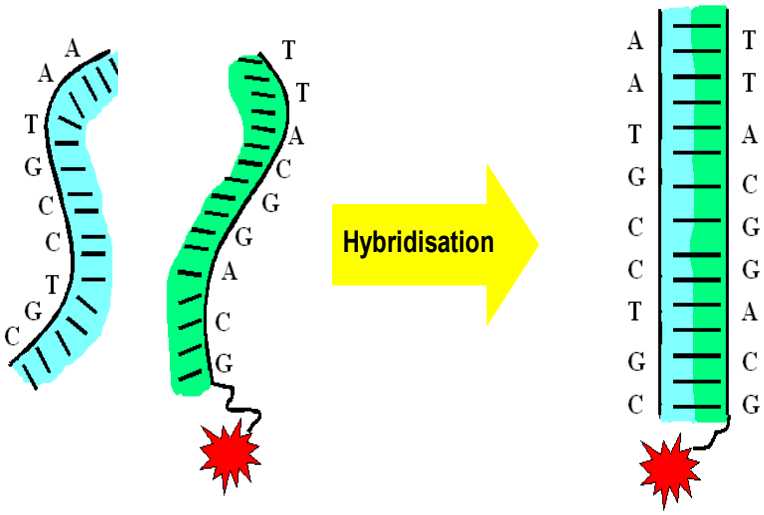
DNA Oligonucleotide Hybridisation

Automatic Detection of Fluorescence Lifetime with ULTRA Evolution



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Hybridisation of two complementary DNA single-strands into one double strand is a fundamental biochemical reaction. The extent of this reaction can be inhibited or modulated by modifications of one of the binding partners (e.g. single nucleotide polymorphism) or by adding extraneous chemicals or proteins.

Within this technical note we utilise the well understood hybridisation reaction as a model system to demonstrate how to use Fluorescence Lifetime as a signal for monitoring (bio-)chemical reactions. Elsewhere we laid out the theoretical and technical basis for lifetime experiments (ref 1). In short, the light emitted from fluorescent labels is characterised by several parameters, among which one is the Fluorescence Lifetime. This parameter is highly susceptible to changes in the immediate physical environment of the label. When this environment changes during a chemical reaction, then the lifetime determined will change accordingly. Hence, fluorescence lifetime can be used to monitor such reactions. A prerequisite is a suitable label and it's positioning on one of the reaction partners, and technical means of reading the parameter, fluorescence lifetime.
Tecan's new ULTRA Evolution microplate reader with FLT option allows the automatic determination of fluorescence lifetime parameters. We have chosen complementary 15-mer oligonucleotides, one of which was conjugated on its 3'-end with a red-absorbing fluorescent label, TAU670. The following detailed discussion of the data reduction process aims to provide practical assistance into (i) how to retrieve as much information as is available from one measurement point; and (ii) how to assess the quality of this information.

1. Data analysis

Fluorescence lifetime measurements performed on the ULTRA Evolution result in a multitude of parameters. But where is the information

about the sample? Elucidating this is the subject of the following paragraphs.

The primary data as retrieved from each sample is a fluorescence decay curve, an example of which is shown in Figure 1. The decay curve can be understood as a measure of how many excited molecules are present in the sample. After a short flash has excited a large number of fluorescent labels, the latter will eventually release their excitation energy and return to the ground state. The time this takes for each individual molecule cannot be predicted. On average, the molecules remain in the excited state for a time span that is referred to as the *fluorescence lifetime*. The complete decay curve represents the temporal course of this statistical process.

The information about the sample is contained in the shape of the decay curve.

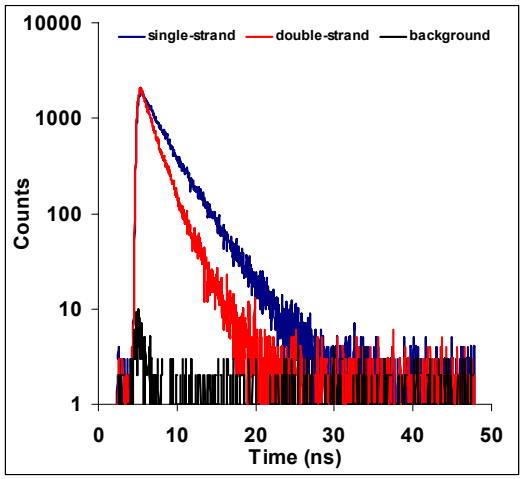

Figure 1: A typical FLT curve. The labelled single-stranded oligonucleotide is characterised by a longer lifetime (less steep decay) as compared to its double-stranded form. As a control, the fluorescence decay curve of the blank is included in the plot.

In order to retrieve the information in one numerical value, mathematical models are used and automatically applied by the ULTRA Evolution's control software. Initially, this results in the aforementioned multitude of parameters. In reference 1 the meaning and relevance of those parameters are discussed. We will now apply the various theoretical models to our test system of oligonucleotide hybridisation.

Figure 2 shows a schematic titration curve of the hybridisation event. When adding increasing amounts of complementary single-strand oligonucleotide2 (referred to as oligo2) to a set amount of labelled oligonucleotide1 (oligo1), more and more double-strand DNA will result. In general, each sample contains three species, the single-strand labelled oligo1, the single-strand oligo2, and the labelled double-strand DNA. Obviously, both of the labelled species contribute to the fluorescence signal and therefore, the shape of the fluorescence lifetime decay curve of any sample must represent the mixed population.

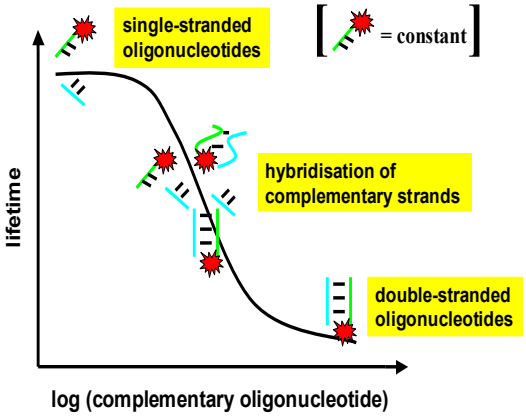

Figure 2: DNA hybridisation. Detection is based on the change of fluorescence lifetime of the label.

1.1. The mono-exponential fit

Despite the fact that more than one lifetime component must be present in the decay curve, it is worth trying to use the simplest mathematical model possible, the mono-exponential model. Here the shape of the curve is distilled into one parameter, the mono-exponential lifetime. In a semi-logarithmic plot, like the one shown in Figure 1, the mono-exponential lifetime is the slope of a linear regression through the decay curve. Even if the curve itself is bent, this very simple description will yield some information about the general shape of the decay curve. In practise it turns out that this simplistic description often is good enough to yield the information about the chemical state of the sample. Furthermore, the determination of this single lifetime is usually extremely reproducible, with relative errors of far below one percent.

N.B. Comparing this straightforward method with other techniques like Fluorescence Intensity or Fluorescence Polarisation shows immediately the advantages: in those integrative measurement modes one cannot distinguish between the different signal sources. Here too, the sample will contain a mixture of two species. Only an average figure can be generated. And yet, the single figure produced can be used to monitor the chemical state of the sample. In FLT the deviations of the decay curve data points from the linear regression yield the information about the state of the mixture.

Figure 3 shows a binding isoterm utilising the mono-exponential lifetime as the signal. The lifetime value changes from just under 3 ns to 1.8 ns, thus giving a dynamic range of 1.2 ns. As mentioned above, the relative error on the lifetime readings are extremely small, in this case around 0.4%. Assessing the assay by the z'-value it turns out very high, at 0.95. This was achieved at a label concentration of 6 nM and 1 second integration time per sample well. The dependencies on both, label concentration and readtime, will be discussed later.

1.2. The bi-exponential fit

A bi-exponential model tries to resolve the individual components stemming from each subspecies in the mixed population. The shape of the curve in this case is described by four parameters, two lifetimes, τ_1 and τ_2, and two corresponding partial amplitudes, α_1 and α_2.

Trials to use any one of these four parameters as a signal indicating the chemical state of the sample fail. This becomes most apparent when looking at the z'-values, which lie between -1 and 0.3. Negative z'-values mean the free and the bound states are indistinguishable, and only values above 0.6 make for a robust and acceptable assay.

Consequently, this necessitates the combination of all four parameters to retrieve the full information. Generating a single numerical value representing the chemistry, three possibilities arise: (i) the average lifetime $<\tau>$ truly combines all four parameters, whereas the relative amplitudes either (ii) require *a priori* knowledge about the lifetimes of the subspecies or (iii) discard some information. All three ways of data interpretation will be presented in detail below.

Average Lifetime

Averaging the two lifetimes from a bi-exponential fit according to their specific weight yields the Average Lifetime. A weighted averaging is necessary as easily shown in a hypothetical example. Assume a situation where two subspecies and therefore two lifetime components contribute to the decay curve. Both subspecies shall emit about equal amounts of photons (i.e. have similar intensities). Contrast this situation with one where the two lifetimes are the same as before, but the contributions are such that one subspecies produces 90% of the light. Obviously the averaging must not yield the same value in both cases.

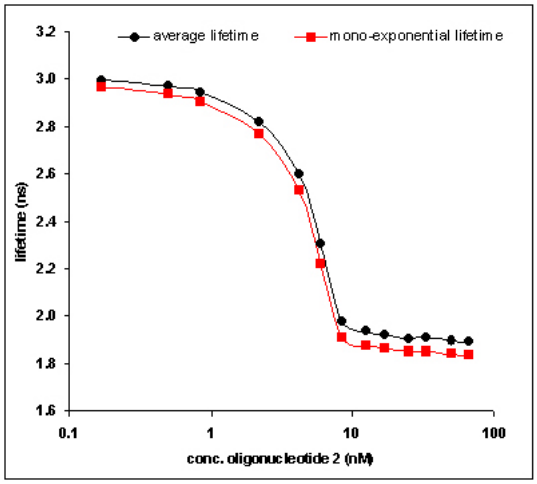

Figure 3: Mono-exponential fit versus average lifetime $<\tau>$

Figure 3 compares the binding isoterms generated from one data set, i.e. from one set of decay curves. Mono-exponential lifetimes as well as the average lifetimes were determined. The binding curves look very similar to each other in shape as well as in position. Both signals apparently monitor the same physical change of the label's microenvironment during the hybridisation reaction. Again, relative errors and the z'-value (0.94) were excellent.

Relative Amplitudes with *A Priori* Knowledge

In the case when the two subspecies can be described by a mono-exponential lifetime each, the knowledge of these two limiting lifetimes can be used in the bi-exponential fit. Any decay curve resulting from a mixed population must be an overlay of the two lifetime components, varying only in the partial amplitudes. The relative amplitudes then reflect directly the relative concentration of the subspecies in the mixture.

Figure 4 shows both of the relative amplitudes, one belonging to the single-strand oligo1, the other to the double-strand product. Again, the shape of the binding isoterm as well as its position resembles the previously described curves. The two limiting lifetimes assumed were 1850 ps for the double-strand DNA, and 3000 ps for the single-strand oligo1. The z'-value of 0.95 indicates the same high quality of data. In essence, despite using only two parameters (amplitudes α_1 and α_2) out of the full set of four to describe the shape of the decay curve, the added information about the limiting lifetimes allows for a precise determination of the binding curve.

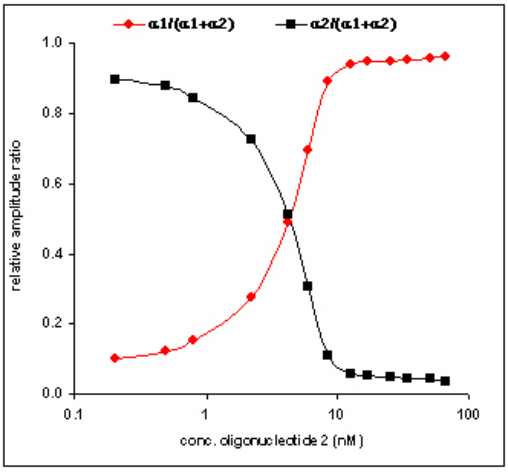

Figure 4: Hybridisation based on the analysis of the relative amplitude ratio. The bi-exponential data set is used.

Relative Amplitudes

What happens if this pre-knowledge is not available or is not used? Above it was mentioned that trying to resolve the binding event from single parameters of the bi-exponential fit yields very poor data. Using those same parameters but calculating the relative amplitudes, means combining two primary parameters in order to get the signal. The information content that is held in the lifetimes is disregarded and therefore lost. Still, binding curves can be retrieved, even though at a lower quality as indicated by a z' of 0.54.

1.3. Conclusion

The biochemical hybridisation reaction can be monitored by fluorescence lifetime. Various "signals" can be calculated from the primary parameters. As long as all the information available is used, extremely reliable and robust ways of describing the chemical status of the sample are provided. For the actual biochemical example discussed in this technical note, all three methods -- mono-exponential lifetime, average lifetime calculated from a bi-exponential fit, and the relative amplitudes for fixed lifetimes -- return similarly good results. In a different scenario, that is a different biochemical system, one or the other signal may turn out better suited to monitor the reaction.

2. Data aquisition

The data analyses discussed above were applied to one data set obtained at a fixed label concentration and measurement time per well. How does a variation of these two assay parameters affect the data quality?

In assay development the concentration of the labelled binding partner must be chosen such that firstly the biochemical requirements are fulfilled (typically one aims for concentrations at least ten times below the affinity of the binding reaction), and secondly the assay performance is sufficient, i.e. the obtained data quality yields a z' of above 0.6 or better.

On the other hand, even at a given label concentration, there will be small variations of this due to pipetting errors, evaporation, adsorption to the plate, etc. In some assay formats, like in Fluorescence Intensity, the small variations will distort the final data. How does Fluorescence Lifetime cope with it?

2.1. Effects of label concentration

While the so far discussed data where obtained from samples of 6 nM labelled oligo1, we now show the dependence of the data quality upon varying this concentration between 600 pM and 12 nM. Binding isoterms of four experiments are shown in Figure 5. Their appearance is very similar in terms of shape, dynamic range, and noise. Only the position of the binding curves shifts, and the mid-point resembles closely the concentration of the labelled binding partner.

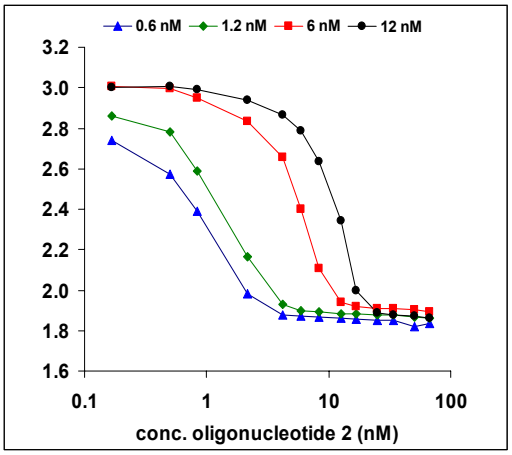

Figure 5: Hybridisation curves for different concentrations of labelled oligonucleotide 2.

N.B. The hybridisation event can be regarded as quasi-irreversible second order binding kinetics. Such reactions will appear as having an extremely high affinity. The observed affinity as measured by the mid-point of the binding isoterm must be called *apparent affinity,* since it only mirrors the concentration of the fixed binding partner.

Figure 6 gives an impression of the data quality obtained. Even at the lowest concentration tested the z'-value already reaches 0.94.

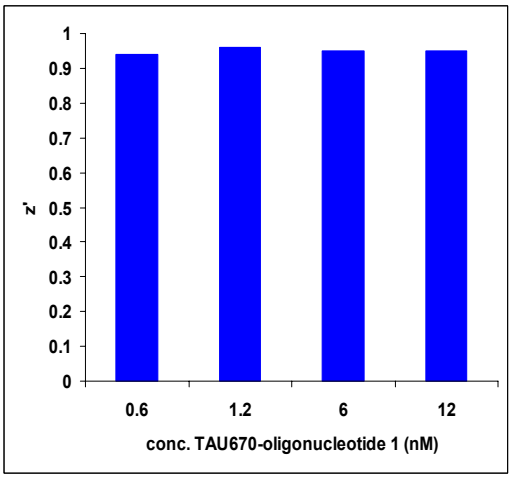

Figure 6: Robustness of FLT measurements against label concentration.

In summary, the fluorescence lifetime signal itself does not depend on the label concentration. This is a result of the fact that it monitors an intrinsic property of the label and not the number of labels in any given state. Hence, Fluorescence Lifetime as an assay readout is highly robust against any changes of the label concentration. Furthermore, even at relatively low concentration it yields an excellent data quality.

2.2. Effects of measurement time per well

In Time-Correlated Single Photon Counting (TCSPC) the total number of photons counted is proportional to the measurement time. The higher this number is, the smoother the decay curve will be (Figure 7), and the more reliable is the mathematical fit to the data (see reference 1). Therefore there is a correlation between the measurement time per well and the data quality. For HTS, measurement times longer then 100 ms or 200 ms are impractical. We have tested various measurement times between 25 ms and 10 seconds per well. The longer times were included in order to determine the methods limits, and the shorter times to demonstrate its HTS capabilities.

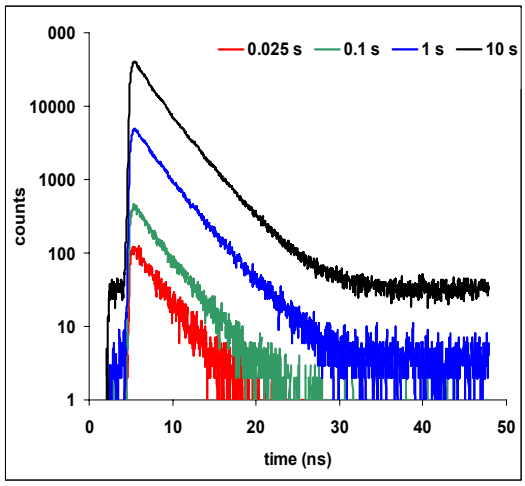

Figure 7: FLT decay curves versus measurement time.

Figure 8 shows a comparison of the obtained z'-values for various acquisition times. The base for this is a mono-exponential lifetime determination. Even at the shortest times tested the precision of the data is already very close to the optimum.

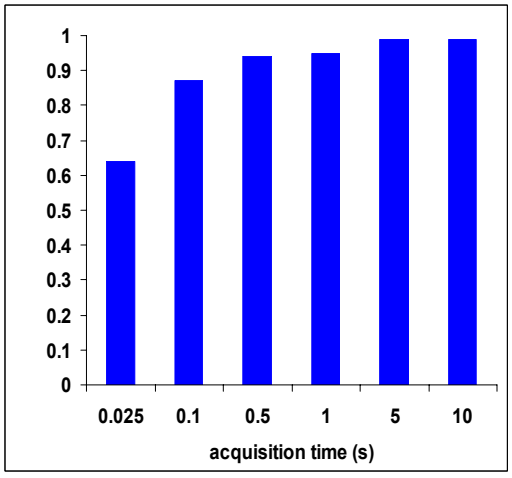

Figure 8: z'-factor versus acquisition time. TAU670-oligonucleotide 1 was used at concentration of 6 nM.

For any given assay situation, optimising the data acquisition process in terms of read time and label concentration can be carried out as demonstrated here. Fluorescence Lifetime offers an outstanding performance allowing rapid measurements at low label concentrations.

3. The z'-value

In any assay situation the question arises whether a particular test well produces a signal change. Negative and positive controls commonly are employed to determine the maximum signal change, which is called the dynamic range of the assay readout. Replicates of the control samples will yield information about the precision of the signal determination. Only if the signal change is considerably larger than the standard deviations of the control signals, only then the assay readout is suitable to monitor the reaction scrutinised.

The so-called z'-value was developed (2) in order to have a simple way of assessing the suitability of data. Unlike other simple statistical parameters, e.g. signal-to-noise ratio, or signal-to-background ratio, the z'-value combines the dynamic range and the standard deviations of the control signals. It is defined as

$$z' = 1 - \frac{3 \times (\sigma_{high} + \sigma_{low})}{\tau_{high} - \tau_{low}}$$

where σ denotes the standard deviation, and the subscripts high and low, respectively, denote the control signal levels. For HTS applications, a z'-value of greater than 0.6 is frequently required.

4. Materials and methods

Experiments were performed with appropriate diluted solutions of TAU670-oligonucleotide 1 and complementary oligonucleotide 2 in PBS buffer, pH 7.4 with 0.01% TritonX-100. Both oligonucleotides were synthetic 15-mer DNA oligonucleotides, oligonucleotide 1 was 3'-aminomodofied prior to labelling with NHS-activated TAU670 label. The measurements were started after incubation for 30 min at room temperature and were performed on Tecan's ULTRA Evolution in lifetime mode (Ex/Em =635 nm/680 nm). If not stated otherwise, acquisition times of 1 s and laser repetition rate of 20 MHz were applied for data collection. For z' calculation, the ten repeated measurements were taken.

5. Conclusion

Tecan's multifunctional micro-plate reader ULTRA Evolution now offers an optional Fluorescence Lifetime mode. The exemplary biochemical reaction of oligonucleotide hybridisation is a model system that allows to the capabilities and benefits of this new detection technique to be visualised:

- high-content data generation;
- extremely reproducible;
- independent of many known assay interferences.

This Technical note is the third in a sequence of three notes describing the new technique Fluorescence Lifetime.

References

(1) Technical note "Fluorescence Lifetime Measurements: an Introduction to the technique", Tecan Austria, 2003

(2) Zhang et al., *J. Biol. Screening*, **4**, 67 (1999)

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