Similar to the scenario pointed out by other authors, charge separation creates a guanine radical cation, G⁺, which can react with oxygen and water [42, 43, 51–53]. Further secondary reactions include intrabase pair proton and charge transfer, the latter oxidising other G in the hybrid, and most likely, also in the overhang giving rise to product formation at distant sites [37, 47, 53–57]. It was shown that after charge separation, the charge proceeds to the closest guanine base with subsequent relaxation to G dimer or trimer which are creating a trap for the positive charge due to their lower oxidation potentials [28, 37, 45, 47, 58]. Thus,
chemical reactions preferentially take place at these sites. Consequently, in oligonucleotide hybrids containing more than one G a greater number of products can be created. Based on own SPR measurements of oligonucleotides having a base modification or a mismatch and literature data, it is safe to assume that these products have different hybridisation kinetics depending on nature and position of the modification created [20, 59–61]. Whereas the analyte is leaving the observation volume of the ZMW after dissociation and escapes further analysis, irreversible reactions in the immobilised probe lead to changes in fluorescence pulse pattern. A reaction scheme is shown in Figure 12. For reason discussed below, the exact energy of the CS state is unknown. However, the state must be located below \( T_1 \) of CY3 in order to allow efficient charge separation.

In single-molecule fluorescence traces, product formation is revealed by irreversible changes of the original pulse pattern. A pulse pattern is characterised by PW, IPD, and pulse intensity, thus representing a train of pulses that escapes further analysis, irreversible reactions in the immobilised probe lead to changes in fluorescence pulse pattern. A reaction scheme is shown in Figure 12. For reason discussed below, the exact energy of the CS state is unknown. However, the state must be located below \( T_1 \) of CY3 in order to allow efficient charge separation.

Single-molecule fluorescence traces show the same features for hybridisation of 3′ and 5′ CY3 labelled analytes, respectively (data not shown).

### 4.1. Hybridisation of Bio34 and derivatives

Surprisingly, only a minority of fluorescence traces of Bio34/CY3-7 hybridisation showed undisturbed trains of pulses (Figure 3(a)). Very frequently, the transition to different pulse pattern was observed that are characterised by short PW and different IPD and pulse intensities, respectively (Figures 3(b)–(d)). We attribute these changes to various products being created by secondary reactions at the primary G donor and the remote G dimer, respectively [37, 47]. If a reaction occurs at the primary donor G, product formation is most likely accompanied by a change of fluorescence intensity as demonstrated by the dependence of fluorescence intensity on the nature of the stacking base pair (Figure 11), and for additional probes containing guanidinohydantoin (Gh) and spirodihydantoin (Sp) modifications (A. Fleming, C. Burrows, J. Sobek, to be published). Examples are shown in Figures 3(b) and (c) where the original pulse pattern changes to short pulses having a smaller and higher intensity, respectively. In contrast, if a reaction takes place at the remote 5′GG dimer, there is no intensity change since after CT the dye is stacking to the unaltered C/G base pair (Figure 3(d)). The varying intensities in the pattern in Figure 3(c) are presumably a result of altered stacking properties of the dye to the created modified base pair, indicating a more flexible stacking conformation. In about half of all traces, the train of pulses terminates within 25 mins as a result of formation of a product that has lost its affinity (Figure 3(e)). The characteristic pulse pattern change in Figure 3(f) arises from formation of 8-oG in the stacking base pair. The assignment is supported by a corresponding intensity change in the fluorescence spectra of CY3-26 when hybridised with TLG and TLoG, respectively, and the similarity of hybridisation kinetics of 8-oG modified oligonucleotides measured with SPR. This feature was observed in all probe/analyte systems in which the dye, attached to the 5′ end, respectively, was stacking to a C/G base pair (supplement Figure 2), but not in pure AT sequences. Further, single-molecule hybridisation using an 8-oG modified oligonucleotide starts with a pulse pattern of low intensity that rapidly changes as a result of the high reactivity of 8-oG compared with G due to a much lower oxidation potential (supplement Figure 3) [63]. The lifetime of 8-oG created was typically on the timescale of a
few minutes. It changes, presumably by further oxidation, to a product characterised by a short pulse pattern (figure 3(f)) or termination (supplement figure 4) indicating the existence of different reaction pathways. Characterisation of reaction products using oligonucleotides modified with oxidation products of 8-oG including Gh and Sp, and the precursor for 2-aminoimidazolone (Iz), 8-methoxydeoxyguanosine, is in progress (H. Sugiyama, K. Kawai, J. Sobek, to be published) [64].

4.2. The role of G as electron donor
The study of PET in acceptor labelled ds DNA revealed that in the presence of G no other bases were oxidised [65]. The positive charge created by charge injection was found to be transferred to distant G, G dimers and trimers [37, 47, 66]. In oligonucleotide hybrids that we investigated there is convincing evidence that a PET from G to the excited dye is the origin of multiple reactions finally leading to the changes observed in single-molecule fluorescence traces. The overall effect can be visualised by time interval analysis as well as by pattern analysis of a larger number of fluorescence traces.

The presence of a single G donor in an oligonucleotide triggers a multitude of pulse pattern changes. This manifests in a dramatic decrease of fluorescence traces containing continuous trains of pulses in favour of traces featuring short pulses and termination, in contrast to the AT sequence, BioAT15. Removing the G from the stacking base pair as in Bio34ap mainly ceases reactions compared to Bio34. The decrease of the donor oxidation potential by a G trimer that increases the free energy of PET has the opposite effect as observed in Bio34G3 compared with Bio34, and in oligonucleotides having an 8-oG modification [28, 67]. Bio34/CY3-7T shows a distance dependence for the dye stacking to a T:A base pair adjacent to the G donor. Shifting the primary donor by one base pair doubles the number of continuous trains of pulses compared with Bio34/CY3-7. The larger distance to the donor G reduces the electronic coupling between excited dye and G thus decreasing the probability of PET. Finally, results of SMRT sequencing after hybridisation with CY3-7 revealed that chemical reactions mainly occur at the distant G dimer within the hybrid which is substantiating the proposed reaction scheme.

It is important to note that the AT sequence, BioAT15, shows similar but much less frequent pulse pattern changes compared with BioAT15G, except for the lack of the characteristic intensity reduction attributed to the formation of 8-oG. The lower reactivity of A and T arises from their higher oxidation potentials compared with G. It further implies that double strand formation gives rise to a sufficiently large gain of free energy to enable the oxidation of A and T, respectively [65, 68].

4.3. Time interval analysis
Since a single fluorescence trace is only of limited significance, measurements gain validity by comparing features found in a larger number of trace files. The particular strength of the RSH+ lies in the more than 10000 single molecules typically immobilised on a chip which facilitates the analysis, particularly of rare
Table 2. Kinetic data from SMRT measurements calculated from the first 100 s time interval. The standard deviation is given in parenthesis.

| Probe      | Analyte | IPD Weight | PW Weight | $k_{on}/10^9\text{ M}^{-1}\text{s}^{-1}$ | $k_{off}/10^9\text{ M}^{-1}\text{s}^{-1}$ | $k_{on}/\text{s}^{-1}$ | $k_{off}/\text{s}^{-1}$ |
|------------|---------|------------|-----------|--------------------------------------|--------------------------------------|------------------------|------------------------|
| Bio34      | CY3-7   | 1          | 0.56      | 15.82(30)                            | —                                    | 0.0875(24)             | 1.11(9)                |
| Bio34      | CY3-7T  | 0.69       | 0.79      | 11.38(53)                            | 24.25(280)                           | 0.213(4)               | 0.868(102)            |
| Bio34G3    | CY3-7   | 0.78       | 0.59      | 9.93(47)                             | 21.89(100)                           | 0.0778(2)              | 0.771(39)             |
| Bio34ap    | CY3-8   | 0.85       | 0.83      | 14.55(37)                            | 41.75(570)                           | 0.260(4)               | 1.57(36)              |
| Bio15AT    | CY3-TA12| 1          | 0.88      | 3.43(5)                              | —                                    | 0.150(3)               | 0.863(226)            |
| BioAT15G   | CY3-TA12C| 0.61       | 0.74      | 4.11(26)                             | 9.96(116)                            | 0.0994(2)              | 0.946(54)             |

Figure 10. Time interval analysis of BioAT15G/CY3-TA12C measured in HBS buffer.

Figure 11. Fluorescence intensity of CY3-26 and hybrids with TL derivatives (TLX). Non-matching ON34 was used as a negative control.
events. On the other hand, calculation of rate constants from PW and IPD for short time intervals permits to gain a global view of ongoing processes. As described previously, calculations are based on a sum of two exponentials kinetic models [18]. The slow components of IPD and PW arise from the association and dissociation step, respectively, and refer to the original pulse pattern of hybridisation. The additional fast components are caused by a number of processes due to dye effects and in particular chemical reactions proceeding after charge separation which typically result in a loss of affinity. In the sum of two exponentials models, all additional processes and products created are described by a single rate constant, $k_{on}$ and $k_{off}$, respectively. Calculated rate constants were found to be time dependent as a result of irreversible chemical reactions changing the hybridisation kinetics of the immobilised molecules. Depending on the number and the amount of products accumulating in the course of a measurement, different rate constants and weights were calculated for time intervals. The differences of rate constants over time intervals were caused by the kinetic model which is not suited for the evaluation of PW and IPD data affected by product formation except for a short period at the beginning of a measurement. Strong variation of the calculated rate constants and large error bars demonstrate excessive variances due to formation of different products which cannot be adequately reproduced with only two fit components. Changes of the number of counts are mainly caused by reactions leading to a strong loss of affinity that terminates a pulse pattern. A comprehensive quantitation would require a kinetic model taking all products into account which is not feasible for the large number of products and the fact that calculations would not converge. Therefore, for a comparison with SPR, kinetic data are only meaningful within a limited period of time which comprises the first time interval of 100 s. This period was chosen such that rate constants are not significantly affected by proceeding reactions but must be sufficiently longer than the average PW and IPD. In a previous publication, rate constants were calculated for measurements over 5 min and found to agree well with SPR data [18].

Results show that time interval analysis is a valuable tool to visualise the extent of changes of PW, IPD, and the number of events in different probe/analyte systems and in measurements conducted under different conditions. For example, time interval analysis allows us to visualise differences in reactivity caused by the presence of a single G in the sequence (figures 9, 10). The effect of solvent ($\text{H}_2\text{O}$, $\text{D}_2\text{O}$), additives including DTT and an oxygen scavenger/triplet quencher mixture (PCD/PCA/TSQ) was clearly demonstrated (figures 4(a)–(d)). It has to be noticed that time interval analysis is semi-quantitative. The analysis does not take into account the effective irradiation time as a function of affinity and analyte concentration as well as spectral properties in the case of comparing measurements with different dyes.

4.4. Preconditions of a photoinduced electron transfer

According to Marcus theory, the preconditions for an efficient electron transfer are a negative Gibbs free energy and electronic coupling [69]. Electronic coupling is determined by the overlap of wave functions of donor and acceptor states which can be reached by a through-space interaction due to close contact of their electronic systems. This is ensured by the dye stacking to a base pair as shown in NMR measurements of a ds oligonucleotide with CY3 and CY5 attached at blunt ends [70]. For 7 nt oligonucleotides, stacking was found to decrease $k_{off}$ by an order of magnitude compared to the non-labelled compound which refers to a stabilisation by 7–8 kJ mol$^{-1}$ as calculated from affinity measurements [18, 20]. Single-molecule dissociation rate constants indicate that stacking interactions also exist in the excited state.

The Gibbs free energy of electron transfer, $-\Delta G_{\text{ET}}$, can be calculated by means of the Weller equation [71]. Whereas the dye excitation energy can be obtained from optical spectra, determination of redox potentials for oligonucleotides by classical cyclic voltammetry is impeded by proton and CT processes.

![Jablonski diagram](image)

**Figure 12.** Jablonski diagram (energy level not drawn to scale) to visualise processes after light excitation of CY3. The oligonucleotide sequence is reduced to the G to which the dye is stacking. ISC: intersystem crossing, CS: charge separation, CR: charge recombination, F: fluorescence.
and subsequent chemical reactions leading to irreversible electrochemical cycles. As an estimate, the oxidation potential of the free nucleosides was used for which a slightly positive free energy was calculated [24]. However, base stacking and hydrogen bonding, dye conjugation and stacking as well as stabilisation by secondary reactions were shown to have a strong effect on redox potentials [27, 72, 73]. Experimental data showed that C:G base pair formation reduced the oxidation potential by 0.34 V [26, 27]. Calculation of the structures and stability of base pair cations revealed a specific effect stabilising the G⁻:C pair by 0.75 eV as a result of the contribution of a proton shifted resonance structure [27]. This structure is in agreement with later findings of an intrabase proton transfer [73].

On the basis of these data, the energy difference between excited state of the dye and the charge separated state should be sufficiently negative to enable PET as the first step of our proposed reaction sequence.

4.5. Product formation

Product formation originating from G⁺ has to compete with fast charge recombination (CR) that rapidly recovers the initial state [69, 74, 75]. The fact that subsequent reactions can efficiently compete with back electron transfer suggests that additional fast processes must exist that increase the lifetime of the charge separated state [76]. A possible reaction is that of the CY3 anion with oxygen as a good electron acceptor being present at a concentration of about 220 μM in buffered solution (R. Meyer, PreSens, Regensburg, Germany, personal information). Since deactivation of excited singlet states is too fast, this process can only be efficient in the dye triplet state [10, 74, 77]. The quantum yield of triplet formation for sulfo-CY3 was determined to be 0.03 [10]. The contribution of the triplet state in the reaction sequence shown in figure 12 can be demonstrated by time interval analysis of Bio34/CY3-7 carried out in the presence of a triplet quencher (figure 4(d)). TSQ efficiently deactivates the dye triplet state and thus strongly reduces products formation.

Further, intramolecular CT from remote G can create a long distant CS state [47, 66]. Due to a much slower CR, chemical reactions at distant sites become more likely [49]. CT was shown to proceed on the ns and ps time scale depending on the number of bridging nucleotides, the redox properties of the donor, the bridging A-T base sequence, and the presence of mismatches [66, 78–83]. From the observation of product formation in probes having a single G we conclude that the (triplet) CS state must be living long enough even for relatively slow reactions to proceed in agreement with experimental findings, which demonstrate that 8-oG formation in ds DNA is very efficient [52]. Likewise, results of SMRT sequencing support the idea of CT to the distal G dimer followed by irreversible chemical reactions. The slow components of IPD and PW arise from the association and dissociation step, respectively, and refer to the original pulse pattern of hybridisation. The additional fast components are caused by a number of processes due to dye effects and in particular chemical reactions proceeding after charge separation which typically result in a loss of affinity.

The reaction scheme is complicated by the observation that the guanine radical is not a cation but a neutral species [54, 73]. Owing to the low pKₐ, in ds DNA a proton transfer to C in the base pair occurs creating G(-H):C(+H)⁺ in an equilibrium with G⁺ [54]. This equilibrium also plays a role in CT processes since the hole hopping process is coupled to proton transfer reactions [58]. G⁺ reacts slowly with water giving rise to the formation of 8-oG [83, 84]. The neutral radical does not react with oxygen but with superoxide radical anion, O₂⁻ [85–87]. Since there is a clear oxygen dependence of product formation, we suggest that the reactive species in our reaction sequences is O₂⁻ created by the reduction of O₂ by the triplet state of CY3 or CY3 anion.

4.6. The effect of oxygen

Dissolved oxygen generates a series of effects affecting both the dye and the nucleobases [77, 88–91]. In single-molecule fluorescence measurements the absence of oxygen is a mandatory precondition in order to work under stable conditions. One reason is the formation of reactive oxygen species (ROS) which typically originate from bimolecular reactions in the triplet state [31, 92, 93]. This is usually prevented by the addition of an oxygen scavenger and a triplet quencher [29, 30, 91]. In particular, triplet states are known to sensitize the production of singlet oxygen, ¹O₂, which can react with cyanine dyes and nucleobases [90, 94, 95]. Further, electron transfer from dye triplet state was shown to create superoxide radicals. Downstream reactions create further ROS including hydroxyl und superoxide radicals [77]. The formation of ROS facilitates type I and II photooxidation including the oxidation of G to 8-oG via [4 + 2] cycloaddition, and subsequent reactions [63, 95–97]. Under reductive conditions, the formamidopyrimidine FapyG can be created by reaction of G with hydroxyl radicals [98]. It was also demonstrated that reaction of G⁺ with superoxide can create singlet oxygen [51]. The variety of reactions of ROS with nucleobases is reflected by the large number of reaction products identified by their pulse pattern.

The effect of singlet oxygen can be investigated in D₂O as a solvent. The decrease of radiationless deactivation in D₂O increases the lifetime of triplet states and singlet oxygen [99, 100]. Therefore, ET and bimolecular reactions with oxygen and other reactive species become more likely. Time interval analysis for Bio34/CY3-7 measured in H₂O and D₂O,
respectively, reveals a number of interesting findings. First, rate constants of hybridisation, $k_{1\text{on}}$ and $k_{1\text{off}}$, for the first time interval are not affected in D$_2$O, in the presence of DTT, and an oxygen scavenger/triplet quencher system (PCD/PCA, TSQ) (figures 4(a)–(d)). Second, the fast component of the dissociation rate constant is also not affected. This component contains all additional processes taking place in the hybrid including product formation and dye effects. This led us to conclude that singlet oxygen does not significantly react with the dye which is consistent with a similar finding for the oxidation of CY3 [77]. In contrast, the large error of the association in D$_2$O suggests the formation of a greater number of products presumably by reaction with G in the probe sequence. The enhanced creation of ROS along with the triplet charge separated state which is expected to live longer in D$_2$O gives rise to the enhanced product formation that is observed during the further course of the measurement.

The reduction of oxygen concentration results in a general stabilisation of the system under study. The presence of DTT increases the number of continuous trains of pulses and reduces terminations. At low oxygen concentration, reaction with water becomes competitive revealed by an increase of formation of 8-oG at the expense of formation of low affinity products. The use of an enzymatic oxygen scavenging system in combination with a triplet quencher ceases most reactions which underlines the importance of oxygen and the dye triplet state for the formation of reaction products.

4.7. Fluorescence quenching
Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample [101]. Due to the close proximity of the electronic systems of dye and nucleobases, stacking interactions with a terminal base pair result in the formation of a weak ground state complex that manifests in a hybrid stabilisation by 7–8 kJ mol$^{-1}$ [20]. As discussed above, the redox properties of nucleobases dramatically change upon base pairing and stacking. Therefore, nucleobases in ds DNA are much better electron donors and dye stacking to a base pair has two opposite effects on fluorescence efficiency. First, a stronger blocking of the trans-cis isomerisation, and second fluorescence quenching. Which effect prevails is determined by details of complex formation including the linkage between dye and nucleobases, the number of sulfonic acid groups in the dye, and the sequence context [3, 5, 15, 102].

The observed dependence of fluorescence quenching on the donor oxidation potential in a series of structurally similar purine nucleobases (TLI < TLA < TLG < TLOG) and results from single-molecule measurements led us conclude that fluorescence quenching happens via ET in the excited state represented by CS in figure 12 [20]. The concept of electron transfer quenching was used to explain an additional fluorescence lifetime component in a hybrid of a thymidine labelled oligonucleotide, and the reduced fluorescence intensity in fluorescein conjugated oligonucleotides [103, 104]. The data in figure 11 suggest that there is a contribution of static fluorescence quenching, which is driven by hydrophobic and π-stacking interactions in ground state complexes [105]. An interesting observation relates to the behaviour of TLIap. The quenched fluorescence suggests that the dye is interacting with adjacent nucleobases. Further investigation of these effects is in progress.

4.8. Investigation of other dye labels
Hybridisation experiments of Bio34 were also conducted with derivatives of CY3–7 including CY3B, DY547, and rhodamines including ATTO532 and ATTO550 (data not shown) [18]. Basically, fluorescence traces show comparable features with regard to IPD, PW, and intensity changes. It can be concluded that similar reactions proceed after electronic excitation as for CY3 [106]. SPR data of Bio34/dye-7 also demonstrate the stabilising effect in comparison to the unlabelled oligonucleotide (data not shown). This confirms results of other authors stating that stacking is an intrinsic property of cyanine dyes [8, 102, 107]. Our findings support the assumption that chemical reactions that proceed after light excitation are a general property of dyes that can function as electron acceptor and are able to create ROS. The results of our study are in line with the large number of DNA systems investigated which utilize a covalently linked acceptor for charge injection including rhodium and ruthenium complexes, stilbenes, anthraquinones, and others [38, 49, 75, 108–111].

5. Conclusions
Experiments conducted with the RSII + sequencer are a rich source of information about chemical and dynamic processes taking place in single molecules in a time range which is not easy to access experimentally. We have shown that CY3 attached to the 5’ end (and also to the 3’ end) of ss DNA functions as an electron acceptor that is able to oxidise nucleobases. The reason is the special conditions in ds DNA, which strongly alter the redox properties of the nucleobases on base pairing and stacking. Our data indicate that this provides a sufficient driving force for photoinduced electron transfer from triplet state enabling the oxidation of G and also pure AT sequences. Reaction products can be identified by comparing kinetic data obtained from PW and IPD analysis, respectively, with SPR data using oligonucleotides that carry those base modifications which are expected to be created in the reaction sequence, and through analysis of static fluorescence spectra.
The findings of this study answer questions raised in a previous publication [18]. The additional components in kinetic hybridisation data are caused by processes initiated on electronic excitation of the dye and a PET, followed by chemical reactions originating from a CS state. This leads to the formation of base modifications that change the hybridisation kinetics of the immobilised probe. Reaction products can be analysed by a comparison with data obtained through SPR and static fluorescence spectroscopy as shown on the example of 8-oG. Time interval analysis allows to estimate the extent of chemical reactions in the probe on a global scale. The opportunity to monitor the fate of some ten thousand of isolated molecules over a long period enables us to follow sequences of chemical reactions and reaction pathways, including the determination of product yields.

The strongly enhanced donor properties of nucleobases in ds DNA affect the fluorescence efficiency of the stacking CY3. The concept of an enhanced fluorescence quenching upon stacking to a base pair is a suitable approach to explain the particular behaviour reported in the literature. Due to the sensitivity to the environment, fluorescence of CY3 and other dyes allows to monitor chemical reactions proceeding in the stacking base pair and the adjacent nucleotide in the overhang.

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Competing interests

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