# Utilizing Blinking Dynamics for Multiplexed Super Resolution Imaging

A thesis submitted in partial fulfillment of the requirement
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by

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Accepted

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# Contents

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**Abstract**

We are looking at a novel approach to multiplexed optical imaging. This method will utilize the unique blinking patterns of single molecule emitters. To do this, single molecule microscopy is used to quantify the variation in blinking dynamics of two emitters with the same spectral emission, Rhodamine 6G (R6G) and Quantum Dots (QD). When single molecules are under constant excitation they fluctuate between emitting and non-emitting events, creating a pattern defined as blinking. It has been shown that there are consistent trends in the blinking unique to emitters. The change point detection method (CPD) was used to quantify the blinking variables that best described the unique characteristics of the emitters' blinking patterns. Distinctive blinking dynamics were combined to create a new metric (M) to identify unknown emitters based solely on their blinking. Metric $M$ was able to correctly identify 92.4% of the known R6G and QD. $M$ was tested by using it to identify emitters based on blinking in three mixed sample dilutions. The dilutions of R6G:QD were 30%:70%, 50%:50%, 70%:30%. Through analyzing the data from the blinking traces with our metric the dilutions for these data sets was found to be: 29.25%:70.75%, 51.03%:48.97%, 66.06%:33.94% respectively. Thus, our created metric is accurately able to capture the identity of the emitters based solely on the blinking statistics.

Our research is now focusing on applying the proof of concept of blinking based multiplexed (BBM) imaging to a medium that is more applicable for the goal of biological systems. The next step for this project is replicating our experimental process on a polyvinyl alcohol (PVA) solution.

# Chapter 1

# Motivation

## 1.1 Super Resolution Imaging

Biological imaging is crucial to understanding cell structure, function and interactions. There are many ways images can be generated; common procedures include ultrasound utilizing sound waves, MRI using magnetic resonance, and microscopy or examining samples through a microscope. Each of these highlights certain aspects of anatomy and can demonstrate different insights. They also each have their drawbacks. Microscopic imaging is constrained by the diffraction limit of light, or 250-300 nm [1]. Subcellular molecular complexes, viruses, chromatin complexes, and cytoskeletal filaments, which can be around 100 nm in size, are beyond the scope of microscopic imaging [2]. Super resolution imaging offers an alternative method to resolve this issue by allowing imaging beyond the diffraction limit of light. Instead of magnifying an image, super resolution imaging utilizes a new process to generate an image. Scientists use fluorescing molecules as markers that can be localized. After locating many single molecules, an image is generated through compiling all of these single points [3] [4] [5] [6]. Single molecules are localized through induced fluorescing, and then turned off or photobleached [6]. Through many repetitions, a refined image is generated from these pinpoint locations as shown in Figure 1.1. Additionally, super

resolution imaging can be used to create multicolor single molecule imaging. Utilizing multicolor super resolution imaging highlights interactions between biomolecular pathways and how structures connect. Multiple dyes can be distinguished by using spectrally distinct emitters [5] [6] [7]. The single molecules are localized in the same process as Figure 1.1, but then are additionally identified by their spectral distinction to determine which emitter it is.

One limitation of multicolor imaging as it is conducted today, is the potential for spectral crosstalk between emitters [6]. Furthermore, there is limited availability of combinations of emitter that match the necessary imaging conditions needed [7]. This issue is further exacerbated when using more than two dyes. It becomes more difficult to find spectrally distinct dyes that can be identified, and creates more possibilities for error. Acquiring information sequentially or exchanging the buffer medium between scans can reduce error, but this is not ideal as it can lead to a reduction in the number of emitted photons collected, creating a less optimal image. [7].

One way to overcome the issues currently faced by multicolor imaging is to not rely on spectral distinction to identify emitters. We propose an alternative way to approach multicolor imaging. It was demonstrated in our lab's previous research that Eosin Y (EY), an organic emitter, could be distinguished from other Xanthene emitters based on blinking alone. The unique blinking properties inspired the concept of imaging based on blinking dynamics instead of spectral uniqueness.

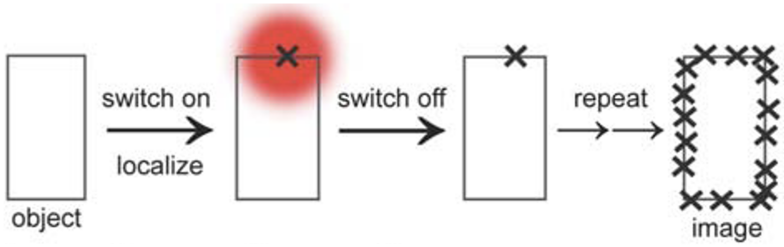


Figure 1.1: The process through which an image is create through the process of Super Resolution Imaging.

# Chapter 2

# Theory

## 2.1 Blinking

Diluting emitters to a single molecule level allows us to examine properties unseen when in an ensemble collection. One aspect that is only seen on the single molecule level is a behavior called "blinking." When a single molecule emitter is under constant excitation by a laser it absorbs light to generate an excited state, which is indicated as $S_1$ in Figure 2.1. The single molecule then returns to the ground state, $S_0$. As seen in Figure 2.1, there are two transition mechanisms to return to the $S_0$. The pathway $k_{21}$ produces an observable fluorescence. The alternative pathway $k_{31}$ is a "dark state," and does not result in emission [6]. These two options cause the emitter to quickly alternate between excited "on" and non-excited "off" states while under constant excitation. The random fluctuations of the emissive and non emissive events is known as blinking [6]. Figure 2.1 also illustrates the potential of photobleaching, a phenomenon caused by the emitter moving to a permanent off state caused by either an electronic change or chemical reaction [6]. The blinking patterns of every emitter is random, but follows trends based on the identity of the emitter.

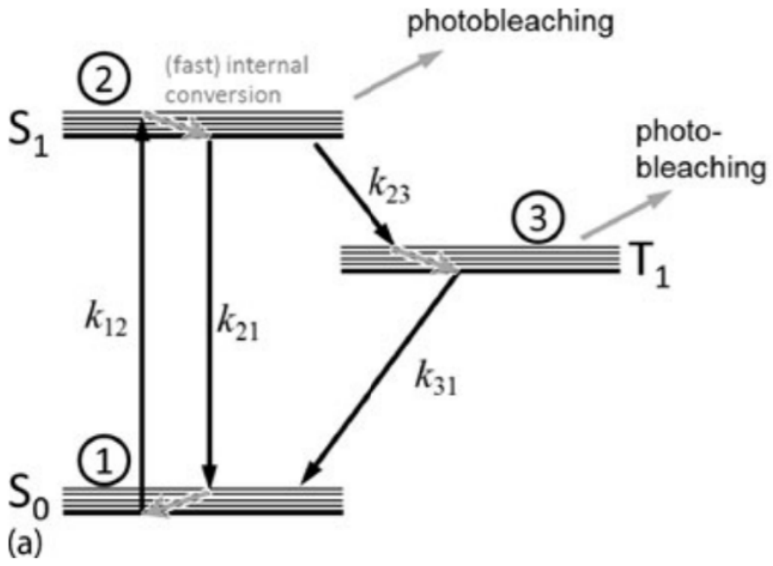


Figure 2.1: A Jablonski diagram depicting the transitions between the ground, excited fluorescing, and excited dark states [6].

## 2.2 Change Point Detection

Blinking dynamics can be analyzed using the Matlab program Change Point Detection (CPD). CPD is follows an outline written by Watkins and Yang and has been adapted to best suit our analysis by other members in the lab [8] [9] [10]. CPD generates an image of the blinking as well as quantitative information on the number of intensity states, number of on and off segments, number of on and off intervals, the threshold of one standard deviation (the sum of the minimum intensity and the square root of the minimum intensity), threshold 20 max (20% of the maximum intensity), the event frequency (number of events in 200 seconds), average intensity, average on and off segment time (the average amount of time between the emitter switching between excited fluorescing states and excited dark state), average on and off interval time (the average amount of time the emitter is in an excited fluorescing

state or in an excited dark state), and average event frequency interval (the average interval between the changes in intensity). In addition to this quantitative data, CPD produces a visual analysis of the blinking trace collected. Figure 2.2 shows an example blinking trace analyzed by CPD. The blinking trace captures the intensity of emission over a 200 second interval. The black segment in Figure 2.2 is the emission intensity being captured by our detector from a single molecule emitter. The green overlay on top of that is what CPD generates. The green shows the segments between where CPD has identified change points in the intensity of emission. Each red dot at the top is where along the x axis a change point is detected. The number in the top right, in this example a 9, is the number of unique intensity states. In Figure 2.2 you can see how the program is able to captures the quick and random switching of on and off states seen by a single molecule emitter.

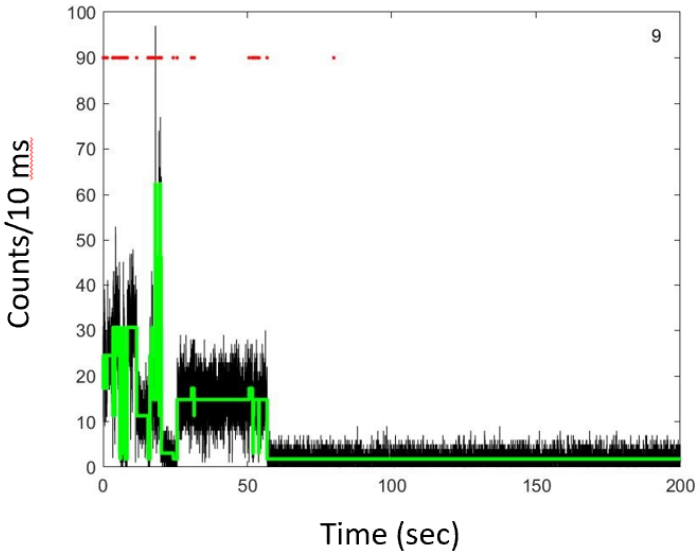


Figure 2.2: Blinking trace of Rhodamine 6G, demonstrating the makeup of the collected information with the CPD analyzed green overlay.

## 2.3 Blinking Based Multiplexing (BBM)

Super resolution imaging utilizes fluorescing to localize many molecules and create an image from these single points. Multicolor imaging builds upon super resolution imaging, by localizing single molecules and also identifying them. As discussed in the motivation section though, there are currently limitations for multicolor imaging. These issues stem from trouble identifying emitters. Emitters are traditionally identified by spectral distinction. We propose an alternative method to creating a multicolored image. Instead of using spectral distinction, spectrally overlapped emitters can be identified using the unique blinking patters, or Blinking Based Multiplexing (BBM.) This approach is illustrated in Figure 2.3. Each single molecule is localized in the first square. Using blinking it is then identified as its respective emitter. Identifying the emitter based on blinking would then allow multicolor imaging.

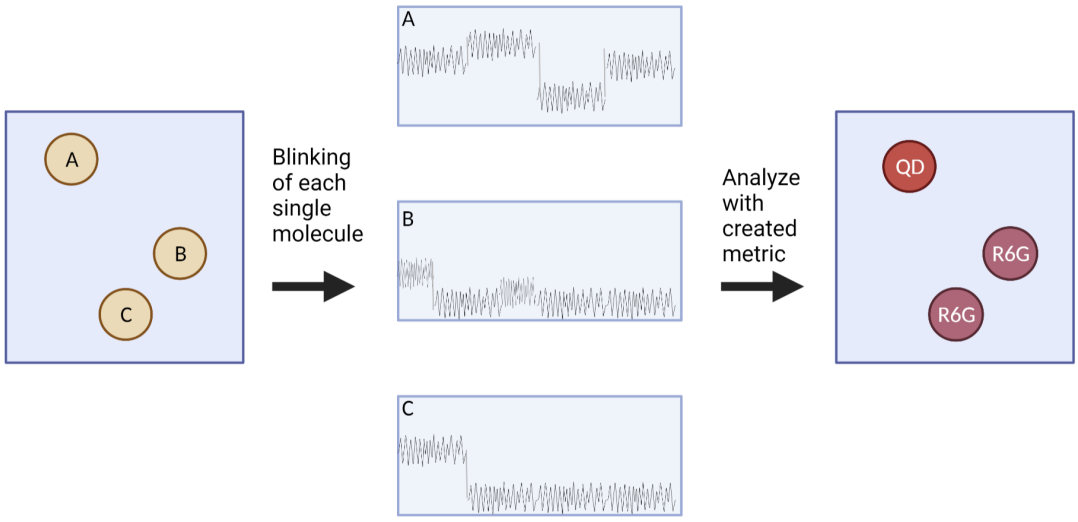


Figure 2.3: Schematic depicting the step-by-step process of how blinking dynamics can be applied to identify emitters for BBM.

# Chapter 3

# Experimental Technique

## 3.1 Single Molecule Spectroscopy (SMS)

A confocal microscope with a 532 nm wavelength laser is used to generate images on a micrometer scale. Emitters are diluted to a nanomolar scale and spin coated onto glass slides. A 6×6 µm image of the diluted sample of the molecule of interest is captured to show single molecules. Figure 3.1 shows an example of a 6×6 µm scan that has been false colored. Each bright spot indicates the presence of a single molecule. The laser is then focused on each molecule one by one, and the blinking statistics of these is captured. All scans are taken with our slide under nitrogen. Figure 3.2 is a schematic of the equipment used to generate these scans.

## 3.2 Emitters Used

Blinking dynamics of the emitters Rhodamine 6G (R6G) and Quantum Dots (QD) were examined. These emitters were chosen as they both have similar emission spectra and fluoresce under the same wavelength and power conditions.

QD demonstrates a lot of potential for the single molecule imaging uses. They fluoresce at high intensity and for long intervals and typically do not experience photobleaching [12]. The unique imaging opportunities that the fluorescing abilities of

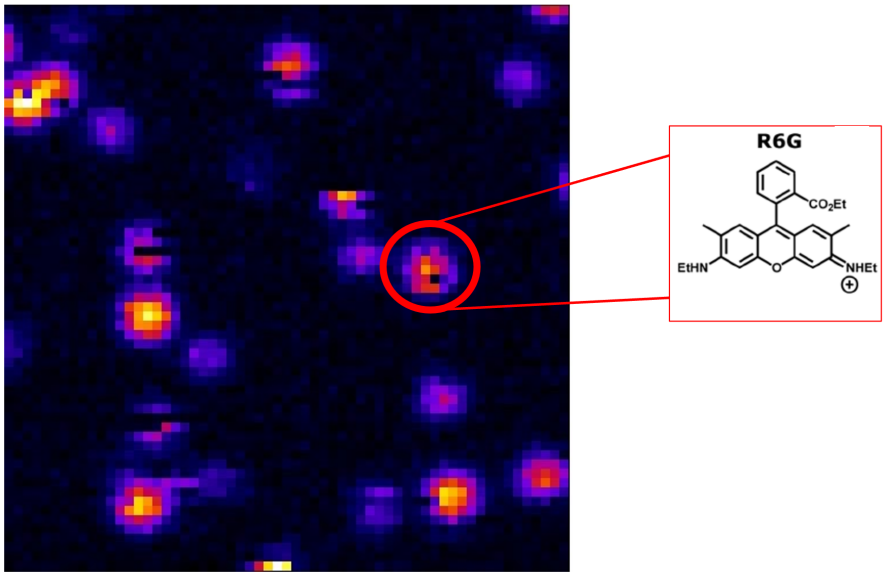


Figure 3.1: A 6x6 micrometer scan capturing single molecules of R6G, indicated by the bright spots.

QD provides make it extremely promising. QD are nanoparticles with a semiconductor core and a shell that protects them from oxidation [13] [12]. In biological imaging typically a CdSe core is used and surrounded by a ZnS outer layer. A thinner outer layer increases the level of photoluminescence while a thicker outer layer is useful for additional protection from oxidation and more intense environments [14]. The ability to adjust the size is also useful as it impacts the resulting fluorescent emission. Another benefit of QD is that they are slightly larger than organic dyes and so they are not transferred between cells until they fuse [15].

Previously QD have been used for imaging lymph nodes, cellular trafficking, and vascular mapping, along with other promising results in vivo [15]. The toxicity of QD, though, is still a concern. Cd, Zn, and other common QD components such as Se, Te, Hg, and Pb are all toxins that can damage the liver, nervous system, and DNA. It has been shown that a thicker outer layer can decrease the amount of core

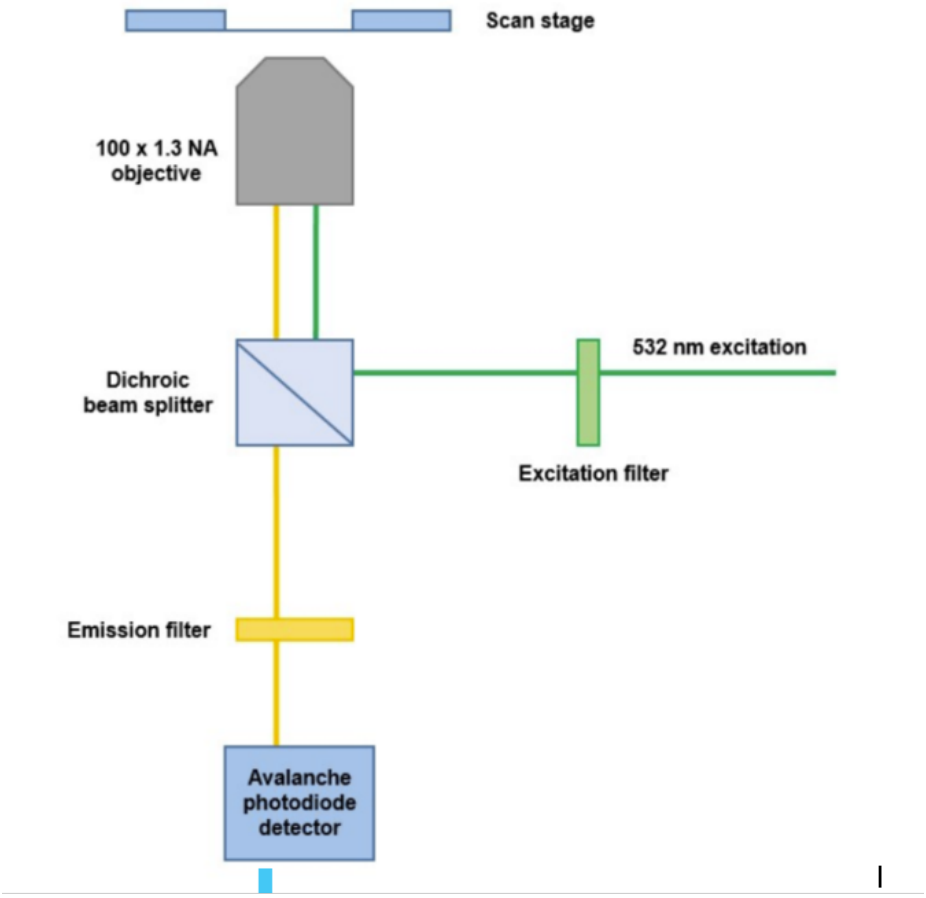


Figure 3.2: A schematic of the instrumentation used to collect the blinking traces. [11]

ions absorbed, but there have not been any long term animal studies on this process [14]. A lot is still unknown and more will have to be done to study any concerns around cytotoxicity [13].

R6G is an organic fluorophore that is small enough to be applicable on a nanometer imaging scale and which fluoresces brightly when under excitation from a laser [5]. It is a xanthene, meaning it has a characteristic grouping of two benzene rings connected by an oxane [16]. Rhodamine absorption is between 440 nm and 570 nm,

and emission is between 569 nm -610 nm [17]. R6G has a high fluorescence quantum yield and photostability making it a useful dye for single molecule imaging [16].

As shown in Figure 3.3, R6G has a distinct blinking pattern in its mildly high peaks at short intervals and often occur within the first 50 seconds of the scan. The molecules then often experience photobleaching and stop blinking for the remainder of the scan. QD have a very different behavior, as illustrated in Figure 3.4. QD are predominantly in an "on" state and emit for the majority of the scan due to their resistance to photobleaching.

Additionally, eosin (EY) also offers blinking imaging opportunities. EY is another organic fluorophore that demonstrates blinking uniqueness while falling in the same spectral emission range as R6G and QD. It is a xanthene like R6G, but has different groupings coming off of the fundamental structure of two benzene rings connected by an oxane. EY provides a useful extension of the research because organic dyes do not have the same issues of cytotoxicity seen in QD.

## 3.3 Biological Medium: Polymer Film

In order to test single molecule blinking in a medium to replicate in vivo imaging, we are currently studying the best way to create a polymer film. While still under development, the film is spin coated onto a glass slide, and then our single molecule dilution is layered on top. There has been limited success with creating an effective film so far, but we have learned ways to improve our methods.

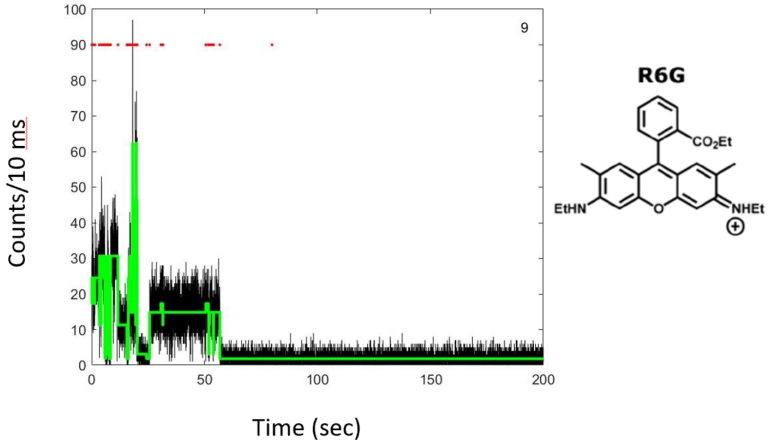


Figure 3.3: A blinking trace demonstrating the characteristic quick peaks and photobleaching around 50 s seen in R6G blinking.

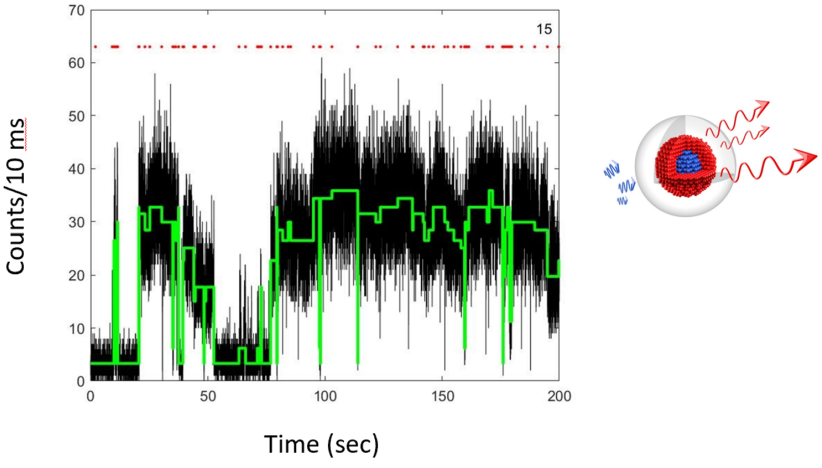


Figure 3.4: A blinking trace demonstrating the characteristic long on segments and lack of photobleaching seen in R6G blinking

# Chapter 4

# Results and Discussion

## 4.1 Developing a Metric to Identify R6G and QD

Data were collected on the blinking dynamics of over 100 emitters of both QD and R6G to create representative data sets. All data were normalized against the entire R6G and QD dataset and multiplied by 100. The uniqueness seen visually in the blinking was demonstrated in the data CPD produced as well. Our goal was to find a way to use the data to identify each blinking trace as the correct emitter, either R6G or QD. The attributes that best distinguished R6G and QD were:

- Number of intensity states $N_I$
- Threshold one standard deviation $I_{min}$ (the sum of the minimum intensity and the square root of the minimum intensity)
- Event frequency $\nu_E$ (number of events in 200 seconds)
- Average intensity $< I >$
- Average on interval time $< T_{on} >$ (the average time the emitted is recognized as being 'on' in its blinking)

These average values for each of these by emitter can be seen in Table 4.1.

| **Blinking Measurement** | **R6G** | **QD** |
|---|---|---|
| $N_I$ | 6.04 | 17.08 |
| $I_{min}$ | 3.99 counts | 15.38 counts |
| $< I >$ | 4.71 counts | 53.76 counts |
| $< T_{on} >$ | 5.597 s | 13.97 s |
| $\nu_E$ | 0.17 $s^{-1}$ | 1.45 $s^{-1}$ |

Table 4.1: Data demonstrating the difference in blinking dynamics between R6G and QD.

Figure 4.1 and 4.2 demonstrates two of the metrics that showed a strong difference between R6G and QD. The number of intensity states $N_I$ was the only attribute that created two distinct bell curves. The other attributes demonstrated more of a difference with R6G having a small range and QD having a wider range with a right skewed curve similar to threshold one standard deviation $I_{min}$. All of these, though, showed a trend of being able to differentiate some emitters as a distinct molecule if above or below a certain threshold. There is a middle area though where given the data, the emitter is unable to be accurately identified. Many variations of combining the representative blinking dynamics in Table 4.1 were examined. The one that resulted in the highest amount of emitters accurately identified was:

$$M = N_I I_{min} \nu_E^2 < I >^2 < T_{on} > \tag{4.1}$$

Our Metric ($M$) created two distinct groups, enabling us to create an upper limit where all data points above were identified as QD, and a lower limit where all data points below were identified as R6G, with a few outliers. $M$ was able to accurately capture 90.4% of the data, leaving 9.6% of the data unidentified between these limits. We then relied on visualization of the blinking traces to look for qualitative attributes

that would exemplify the characteristic blinking for each. R6G is distinct in its mildly high peaks that are short and often occur within the first 50 seconds of the scan. 87.2% of R6G does not have any blinking after 150 seconds. The molecules then often experience photobleaching and stop blinking for the remainder of the scan, as discussed before. QD are predominantly in an "on" state and emit for the majority of the scan, distinguishing them from R6G. Therefore, only for the emitters indistinguishable by $M$, they were visually examined for blinking after 150 s. If blinking was present they were classified as QD, if not they were classified as R6G. The addition of examining for blinking after 150 s allows our data to accurately identify 92.5% of the emitters.

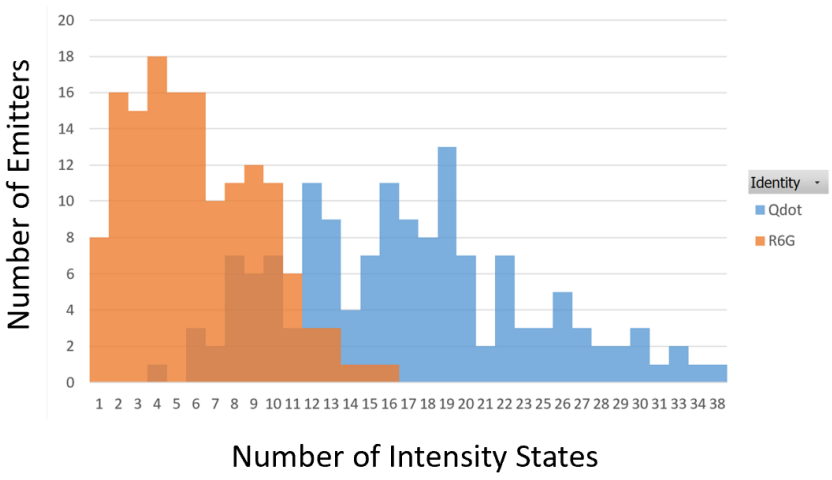


Figure 4.1: The number of intensity states show two distinct bell curves, but there is still overlap where the identity of the emitter cannot be distinguished using only this measure.

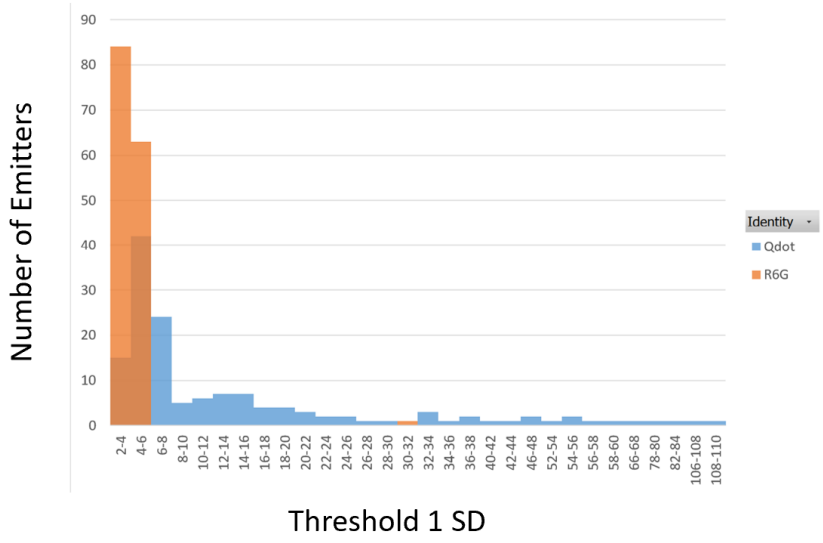


Figure 4.2: Threshold 1 standard deviation ($I_{min}$) shows a large amount of R6G below a certain level, and QD with a wide range. This somewhat differentiates them, but not enough to identify all of the data on its own.

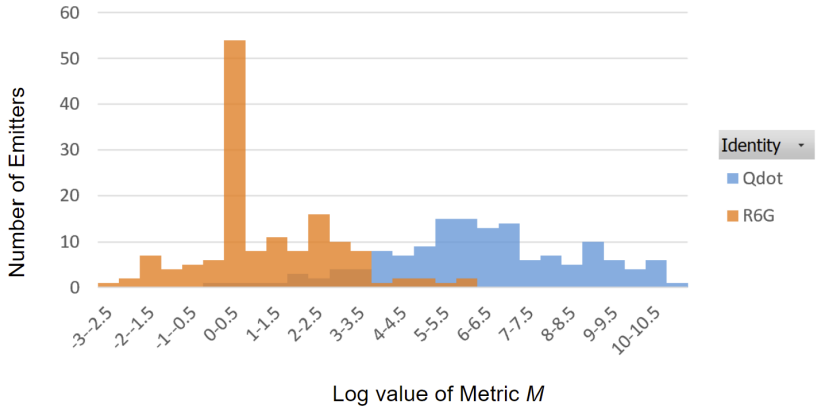


Figure 4.3: The final metric $M$ was able to combine the most differentiating blinking variables and correctly identify 90.4% of the data. The ones not correctly identified are seen here in the overlapped region.

## 4.2 Testing our Metric

To test our metric, it was used to analyze three different mixed dilutions of R6G and QD. Over 100 data points for each dilution 30% QD:70% R6G, 30% R6G :70% QD, and 50% R6G:50% QD were collected. All the data for each trial were run through the same CPD program and collected data on the same attributes. The same steps were followed for normalizing by dividing all data by the maximum of the respective variable, and multiplying by 100. For each dilution our metric was used to identify each molecule as either a QD or R6G. For molecules unidentified by metric $M$ we assigned them based on if there was blinking present after 150 seconds.
The ratios calculated by our metric are all highly similar to the dilutions that were tested (see Table 4.2). All predicted compositions are within 1%-4% of the expected compositions. This shows $M$ was able to accurately identify an unknown molecule as either R6G or QD based solely on a blinking trace.

### 4.2.1 Cleaning Data

Before analyzing our data to create our Metric $M$, we wanted to ensure our data was accurate. To do this we clean that data. The application ImageJ was used to find the width of each emitter using the 6×6 micrometer scans. If an emitter was too large it could indicate it is not actually the intended emitter and rather a contaminant, or is two emitters together. Both of which would create inaccuracies in our quantifying of characteristic blinking. Molecules that had a width of greater than 5 µm were discarded.

### 4.2.2 A Note on the 50% R6G:50% QD Dilution

The data in Table 4.2 reflects a redone trial of the 50% R6G:50% QD dilution. The original data was found to have unexpected trends in the data. The data was

expected to be random mixtures of the emitters around approximately 50:50 of each emitter. When collecting data an unexpected trend was found in the data having trends day by day of a high majority of either R6G or QD. Very few scans, or even days of data, resulted in approximately 50:50 data. The skewing could have been due to the aging of the QD, as the 50:50 dataset was our last collected. Aging could have led to an unexpected interaction between the QD and R6G when mixed in the dilution. The 50%:50% dilution was repeated with new QD and we created a new data set of approximately 100 molecules and we repeated the same process of analysis.

| | Dilution Concentrations | | |
|---|---|---|---|
| | **70%QD:30%R6G** | **50%QD:50%R6G** | **30%QD:70%R6G** |
| Number of Emitters Metric Identified as QD | 75 | 74 | 38 |
| Number of Emitters Metric Identified as R6G | 30 | 71 | 77 |
| Metric Identified by % QD:R6G | 71:29 | 51:49 | 33:67 |

Table 4.2: Results of our experiment with the experimental dilutions reflecting closely the predicted dilutions.

### 4.2.3 Creating a Polymer Film

All of the results reported above were done on glass. While glass provides a useful proof of concept, further research must be done to demonstrate this concepts' utility in an in vivo system and for its ultimate purpose of biological imaging. Thus, to synthesize this, polyvinyl alcohol (PVA) is used to replicate a biological medium. The same procedure of collecting and analyzing blinking dynamics of QD and R6G on glass is going to be replicated on PVA.

PVA is a water-soluble crystalline structured polymer. It is created artificially through hydrolysis of polyvinyl acetate to provide a biodegradable imitation of natural polymers. There are various grades of PVA that vary based on the percent hydrolyzed

and the molecular weight [18]. A low percent hydrolyzed results in longer chains of polymers. Figure 4.4 below demonstrates the base structure of PVA. Hydrogels, such as PVA, are often used in the biomedical field to replicate physicochemical aspects of in vivo environments [19]. PVA is specifically useful in our case as there has been verification of the effectiveness of the ability to detect single molecule emitters in PVA [20].

First the polymer film layer for our in vivo replication has to be created. The main issue currently being addressed is how to fully dissolve the PVA in a solution. PVA is water soluble. Initial dilutions have tested 1% PVA by weight dilutions in Milli-Q and 1:1 Milli-Q methanol solutions. Additional methods that have been tested are crushing and not crushing the PVA before combining, heating at various temperatures (60-90° C) and various heating durations (1-5 hours.) In addition, the PVA layer has been left to dry on the glass slide overnight. Lastly various volumes of the solution on glass have been spun coat onto the glass slide. None of these have resulted in a functional film. Although some appear visually to be fully dissolved, once scans are done on the nanometer scale it is evident that there are still aggregates present that have to be further dissolved. Figure 4.5 illustrates the aggregates that are present. This is a scan of 100 microliters of a dilution of 1% by weight PVA in 1:1 Milli-Q Methanol heated to 90° for approximately 5 hours which was spin coated on glass. The dilution looked fully dissolved visually but the areas of high counts indicate that there are still aggregates of PVA present that have to be further dissolved for this to be an effective medium.

Going forward, we are looking to use a different grade of PVA. So far the grade used has been a 98.0% - 98.8% hydrolyzed, 50,000-85,000 molecular weight PVA. Going forward a less hydrolyzed PVA should help reduce the amount of aggregates.

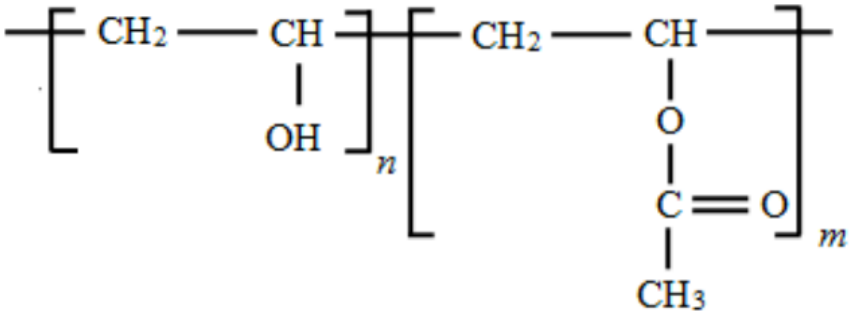


Figure 4.4: Chemical structure of PVA.

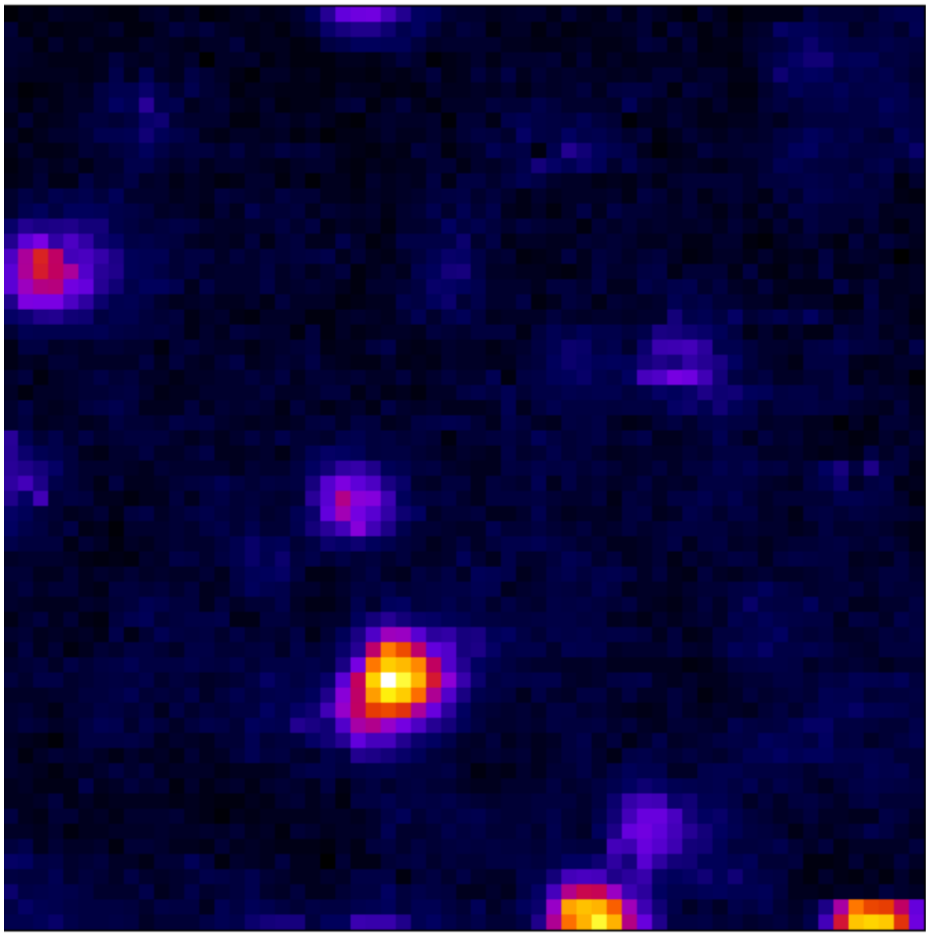

Figure 4.5: An example 6x6 micrometer scan of only PVA on glass in nitrogen.

### 4.2.4 EY Dataset and Troubleshooting

EY is an organic fluorophore that visually demonstrates uniqueness in blinking patterns. EY provides a great addition where the previous methods can be used to quantify the uniqueness in blinking. So far, an issue with the EY data collection has been there is a much lower concentration of emitters on each scan. Both the QD and R6G data sets were collected at a concentration of [1 nM]. The concentration may need to be increased for the EY data set collection, and the mixed dilutions adjusted accordingly. Another issue was malfunctions with the detector, which hindered our collection of EY. The detector issues created difficulty collecting accurate counts of the emission intensity. This difficulty was an equipment issue and not due to the fluorophore itself, but slowed data collection significantly.

EY demonstrates a lot of potential to be incorporated in a new metric. The scans collected demonstrate that EY blinks in a different way than the other dyes, as seen in Figure 4.6. The blinking traces indicates that there is strong potential to differentiate EY using CPD or in a mixed metric, again combining quantitative and qualitative attributes.

## 4.3 Plans for Future Work

Our results are very promising for the applications of biological imaging utilizing the blinking traces to use dyes with similar spectral emission under the same laser power conditions and wavelength. The next important step is to test our BBM concept in a more representative medium. The first thing that must be accomplished is finding an effective dilution of PVA to create a polyvinyl film to utilize as this medium. In addition, a full dataset of EY blinking scans on the same power settings needs to be collected.

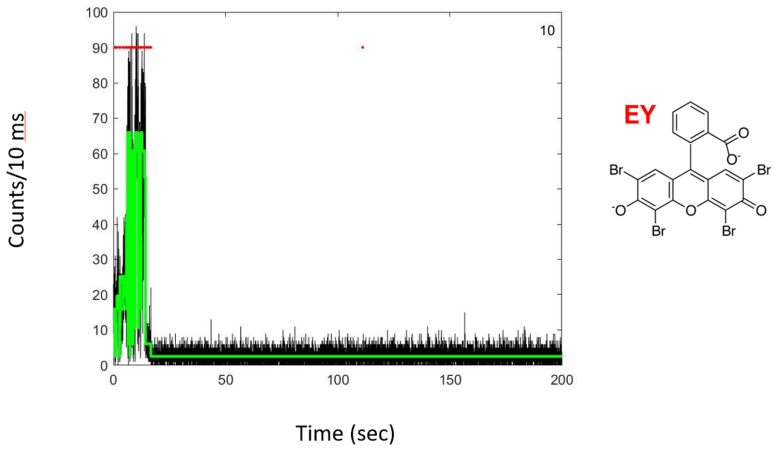


Figure 4.6: A blinking trace of EY demonstrating the quick peaks and photobleaching characteristic of this emitter.

# Chapter 5

# Conclusion

has been a large amount of progress made on our research of Blinking Based Multiplexing (BBM) so far. We have found an effective way to distinguish emitters based solely on their blinking patterns. The metric created through the unique trends seen in blinking seen by emitter was able to accurately identify 92.4% of the known R6G and QD. In addition, in mixed dilutions it upheld in effectiveness and identified dilutions of R6G:QD 30%:70%, 50%:50%, 70%:30% as very similar rations: 29.25%:70.75%, 51.03%:48.97%, 66.06%:33.94% respectively. Going forward, there are several directions this project can go to work on the practical application of BBM as well as improve its process. First, studying the medium we use. As discussed, PVA provides a great opportunity to study further the concept we have established by proving its utility on a biological medium. In addition to the medium used, expanding the emitters used can further proof of utility. This can be useful in multicolor imaging with more than two emitters, and helps resolve any concern of cytotoxicity with QD. Lastly, to improve the process of identifying the emitters, we are looking into using machine learning to analyze and identify emitters. This process is very promising to streamline the process of making an identifying metric and is more efficient than figuring out a metric manually. These aspects are all continuing to be researched in our group and will help further the established concept of BBM.

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