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Understanding how CaMKII holoenzyme dynamics facilities activation-triggered subunit exchange

Ana P. Torres-Ocampo
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Understanding how CaMKII holoenzyme dynamics facilitates activation-triggered subunit exchange

A Dissertation Presented

by

ANA PAMELA TORRES-OCAMPO

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2021

Molecular and Cellular Biology
Understanding how CaMKII holoenzyme dynamics facilitates activation-triggered subunit exchange

A Dissertation Presented
by
ANA PAMELA TORRES-OCAMPO

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DEDICATION

To my parents Maria Eugenia Ocampo-Vera and Jesus Fernando Torres-Mancilla, who supported me through this whole process without ever doubting my capabilities as a scientist, even when I questioned them. Thank you for being an endless source of support, for loving me, and for making me who I am today.

And to my grandparents, Maria del Refugio Vera-Romo and Guillermo Ocampo-Gastaldi, who are not here with me right now, but I know they are watching and celebrating my triumphs.

“He said he had contemplated, from above, human life.

And said that we are a sea of little fires, tiny flames

The world is that—he revealed—

A cluster of people, a sea of little fires.

Each person shines with their own light among all others.

No two fires are alike.

There are large fires and small fires

and fires of all kinds and colors.

There are people of serene fire, unaware of the existence of wind,

and people of crazy fire, who fill the air with sparks.

Some fires, foolish fires, do not shine or burn;

but others burn life so heartily you cannot observe them without stopping to blink,

and whoever gets close, flares up”

Eduardo Galeano

The book of hugs
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Thank you to the MCB family, who was always there. A heartfelt thank you. 😊
ABSTRACT

Understanding how CaMKII holoenzyme dynamics facilitates activation-triggered subunit exchange

SEPTEMBER 2021

ANA PAMELA TORRES-OCAMPO, B.SC., UNIVERSITY OF PUERTO RICO-RÍO PIEDRAS CAMPUS

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Directed by: Professor Margaret M. Stratton

Long-term memory and learning are still poorly understood from a molecular and cellular standpoint. Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) is an oligomeric kinase that is involved in this remarkable process. However, the molecular details of its specific roles in these processes remains elusive. CaMKII activation-triggered subunit exchange presents a novel possible mechanism involved in long-term memory and learning by exchanging active subunits with other CaMKIIs. CaMKII subunit exchange also shows that exchanged CaMKIIs spread their phosphorylation state to newly synthesized CaMKIIs. This provides a long-lasting signal that might possibly be involved in long-term memory by escaping a cell’s protein turnover. In this thesis work, I expanded understanding of CaMKII activation-triggered subunit exchange to the other CaMKII human genes, CaMKIIγ and CaMKIIδ. I also characterized CaMKIIα holoenzyme stability. I also uncovered a potential new role for CaMKII oligomerization and how it is related to activation properties. Lastly, I used CaMKII FRET biosensor Camui for a proof of concept using microscopy technology FLIM-FRET.
# TABLE OF CONTENTS

ACKNOWLEDGMENTS ............................................................................................................................ v

ABSTRACT ............................................................................................................................................... viii

LIST OF TABLES ..................................................................................................................................... xiv

LIST OF FIGURES ................................................................................................................................... xv

CHAPTER

1. Ca\textsuperscript{2+}/Calmodulin-dependent protein kinase (CaMKII) role in long-term memory through activation-triggered subunit exchange and holoenzyme stability .... 1

   1.1 Abstract .......................................................................................................................................... 1

   1.2 Introduction ...................................................................................................................................... 1

   1.3 The role of CaMKII in memory and learning .................................................................................. 2

   1.4 Francis Crick’s postulate of molecular memory .......................................................................... 3

   1.5 CaMKII as a candidate for memory molecule ............................................................................. 3

   1.6 CaMKII structure and oligomerization ......................................................................................... 4

   1.7 CaMKII Conclusions ..................................................................................................................... 5

2. Activation-triggered subunit exchange in CaMKII\text{y} and CaMKII\text{δ} ........................................ 7
3.5 Analysis of CaMKII dissociation using mass photometry .................................................. 32

3.6 Conclusions ................................................................................................................................ 34

3.7 Methods ...................................................................................................................................... 39

3.8 Acknowledgements ..................................................................................................................... 43

4. Studies of CaMKII oligomerization domain and holoenzymes in solution ........ 56

4.1 Abstract ....................................................................................................................................... 56

4.2 Introduction .................................................................................................................................. 57

4.3 CaMKII hub stoichiometry state and thermal stability ......................................................... 59

4.4 CaMKII hub stoichiometry state may predict calcium sensitivity ...................................... 60

4.5 CaMKII chimeras stoichiometry of hub is related to Ca\(^{2+}\) sensitivity ....................... 60

4.6 Conclusions ................................................................................................................................. 61

4.7 Methods ...................................................................................................................................... 62

4.8 Acknowledgements ..................................................................................................................... 67

5. Live-cell FLIM FRET using a commercially available system ........................................... 82

5.1 Abstract ........................................................................................................................................ 82
5.2 Introduction ................................................................................................................................. 83

5.3 Principles of FLIM-FRET ............................................................................................................... 84

5.4 Equipment and materials ............................................................................................................. 87

5.5 Proof of concept 1 - CyclinB1-Cdk1 activity reporter in Drosophila S2 cells .......... 89

5.6 Proof of concept 2 – Camui CaMKII sensor in human HEK293T cells .................................................. 102

5.7 Utilizing the Nikon A1-FLIM platform ................................................................................... 104

5.8 Conclusions .................................................................................................................................. 105

5.9 Acknowledgments ....................................................................................................................... 106

6. Future perspectives on CaMKII subunit exchange role in long-term memory formation ................................................................. 119

6.1 Overview ....................................................................................................................................... 119

6.2 CaMKII subunit exchange .............................................................................................................. 119

6.3 CaMKII holoenzyme stability ....................................................................................................... 121

6.4 CaMKII oligomerization state ...................................................................................................... 121

6.5 CaMKII FRET biosensor .............................................................................................................. 122
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Parts list and cost estimates for the compounds of TCSPCFLIM system</td>
<td>115</td>
</tr>
<tr>
<td>2. Properties of fluorescent protein donors that exhibit mono-exponential decay</td>
<td>116</td>
</tr>
<tr>
<td>3. Lifetime measurements of CyclinB1-Cdk1 reporters in proof of concept 1</td>
<td>117</td>
</tr>
<tr>
<td>4. FRET efficiency measurements of soluble CyclinB1-Cdk1 reporters in proof of concept 1</td>
<td>118</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Figure 2.1 CaMKII protein domains and holoenzyme architecture</td>
<td>21</td>
</tr>
<tr>
<td>2.</td>
<td>Figure 2.2 CaMKIIγ shows activation-triggered subunit exchange with FRET bulk assay</td>
<td>22</td>
</tr>
<tr>
<td>3.</td>
<td>Figure 2.3 CaMKII subunit exchange of homo-oligomeric CaMKIIα/CaMKIIα and CaMKIIγ/CaMKIIγ using single-molecule TIRF</td>
<td>23</td>
</tr>
<tr>
<td>4.</td>
<td>Figure 2.4 Hetero-oligomeric subunit exchange of CaMKIIα/CaMKIIγ with single-molecule TIRF</td>
<td>24</td>
</tr>
<tr>
<td>5.</td>
<td>Figure 2.5 Biophysics of CaMKII subunit exchange</td>
<td>25</td>
</tr>
<tr>
<td>6.</td>
<td>Figure 2.6 CaMKIIδ subunit exchange using FRET bulk assay</td>
<td>26</td>
</tr>
<tr>
<td>7.</td>
<td>Figure 3.1 CaMKII architecture</td>
<td>45</td>
</tr>
<tr>
<td>8.</td>
<td>Figure 3.2 Differential scanning calorimetry of CaMKII domains</td>
<td>46</td>
</tr>
<tr>
<td>9.</td>
<td>Figure 3.3 Crystal structure of the CaMKII kinase domain</td>
<td>47</td>
</tr>
<tr>
<td>10.</td>
<td>Figure 3.4 Mass photometry analysis of CaMKII variants</td>
<td>48</td>
</tr>
<tr>
<td>11.</td>
<td>Figure 3.5 DSC replicates</td>
<td>49</td>
</tr>
<tr>
<td>12.</td>
<td>Figure 3.6 Reorientation of the αD helix</td>
<td>50</td>
</tr>
<tr>
<td>13.</td>
<td>Figure 3.7 Raw MP data: CaMKIIα hub</td>
<td>51</td>
</tr>
<tr>
<td>14.</td>
<td>Figure 3.8 Raw MP data: CaMKIIα-30</td>
<td>52</td>
</tr>
<tr>
<td>15.</td>
<td>Figure 3.9 Raw MP data: CaMKIIα-0</td>
<td>53</td>
</tr>
<tr>
<td>16.</td>
<td>Figure 3.10 Size estimation of CaMKII variants from Mass Photometry</td>
<td>54</td>
</tr>
<tr>
<td>17.</td>
<td>Figure 3.11 CaMKIIα oligomer dissociation</td>
<td>55</td>
</tr>
<tr>
<td>18.</td>
<td>Figure 4.1 CaMKII protein domains and the four hub domains for the four CaMKII variants</td>
<td>68</td>
</tr>
</tbody>
</table>
19. Figure 4.2 CaMKII hubs in solutions show different stoichiometry and different patterns of dissociation at the same concentration (100 nM) with mass photometry.

20. Figure 4.3 CaMKIIα hub dilution from 100 to 70 nM using mass photometry.

21. Figure 4.4 CaMKIIβ hub dilution from 100 to 70 nM using mass photometry.

22. Figure 4.5 CaMKIIδ hub dilution from 100 to 70 nM using mass photometry.

23. Figure 4.6 CaMKIIγ hub dilution from 100 to 70 nM using mass photometry.

24. Figure 4.7 Differential Scanning Calorimetry for all four CaMKII hubs.

25. Figure 4.8 The replicates for DSC CaMKII hubs.

26. Figure 4.9 CaMKIIβ hubs shows stoichiometry of 14-mer and a 16-mer.

27. Figure 4.10 CaMKII holoenzyme stoichiometries using mass photometry.

28. Figure 4.11 CaMKII holoenzymes stoichiometries using mass photometry replicates.

29. Figure 4.12 CaMKII holoenzymes and CaMKII chimeras stoichiometries using mass photometry are related to calcium sensitivity.

30. Figure 4.13 CaMKII chimeras stoichiometries using mass photometry and replicates.

31. Figure 5.1 Schematic of how a decay curve is generated in the time-correlated single photon counting (TCSPC) FLIM technique.

32. Figure 5.2 Schematic showing how a single exponential decay donor behaves in the absence of FRET and when engaged in FRET with an acceptor.

33. Figure 5.3 Layout of the FLIM system outlined in this methods chapter.

34. Figure 5.4 CyclinB1-Cdk1 FLIM-FRET reporter design.

35. Figure 5.5 Lifetime analysis using SPCImage software (Becker & Hickl).

36. Figure 5.6 Layout and principles of a phasor plot.

37. Figure 5.7 FLIM-FRET data analysis and visual display using the phasor plot.

38. Figure 5.8 Camui FLIM-FRET sensor for CaMKII activation.
CHAPTER 1

Ca\textsuperscript{2+}/Calmodulin-dependent protein kinase II (CaMKII) role in long-term memory through activation-triggered subunit exchange and holoenzyme stability

Written by Torres-Ocampo Ana P.

1.1 Abstract

Calcium/calmodulin-dependent protein kinase II is Thr/Ser kinase discovered in the 1970s. To date, behavioral and cellular data pointed to a possible role during Long-term potentiation (LTP), the underlying cellular mechanism for memory formation. However, its specific role remains ambiguous. How memory and learning work at the molecular level remains unclear. Francis Crick proposed a postulate for a memory molecule that is the key to escape a protein’s turnover. CaMKII fits all of his proposed criteria, and in this chapter, it is summarized how CaMKII is a key molecular player in memory formation.

1.2 Introduction

While looking to understand better the regulation of bovine brain hormonal regulation and their mechanism, calmodulin (CaM) was discovered to be the mediator for various signaling processes (Cheung, 1969). Calmodulin is a 16.7 kDa protein that can bind up to four calcium ions and is now widely known as a sensor for intracellular calcium that is tightly regulated in order to maintain cellular homeostasis. It is crucial for the activity of various kinases, ion receptors, among others (Takemoto-Kimura et al., 2017; Urrutia et al., 2019). One of the kinases that require CaM/Ca\textsuperscript{2+} for its activity is calcium/calmodulin-dependent protein kinase II (CaMKII), described by Shulman and Greengard (Schulman & Greengard, 1978).
In the 40+ years since then, extensive research has shown that this oligomeric kinase (Ahmad et al., 1982; Bhattacharyya et al., 2016; Chao et al., 2011; Fukunaga et al., 1982) has been found in various human tissues and plays significant roles in each. CaMKII has four genes in humans: α, β, γ, and δ. Historically, CaMKIIα and β are found in the brain and are involved in memory and learning (Herring & Nicoll, 2016). CaMKIIγ has a preeminent role during egg fertilization (Escoffier et al., 2016; Yoon et al., 2008), and CaMKIIδ is present in cardiac muscle (Backs et al., 2009; Maier & Bers, 2002). CaMKII has four protein domains: it has a kinase, regulatory segment, variable linker, and hub. The hub, or oligomerization domain, oligomerizes the enzyme into dodecamers or tetradecamers that range from 500 kDa to 700 kDa. A CaMKII monomer is 50 kDa to 80 kDa depending on the linker length.

The nature of our research was to describe the biochemical and biophysical properties of CaMKII. We wanted to understand how this might be related to its activity and subunit exchange, a potential mechanism that might be involved in neurons during long-term memory formation (Bhattacharyya et al., 2016; M. Stratton et al., 2014)

1.3 The role of CaMKII in memory and learning

After CaMKII’s initial discovery, tremendous volumes of research started to accumulate describing its potential roles. Giese and colleagues made an intriguing observation where they showed how mice exhibit difficulty remembering and learning after they mutate T286 to T286A, an important phosphorylation site for CaMKII. This mutation inhibits CaMKII’s T286 autophosphorylation and subsequently the ability to gain calcium autonomy in the absence of Ca²⁺ signaling (Giese et al., 1998). Along with T286, CaMKII also has two other essential phosphorylation sites, T305 and T306. When mutated to T305A/T306A, mice with these mutations also displayed learning and recalling deficiencies in mice (Elgersma et al., 2002). This phenotype pointed to a significant role for CaMKII during memory formation and learning.
1.4 Francis Crick’s postulate of molecular memory

How are memories stored for years in our brains? The way memory and learning works at the molecular and cellular level is still poorly understood. It is known that the main drivers of cellular processes are proteins. However, a cell’s proteome is turned over in days, at most weeks. Therefore, the question arises: How is information stored for years within the cells if the proteins that orchestrate cellular processes are turned over much faster than this? Francis Crick came up with a postulate about a memory molecule, most likely a protein, that would be found in cells and would be the key to understanding how memory works at the cellular and molecular level (Crick, 1984). Here are the 4 characteristics proposed for a memory molecule:

1. Its activity is crucial for memory

2. Its activity is modulated by a post-translational modification

3. It is oligomeric

4. It can exchange subunits with newly synthesized protein and share its activation state

CaMKII fits all these criteria, as I will highlight below.

1.5 CaMKII as a candidate for memory molecule

CaMKII is clearly crucial for memory formation. Since its discovery, CaMKII was associated with kinase activity (Yamauchi & Fujisawa, 1980) and later Miller and Kennedy described the process by which CaMKII was being auto phosphorylated in Thr286 after CaM/Ca^{2+} binding to the regulatory segment (Miller & Kennedy, 1986). Therefore, CaMKII fits also the criterion that its activity is modulated by post-translational modification.
From the study from Giese and colleagues (Giese et al., 1998), we know CaMKII has a role during memory and learning. But apart from that, there are a several publications highlighting CaMKII’s presence during LTP, the underlying cellular mechanism for memory formation and learning (Lisman et al., 2002; Otmakhov & Lisman, 2012; Rossetti et al., 2017a). Therefore, CaMKII fits the criterion for its activity being crucial for memory formation.

There was vast evidence that CaMKII was an oligomeric complex (Goldberg et al., 1996; Hoelz et al., 2003; Rellos et al., 2010; Rosenberg et al., 2005; Schulman & Greengard, 1978). Few laboratories had crystallized different protein domains of human CaMKII (Bhattacharyya et al., 2016; Chao et al., 2010; McSpadden et al., 2019; Rellos et al., 2010; Rosenberg et al., 2005; Torres-Ocampo et al., 2020). However, Chao and colleagues were the first to solve the crystal structure of the intact CaMKII holoenzyme (Chao et al., 2011). This opened the door to study its molecular structure in more detail. Therefore, CaMKII fits the criterion of being an oligomeric complex.

Most recently, CaMKII was reported to undergo activation-triggered subunit exchange (M. Stratton et al., 2014). It was reported that upon activation, CaMKII can exchange subunits with other CaMKII holoenzymes, whether the other CaMKIIs are activated or not. And most importantly, after exchanging CaMKII was able to pass it phosphorylation state to the other previously unactivated CaMKII. This provides clues for a possible mechanism that may be able to escape protein turnover since it will be able to have a prolonged signal in the cell. Thus, CaMKII is an ideal candidate as a memory molecule according to Crick’s postulate.

1.6 CaMKII structure and oligomerization
Several labs have elucidated CaMKII oligomeric architecture. We know that CaMKII is a dodecameric/tetrameric complex (Bhattacharyya et al., 2016; Chao et al., 2011). CaMKII is one of the few oligomeric kinases that show this high ordered oligomerization state. Due to this, CaMKII apart from being tightly regulated by Ca\(^{2+}\)/CaM, it also has this extra step of regulation for its kinase activity. CaMKII already exhibits such behavior. For example, it has frequency-dependent activation \textit{in vitro} and \textit{in vivo} (Ardestani et al., 2019; M. M. Stratton et al., 2013). This means that CaMKII responds to amplitude and frequency of calcium spikes which in cells. Therefore, not only can CaMKII get autophosphorylated but also can finely tune its kinase activity according to the calcium spikes it’s exposed to. This is a mechanism that points out how tightly regulated CaMKII can be in the cell.

Furthermore, activation is highly cooperative (Chao et al., 2010; M. M. Stratton et al., 2013), and the hub allosterically regulates kinase activity (Sloutsky et al., 2020). Finally, subunit exchange is possible due to the oligomeric structure of CaMKII (Bhattacharyya et al., 2016; M. Stratton et al., 2014). Nonetheless, we know very little on how CaMKII oligomerizes and/or any K\(_d\) or K\(_a\) for the holoenzyme assembly. Studying the K\(_d\) and K\(_a\) is a complex problem because data suggest they are in the low nM range and techniques that have the resolution at this concentration are lacking (Bhattacharyya et al., 2016). Understanding these biophysical details of this oligomeric complex will allow us to understand more thoroughly how it regulates itself by this complex. Accordingly, it is crucial to study how oligomerization occurs for CaMKII.

1.7 Conclusions

Over 40 years of research have poised us to study subunit exchange, the seemingly last piece of the puzzle that would make CaMKII a great candidate for the memory molecule postulate. Stratton and colleagues (Bhattacharyya et al., 2016; M. Stratton et al., 2014) studied CaMKII\(\alpha\) and CaMKII\(\beta\) subunit exchange. Nonetheless, there are still many aspects
that we still do not understand. Among them is subunit exchange in the other CaMKII genes and how its high order oligomeric nature might regulate its own kinase activity.
CHAPTER 2

ACTIVATION-TRIGGERED SUBUNIT EXCHANGE IN CAMKII GAMMA AND CAMKII DELTA

Written by Torres-Ocampo Ana P., Lee Il-Hyung, Chambers James, Hacker Christina and Stratton Margaret

I performed all of the experiments in each figure; I made all the figures and wrote the manuscript.

2.1 Abstract

Ca\textsuperscript{2+}/calmodulin dependent protein kinase II (CaMKII) is an oligomeric kinase that goes through activated-triggered subunit exchange. After Ca\textsuperscript{2+}/CaM binding to the regulatory segment allows the phosphorylation of Thr286 and turns on CaMKII, CaMKII goes through subunit exchange with other CaMKIIs and this allows CaMKII to spread its phosphorylation state to previously inactive CaMKIIs. This permits a long-lasting signal of CaMKII activation. Previously, this process had been characterized in CaMKII\textalpha and CaMKII\textbeta. However, CaMKII has two other variants, CaMKII\textgamma and CaMKII\textdelta. This body of work presents evidence that these two variants also go through subunit exchange by using a FRET bulk assay and a single-molecule TIRF approach. Lastly, an ATP analog was used to interrogate the activation-triggered mechanism of subunit exchange, and these data suggest an unknown role for ATP during CaMKII subunit exchange.

2.2 Introduction

Ca\textsuperscript{2+}/calmodulin dependent protein kinase II (CaMKII) is an oligomeric Ser/Thr
kinase involved in many critical Ca\(^{2+}\) dependent signaling cascades throughout the body (Backs et al., 2009; Herring & Nicoll, 2016; Yoon et al., 2008). Each subunit of CaMKII is organized as a kinase domain, regulatory segment, linker, and hub domain (oligomerization domain) (Fig 2.2A). There are four CaMKII genes in humans: α, β, γ, and δ. CaMKIIα and CaMKIIβ are canonically implicated in the context of memory formation and learning (Herring & Nicoll, 2016a). CaMKIIγ has an important role in egg fertilization (Yoon et al., 2008), and CaMKIIδ has a crucial role in muscle cardiac tissue (Backs et al., 2009).

CaMKII has peculiar conformation as a dodecamer/tetradecamer kinase (Fig. 2.2b) (Bhattacharyya et al., 2016; Chao et al., 2011; Sloutsky et al., 2020) due to the hub domain. Several papers study how the hub confers remarkable properties to CaMKII. CaMKII has frequency-dependent activation \textit{in vitro} and \textit{in vivo}, high cooperativity, and activation-triggered subunit exchange (Ardestani et al., 2019; M. Stratton et al., 2014; M. M. Stratton et al., 2013). Activation-triggered subunit exchange has been previously studied in CaMKIIα and CaMKIIβ (Bhattacharyya et al., 2016; M. Stratton et al., 2014). Therefore, the focus of this paper will be expanding our knowledge of CaMKII activation-triggered subunit exchange to the other two CaMKII variants: CaMKIIγ and δ.

Stratton et al. showed with Fluorescence Resonance Energy Transfer (FRET) and single-molecule Total Internal Reflection Fluorescence (TIRF) experiments that once CaMKII is activated (by adding ATP, Mg\(^{2+}\), Ca\(^{2+}/\)calmodulin), it can exchange subunits with other CaMKIIIs that are active or inactive (M. Stratton et al., 2014). Therefore, subunit exchange causes a spread of activation state to the other CaMKIIIs (Figure 2.2A). Molecular details of CaMKII activation-triggered subunit exchange of this mechanism are described in (Bhattacharyya et al., 2016). Succinctly, CaMKII becomes active by Thr286 autophosphorylation after Ca\(^{2+}/\)CaM binds to the regulatory segment. After the calcium signal fades, CaM dissociates and allows the phosphorylation on Thr305/306 and blocking
CaM rebinding. The regulatory segment docks in the hub and destabilizes the vertical interface of the hub and allows the release of a dimeric unit that can interact and exchange with another CaMKII (M. Stratton et al., 2014).

CaMKII has a critical role during Long-Term Potentiation (LTP), which is the delicate strengthening of the connection between neurons (Herring & Nicoll, 2016). LTP has been appointed as the cellular cascade responsible for memory formation and learning in hippocampal neurons (A. Lynch, 2004). Once activated during LTP, CaMKII binds to the N-methyl-D-Aspartate (NMDA) receptor and phosphorylates various substrates (Bayer et al., 2001; Opazo et al., 2010; Shifman et al., 2006; W. Zhang et al., 2019). The question becomes, how are LTP and CaMKII activation-triggered subunit exchange possibly connected? Neurobiologists are still struggling to explain how memories are stored for long periods of time. CaMKII needs Ca$^{2+}$/CaM initially to become active; however, once the Ca$^{2+}$ stimulus subsides in the cell, CaMKII could hypothetically remain active by exchanging with newly synthesized CaMKII, thereby escaping protein-turnover. This property could be the key to understanding how memory and learning work at the molecular level since it is known that proteins in cells last days to weeks. However, it remains unknown if CaMKII activation-triggered subunit exchange also has a role during LTP.

mRNA transcripts for all four CaMKII genes have been found in human hippocampal neurons (Sloutsky et al., 2020). CaMKII$\alpha$ and CaMKII$\beta$ are in high abundance. Interestingly, many CaMKII$\gamma$ and CaMKII$\delta$ variants are also present (Sloutsky et al., 2020). This suggests that all four CaMKIIs are there in protein form in the neurons as well, although, further studies need to be done to confirm this. Furthermore, there is evidence that CaMKII$\gamma$ regulates the expression of genes during LTP via the phosphorylation of transcription factor CREB (Ma et al., 2014). This led to the study of activation-triggered subunit exchange in CaMKII$\gamma$ and CaMKII$\delta$. Our results show that CaMKII$\gamma$ exchanges with itself and with
CaMKIIα. We also study the biophysics of subunit exchange and activation-triggered subunit exchange in CaMKIIδ.

2.3 CaMKIIα and CaMKIIβ activation-triggered subunit exchange

As mentioned above, there is evidence for activation-triggered subunit exchange in CaMKIIα and CaMKIIβ. Stratton et al. reported that CaMKIIα could exchange with itself using a FRET bulk assay and single-molecule TIRF. Moreover, Bhattacharyya et al. (Bhattacharyya et al., 2016) reported that CaMKIIβ could exchange with itself and with CaMKIIα to form hetero-oligomers. The half-times of the exchange events were calculated, and we found that CaMKIIα/CaMKIIα subunit exchange was 20 minutes while CaMKIIβ/CaMKIIβ was 22 minutes. CaMKIIα/CaMKIIβ had a half-times of ~30 minutes. This suggests that the hetero-oligomeric subunit exchange is slower compared to homo-oligomeric subunit exchange (Fig 2.2D). Thus, CaMKIIγ and CaMKIIδ were tested since their exchange properties are unknown.

2.4 FRET assay to test homo-oligomeric and hetero-oligomeric subunit exchange for CaMKIIγ

Following protocol with the previous publication, we used fluorescent dyes with a maleimide handle to label CaMKII. The CaMKIIγ variant used is (14, 17, 18b) and has a 57-residue linker, according to the naming scheme proposed by Sloutsky et al. (Sloutsky & Stratton, 2020). From now on, it will be called CaMKIIγ since it is the only CaMKIIγ used here. Using the bulk FRET assay, active and unactivated samples of CaMKIIγ were tested. For both samples, CaMKII labeled with red dye was mixed with CaMKII labeled with green dye. For the active sample, ATP, Mg²⁺, and Ca²⁺/CaM were added. For the unactivated sample, buffer was added to achieve the same final volume. For the homo-oligomeric exchange, it was observed that CaMKIIγ/CaMKIIγ displays an increasing FRET ratio when activated over time when compared with the inactive sample (Fig 2.2B). For the hetero-
oligomeric subunit exchange, CaMKIIγ was mixed with CaMKIIα (14,18) with a 30-residue linker, which we call CaMKIIα from now on because it is the only CaMKIIα variant used here. This also showed the same trend that the active sample has a higher FRET ratio over compared to the inactive sample (Figure 2.2C). However, it is clear that the FRET ratio was not as high as the homo-oligomeric FRET for CaMKIIγ/ CaMKIIγ. Despite the fact that all CaMKII variants have 90% identity in the kinase and 75% in the hub and G470C was mutated for optimal FRET signal, it seems that minor differences in structure in both variants might have contributed to not getting a similar FRET ratio like the homo-oligomeric subunit exchange. It might also point to the fact that hetero-oligomeric exchange is just slower in general because of structural differences in both holoenzymes.

The half-times were calculated and for CaMKIIγ/CaMKIIγ exchange it was 18 mins (details in methodology). For CaMKIIγ/CaMKIIα it was 56 mins (Figure 2.2D). This shows the same trend as CaMKIIα and CaMKIIβ, where the homo-oligomeric exchange seems to be faster than the hetero-oligomeric exchange (Figure 2.2D). In order to confirm the results and show that they were not an artifact of aggregation, the next step was to test CaMKIIγ subunit exchange using single-molecule TIRF.

### 2.5 Single-molecule TIRF measuring hetero-oligomeric CaMKIIγ/CaMKIIα subunit exchange

The protocol from Stratton et al. (M. Stratton et al., 2014) single-molecule TIRF approach was used for this experiment. Briefly, the CaMKII constructs have a C-terminal AviTag (Howarth & Ting, 2008). CaMKII was biotinylated during the bacterial expression (confirmed by mass spectrometry, A1). Once CaMKII has been purified from the bacterial lysate and labeled with the fluorescent dyes, we compared an activated sample (ATP, Mg²⁺, and Ca²⁺/CaM) to an inactivated sample (just buffer added) over time. A sample of each
condition is added to a previously functionalized coverslip with PEG-PLL and PEG-biotin. Streptavidin was then added at single-molecule density in order to get a visualization of each CaMKII molecule separately (Figure 2.4A). Lastly, TIRF was used to image and take a time course of both conditions (active and inactive CaMKII). During the analysis, firstly, a Gaussian fit in each channel selects the labeled CaMKII holoenzyme. Then, the labeled CaMKII holoenzymes in the same location in both channels would be considered colocalized and, we interpret this as subunit exchange. In other words, red and green dyes located within one CaMKII molecule (Figure 2.4B).

Multiple experiments using CaMKIIγ revealed irreproducible data. Over time, we observed that each single particle was reduced in brightness and there was also evidence of aggregated particles that were above our brightness threshold. This observation of decreased brightness led us to question whether CaMKIIγ was dissociating at the low concentrations required for these single molecule experiments (Figure 2.3A and B). Various troubleshooting approaches were used, but no reproducible data was collected likely due to stability issues of CaMKIIγ.

When compared with a CaMKIIα/CaMKIIα homo-oligomeric exchange, the single-molecule TIRF experiment not bulk FRET, it was evident that CaMKIIγ does not behave as well as CaMKIIα in the conditions at which the single-molecule experiment is performed. In order to image single molecules, the CaMKII sample needs to be at nanomolar concentrations during the experiment. Therefore, it was concluded that CaMKIIγ is not suitable for this experiment. [See Chapter 3 for a detailed study of CaMKII holoenzyme stability (Torres-Ocampo et al., 2020).]

We attempted a different approach to the single molecule experiment to observe hetero-oligomeric exchange between CaMKIIγ and CaMKIIα. Due to potential dissociation
issues, the AviTag and linker on the C-terminus of CaMKIIγ was deleted, but it was retained on CaMKIIα (Figure 4C). CaMKIIγ was labeled with Alexa 594 (red) and CaMKIIα-AviTag was labeled with Alexa 488 (green). Only CaMKIIα-AviTag would be affixed to the surface through interaction with streptavidin, so the observance of any red particles would indicate that CaMKIIγ had exchanged into a CaMKIIα particle. After ~40 minutes, and we could very clearly see in the active sample the appearance of many red molecules (211 molecules). The inactive sample had fewer visible red molecules (25 molecules)(Figure 2.4C).

This data supports our previous FRET bulk data in which shows the hetero-oligomeric exchange of CaMKIIγ/CaMKIIα. A complete time course of the experiment still needs to be completed. For the CaMKIIγ/CaMKIIγ homo-oligomeric exchange experiment, other approaches need to be found to test that in another way that does not include diluting CaMKIIγ to nanomolar concentrations, like mass spectrometry or ultracentrifugation.

2.6 Other variables inducing CaMKII subunit exchange

After investigating the exciting dynamic of CaMKIIα and CaMKIIγ during subunit exchange, interrogating the biophysics of subunit exchange was something to investigate further. To date, the data support a model in which subunit exchange is activation-triggered. A higher FRET ratio and a higher level of co-localization in active samples of CaMKII is observed. In the unactivated samples, minimal subunit exchange is observed (Bhattacharyya et al., 2016; M. Stratton et al., 2014). The standard setup for this experiment is adding CaMKII to a final concentration of 5-8 uM and adding ATP 400 uM, Ca^{2+}/CaM 6 uM, Mg^{2+} 8 mM. For the unactivated, it is just the holoenzyme at the same final concentration and buffer.

Thus, in order to test if exchange is facilitated specifically by ATP, a FRET bulk assay was used where ATP was replaced with ATP-γS, a non-hydrolyzable analog of ATP. Surprisingly,
we observed subunit exchange in this sample as well when compared to an active sample (Figure 2.5A and B). Other conditions were tested as well, including ATP-γS + Mg²⁺ with no Ca²⁺/CaM present, and Ca²⁺/CaM + Mg²⁺. Since CaM is not present in these samples, CaMKII should not be active and hence not exchange. In the condition with no ATP present, we also don’t expect CaMKII activation because of ATP absence. Additionally, no background phosphorylation was happening during the expression or purification by doing a western blot for pThr286. We did not detect any phosphorylated Thr286 (A2). Notably, some amount of subunit exchange was detected in all the conditions tried.

As possible explanation for this is that the ATP binding pocket regulates subunit exchange independent of activation. When half-times were calculated, a significant difference was not observed between conditions (Figure 5B). However, when comparing the last time point of the time course, its clear that they have different ratios suggesting that there is still exchange, but something is different (Figure 2.5C).

These experiments shed light on the biophysical properties of CaMKII subunit exchange. Moreover, it shows how much still remains to understand of this process. In order to tease out more of these results, we need do the corresponding single-molecule experiments.

2.7 CaMKIIδ subunit exchange

We wanted to test all CaMKII genes; therefore, we also tested CaMKIIδ. Our previous experiments with CaMKIIα construct had a linker and a C-terminal AviTag (Howarth & Ting, 2008). For CaMKIIδ, we did include the C-terminal AviTag, and, interestingly, we did not observe subunit exchange with itself. However, when we mixed this construct with CaMKIIα with the C-terminal AviTag, we did observe subunit exchange. This piece of data is very encouraging because it is the first evidence showing subunit exchange for CaMKIIδ. However, additional experiments need to be done to confirm subunit exchange within the
CaMKIIδ/CaMKIIδ exchange (single-molecule TIRF) and study more thoroughly how the C-terminal AviTag is affecting subunit exchange and CaMKII dynamics.

2.8 Conclusions

This study was seeking to expand the scope of CaMKII subunit exchange. It was previously shown subunit exchange with CaMKIIα and CaMKIIβ. However, after a few recent publications (Sloutsky et al., 2020) and (Ma et al., 2014), it was time to interrogate if CaMKII subunit exchange is possible in the other CaMKII genes with the previous setup used with bulk FRET and single-molecule TIRF (M. Stratton et al., 2014). CaMKII subunit exchange presents an ideal model for persistent activation in neurons, a mechanism that might be the link to understand long-term memory. Therefore, this publication expands the understanding of CaMKII subunit exchange into CaMKIIγ and CaMKIIδ genes. Hence, in this publication, it is reported that CaMKIIγ can exchange with itself and with CaMKIIα. CaMKIIδ seems to exchange with CaMKIIα but not with itself. The role of the AviTag needs to be studied more carefully.

In order to understand more details about how subunit exchange works, it was investigated by using our FRET bulk assay by adding ATP variants. It was observed that activation might not be necessary for subunit exchange, somewhat an allosteric binding of the ATP pocket, which opens the door to more exciting questions and significantly changes the model of how it was thought that CaMKII subunit exchange works. There is evidence that allostery has a role in CaMKII regulation of kinase activity by the hub (Sloutsky et al., 2020). This was surprising because it was always thought that the hub was just a protein domain responsible for oligomerization. However, with the data presented, it is observed that the kinase domain may affect CaMKII holoenzyme dynamics by occupying the ATP pocket. This study is just beginning to understand how CaMKII is regulated by allostery in various ways. It is crucial to have a solid understanding of CaMKII subunit exchange in vitro
before transferring our studies to neurons and interrogating the possible importance of CaMKII subunit exchange during LTP or other long-term memory-derived mechanisms in neurons.

2.9 Methods

Molecular Biology

Human CaMKII constructs for all CaMKII genes used in this study. They were cloned into a pSMT3 vector with an N-terminal HisSUMO tag (LifeSensors Malvern, PA). The CaMKII variants that have no linker were deleted using Inverse PCR. The point mutations for the FRET labeling were done using QuickChange (Agilent Technologies, Santa Clara, CA). All CaMKIIs used in this study had the following numbering: CaMKIIα (7-475, C290S, C298S, D365C, Δ473-475), CaMKIIδ (1-445, Δlinker, C298S, D335C) and CaMKIIγ (1-445, Δlinker, C298S, D335C).

Protein expression and purification

We used an ÄKTA Pure system for all of the protein purification described below at 4 °C. We used the protocol described previously (Torres-Ocampo et al. 2020). Briefly, we used Tuner (DE-3) pLysS cell with an added plasmid with λ phosphatase. The cells were grown until OD₆₀₀ 0.4-0.6 and then induced with 1 M isopropyl β-D-1 thiogalactopyranoside (IPTG) and grown overnight at 18 °C. Cells were pelleted and resuspended in Buffer A (25 mM Tris, 150 mM KCl, 40 mM Imidazole 10% Glycerol and 1 mM TCEP pH 8.5) plus (0.5 mM Benzamidine, 0.2 mM AEBSF, 0.1 mg/mL trypsin inhibitor, 0.005 mM Leupeptin, 1 μg/mL Aproptinin, 1 μg/mL Pepstatin) plus 1 μg/mL DNase, and then lysed. Then, clarified lysate was run through a 5 mL Ni-NTA column and eluted with 0.5 M Imidazole. Fractions were pulled together and desalted into Buffer C (25 mM Tris, 150 mM KCl, 40 mM Imidazole, 10% Glycerol, and 2 mM TCEP pH 8.5) and left overnight at 4 °C.
to be cleaved by Ulp1. The sample was then loaded into Q-FF 5 mL column and eluted with a KCl gradient. The cleanest fractions were pooled together, concentrated, and loaded on to Superpose 6 gel-filtration equilibrated with 25 mM Tris, 150 mM KCl, 1 mM TCEP, and 10% glycerol pH 8.0). Samples were subsequently labeled and frozen at -80 °C or just directly frozen.

**Labeling CaMKII with fluorescent dyes**

CaMKIIα (7-475, C280S, C289S, D365C, Δ473-475) had a C-terminal AviTag (Howarth and Ting 2008) followed by a GASGASGAS linker. CaMKII was biotinylated during bacterial expression. Therefore, CaMKII was followed by labeling with Alexa Fluor C₅-Maleimide 488 (Cat#A10254, Thermo Fischer) or Alexa Fluor C₅-Maleimide 594 (Cat#A10256, Thermo Fischer). Each dye was resuspended in 25 mM Tris pH 8.0 and had a final concentration of 8 to 5 mM. CaMKII was mixed individually with each dye for a 3x molar excess of dye and left at room temperature for 1.5 to 2 hrs. Labeled CaMKII was desalted into PD-25 columns (Product#28918007, GE Healthcare) with gel filtration buffer (25 mM Tris, 150 mM KCl, 1 mM TCEP 10% glycerol) to get rid of excess dye. Samples were then concentrated using Amicon filters to a final subunit concentration of 40-30 μM, flash frozen and kept at -80 C. We calculated labeled protein concentration using spectra analysis (Nanodrop, Thermo Scientific, DE).

First, we measured CaMKII protein concentration using A₂₈₀. The extinction coefficients for all CaMKII constructs were calculated using: [http://web.expasy.org/protparam/](http://web.expasy.org/protparam/). For each dye, A₂₈₀ was measured as well as their respective spectra for each dye. The extinction coefficient for the Alexa dyes used: 488 ε = 73,000 cm⁻¹ M⁻¹ and 594 ε = 92,000 cm⁻¹ M⁻¹. Labeled protein was scanned after desalting, and A₂₈₀ was corrected for dye contribution. Labeling efficiency was calculated by
[dye]/[protein]. Our labeling efficiencies were 40-80%. A double labeled CaMKII was made as previously described by adding both dyes at the same time. Final protein concentration and labeling efficiency were calculated as the means of both dyes.

**FRET experiments**

For each FRET experiment, labeled CaMKII was mixed to a final concentration of 10-6 μM. To activate CaMKII, we added MgCl₂ [8 mM], Ca²⁺/calmodulin [6 μM], ATP [400 μM] final concentration. Inactive CaMKII was just mixed and diluted with gel filtration buffer. After both samples were mixed, active and inactive, they were incubated at 37 °C or 25 °C. Each time point a sample form the active and inactive were taken to a final concentration of 1 or 0.5 μM in a 20 μL 384 well plate (Corning Cat#3820) and each reading was taken in a Synergy H1 microplate reader (Biotek). A 500-700 nM spectrum was acquired for each time point and experiments were done in triplicates.

**Calculation of FRET ratio**

FRET ratio was calculated by dividing the emission fluorescence (621 nm) of the acceptor divided by the emission of the donor (521 nM). Then, we plotted that value to see how the ratio increased or not, over time.

**Calculation of half-times for FRET bulk assay**

Prism: “One-way ANOVA followed by Dunnett’s multiple comparisons test was performed using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com”.

KaleidaGraph: Nonlinear curve fit was performed using KaleidaGraph version 4.5.4 for Windows, Synergy Software, Reading, PA, USA. [www.synergy.com](http://www.synergy.com).

*Single molecule*
**Functionalization of Coverslips**

Coverslips were functionalized using PEG-PLL and PEG-Biotin (SuSoS AG) as previously described (Stratton et al 2014). Briefly, round glass (Fisher brand cat#12545102) were sonicated for 30 mins in 1% Helmanex III (article # 9-307-011-4-507), rinsed with ddH₂O and sonicated for 30 mins with 1:1 ddH₂O and isopropanol. The coverslips were rinsed ddH₂O and dried. The circular coverslips holders (Thermo Fisher cat#A7816) were cleaned the same way. Then, the coverslips were cleaned for 5 mins in Harrick Plasma PDC-001 plasma cleaner and assembled into the circular holders. Immediately after, we added the 0.25 mL of PEG-PLL and PEG-Biotin (1 mg/mL; 500:3 ratio of the two solutions by volume, SuSoS AG). Incubated for 30 mins at and then washed carefully with 1 mL of imaging buffer. Just before experiment, Streptavidin (Molecular Probes) was added to a final concentration of 0.1 mg/mL and incubated at 25 °C for 10 mins and then washed carefully with imaging buffer 7 times.

**Mixing experiments**

We activated CaMKII as previously described. In order to image at single-molecule resolution, CaMKII was diluted to 5-8 nM concentrations and then added to the functionalized coverslip, let it incubate for 1 min and then washed with imaging buffer.

**Microscope settings**

Single-particle fluorescence imaging was done using the NSTORM/TIRF microscope housed in the UMass IALS Light Microscopy Facility. A total of 10 images randomly chosen 1024x1024 pixel fields of view (164 × 164 μm) were imaged on each coverslip using the 488 nm laser at 1% and 561 at 15% power with an exposure time of 500 ms. Using the Nikon 100X Plan Apo TIRF objective and a Hamamatsu sCMOS camera with a TIRF angle of 60.7.
Co-localization Calculation

After images were acquired, we analyzed with a script written by Dr. Il-Hyung Lee using MATLAB and Statistics Toolbox Release 2012b, The MathWorks, Inc., Natick, Massachusetts, United States. Briefly, each image needs to get converted to tiff file in each channel. They were identified the same name for each time point as A or B, for example picture number 10, for red channel is 10a and for green channel is 10b. Then, a difference of Gaussian (DoG) is used for each molecule. Lastly, a second analysis with coordinates produced by prior analysis to c-localize molecules in both channels. We filtered out by using a 1-2.5 pixel cutoff to differentiate really close molecules vs co-localized molecules.

2.10 Acknowledgements

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Figure 2.1. CaMKII protein domains and holoenzyme architecture. A) CaMKII protein domains: kinase, regulatory segment, linker region and hub domain. B) CaMKII holoenzyme cartoon depiction of a dodecamer/tetradeamer and visualization of a CaMKII dimer.
Figure 2.2. CaMKIIγ shows activation-triggered subunit exchange with FRET bulk assay. A) Schematic of how subunit exchange works. B) CaMKIIγ/CaMKIIγ homo-oligomer shows higher FRET ratio over time (squares, purple) over inactive sample (circles, purple). C) CaMKIIγ/CaMKIIα hetero-oligomer shows the same trend, active (squares, green) vs inactive (circles, green). D) Shows calculated half-times for each FRET experiment, homo-oligomers and hetero-oligomers.
Figure 2.3. CaMKII subunit exchange of homo-oligomeric CaMKIIα/CaMKIIα and CaMKIIγ/CaMKIIγ using single-molecule TIRF. A) CaMKIIα/CaMKIIα subunit exchange comparing 0 mins vs 90 minutes. In both time points, both look similar in amount of molecules imaged and intensity in fluorescence. B) CaMKIIγ/CaMKIIγ subunit exchange 0 mins vs 90 minutes. Fewer molecules are observed at timepoint 0 mins and at 90 minutes, there is almost no molecules left and there is a trend of very bright molecules and very dim molecules compared to the 0 mins timepoint.
Figure 2.4. Hetero-oligomeric subunit exchange CaMKIIγ/CaMKIIα with single-molecule TIRF. A) Set up for the functionalized coverslips with PEG-PLL and PEG-Biotin with single-molecule density streptavidin. CaMKII holoenzymes with an AviTag attached to the coverslip and later imaged with TIRF. B) Snapshot of data collected for the time course for CaMKII subunit exchange, both CaMKIIs with an AviTag C) Schematic of CaMKIIγ with no AviTag and CaMKIIα with AviTag. Meaning, subunit exchange is being measured through the attachment of CaMKIIα to the functionalized coverslip. Time point 0 minutes and 40 minutes are shown.
Figure 2.5. Biophysics of CaMKII subunit exchange. A) FRET bulk assay with ATP analogs. B) The half-times calculated for each condition in the FRET bulk assay. C) Normalized values of the last time point for the FRET-bulk assay, 105 mins, comparing all conditions. Although, the half-times are similar there are differences in percentage of ratio.

A double-labeled sample was used as 100% exchanged sample (gray) and the active sample was (green) was ~82%. The other conditions ATP-γS + Ca^{2+}/CaM + Mg^{2+} (dark blue), ATP-γS + Mg^{2+} (pink) and Ca^{2+}/CaM + Mg^{2+} (light blue) have ~75% or less.
Figure 2.6. CaMKIIδ subunit exchange using FRET bulk assay. A) CaMKIIδ/CaMKIIδ with no linker. Both conditions, active and inactive, show no subunit exchange. B) CaMKIIδ/CaMKIIα, CaMKIIδ with no linker/no AviTag and CaMKIIα with a 30-residue linker and AviTag, the active condition shows subunit exchange.
CHAPTER 3

CHARACTERIZATION OF STABILITY OF CAMKII ALPHA HOLOENZYME

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I collected all mass photometry measurements and made the figures for that. Wrote, analyzed data and conceptualized manuscript.

3.1 Abstract

Ca\textsuperscript{2+}/calmodulin dependent protein kinase II (CaMKII) is a Ser/Thr kinase necessary for long-term memory formation and other Ca\textsuperscript{2+} dependent signaling cascades such as fertilization. Here, we investigated the stability of CaMKII\textalpha using a combination of differential scanning calorimetry (DSC), X-ray crystallography and Mass Photometry (MP). The kinase domain has a low thermal stability (apparent T\textsubscript{m} = 36 °C), which is slightly stabilized by ATP/MgCl\textsubscript{2} binding (apparent T\textsubscript{m} = 40 °C), and significantly stabilized by regulatory segment binding (apparent T\textsubscript{m} = 60 °C). We crystallized the kinase domain of CaMKII bound to p-coumaric acid in the active site. This structure reveals solvent-exposed hydrophobic residues in the substrate-binding pocket, which are normally buried in the autoinhibited structure when the regulatory segment is present. This likely accounts for the large stabilization we observe in DSC measurements.
comparing the kinase alone to the kinase plus regulatory segment. The hub domain alone is extremely stable (apparent $T_m \sim 90 \, ^\circ \text{C}$), and the holoenzyme structure has multiple unfolding transitions ranging from $\sim 60 \, ^\circ \text{C} - 100 \, ^\circ \text{C}$. Using MP, we compared a CaMKII$\alpha$ holoenzyme with different variable linker regions and determined that the dissociation of both these holoenzymes occurs at a higher concentration (is less stable) compared to the hub domain alone. We conclude that within the context of the holoenzyme structure, the kinase domain is stabilized whereas the hub domain is destabilized. These data support a model where domains within the holoenzyme interact.

### 3.2 Introduction

Ca$^{2+}$/calmodulin dependent protein kinase II (CaMKII) is a Ser/Thr kinase known to be a central signaling molecule in many systems throughout the body. In humans, CaMKII is encoded by four separate genes: CaMKII$\alpha$, $\beta$, $\gamma$ and $\delta$. CaMKII$\alpha$ and $\beta$ are found predominantly in neurons and play a crucial role in long-term memory formation (Herring & Nicoll, 2016b; Rossetti et al., 2017b) CaMKII$\delta$ is expressed in cardiomyocytes and contributes to cardiac pacemaking (Backs et al., 2009; T. Zhang et al., 2007). CaMKII$\gamma$ is found throughout the body in smooth muscle, skeletal muscle and elsewhere, and also plays an essential role in eggs during fertilization (Escoffier et al., 2016; Yoon et al., 2008). All CaMKII genes are comprised of four domains: kinase, regulatory segment, variable linker region, and hub (Figure 2.1A). The kinase domain and regulatory segments are $\geq 90\%$ identical between the four genes and the hub domains are $\geq 75\%$ identical. CaMKII is activated by Ca$^{2+}$/calmodulin (Ca$^{2+}$/CaM) competitively binding to the regulatory segment and dislodging it from the substrate-binding site, making the pocket accessible. The structure of CaMKII has recently been reviewed (Bhattacharyya et al., 2019).
The hub domain of CaMKII is responsible for oligomerization. In this series of experiments, we will focus on human CaMKIIα specifically. The CaMKIIα hub domain alone (Bhattacharyya et al., 2016) and the CaMKIIα holoenzyme (Chao et al., 2011) were both crystallized as dodecameric assemblies. There is agreement in recent studies using both single particle negative stain and cryo-electron microscopy (EM) that autoinhibited CaMKIIα holoenzymes form both dodecamers and tetradecamers (Bhattacharyya et al., 2016; Myers et al., 2017; Sloutsky et al., 2020). There is evidence from the full-length CaMKIIα (with no variable linker) crystal structure (Chao et al., 2011) and single particle cryo-EM (Sloutsky et al., 2020) that the kinase domains directly contact the hub domain.

The hub domain organizes the kinases into an oligomeric conformation, which confers several unique biophysical properties to the enzyme including highly cooperative and frequency-dependent activation, and activation-dependent exchange of subunits between holoenzymes. CaMKII activation is highly cooperative, where binding of one Ca²⁺/CaM potentiates adjacent subunits to bind Ca²⁺/CaM, likely due to interactions between subunits within the holoenzyme ring (Chao et al., 2010; Gaertner et al., 2004; Rosenberg et al., 2005). It has been shown in vitro that CaMKII has a threshold frequency for activation; meaning that above a specific frequency of Ca²⁺, CaMKII will turn on, but below this frequency it will remain off (Bayer et al., 2002; Chao et al., 2011; Paul & Schulman, 1998). Stochastic kinetic simulations suggest that this frequency response is a product of the rates of kinases binding to the hub domain, Ca²⁺/CaM binding to the regulatory segment, and Thr286 transphosphorylation (Chao et al., 2011).

CaMKII activity biosensors, Camui (Takao et al., 2005) and FRESCA (FRET sensor for CaMKII activity) (Ardestani et al., 2019), have been used to monitor CaMKII activity in response to Ca²⁺ oscillations in live cells. These studies indicate that also in vivo, CaMKII activation is cooperative and varies depending on the frequency of
Finally, Ca\textsuperscript{2+}/CaM binding and subsequent phosphorylation of Thr286 results in autonomous CaMKII activity and induces exchange of subunits between holoenzymes (Bhattacharyya et al., 2016; M. Stratton et al., 2014). Significant evidence supports a mechanism where the regulatory segment interacts with the hub domain, resulting in the release of a vertical dimer unit, which can then assemble into a nearby CaMKII holoenzyme (Bhattacharyya et al., 2016; M. Stratton et al., 2014).

Structural regulation of CaMKII activity appears to depend on the combination of variable linker and hub identity. In CaMKII\textalpha, completely removing the variable linker region makes the enzyme much harder to activate (i.e., requires more Ca\textsuperscript{2+}/CaM for activation) (Chao et al., 2011; Sloutsky et al., 2020). Intriguingly, attaching a CaMKII\textalpha kinase domain to the hub domain of CaMKII\textbeta with no linker makes the enzyme easier to activate (Sloutsky et al., 2020). Little is known about the assembly or disassembly of CaMKII holoenzymes, other than the assertion that the vertical dimer is likely the unit of subunit exchange, and possibly also the unit of assembly. Motivated to further understand how the multiple domains of CaMKII influence one another from a biophysical perspective, herein we focus on understanding the stability of the CaMKII\textalpha holoenzyme.

3.3 CaMKII protein domain stability

We employed differential scanning calorimetry (DSC) to measure the thermal stability of CaMKII\textalpha domains in order to dissect how each domain affects the overall stability of the holoenzyme (Fig. 3.2, 3.5). We separately measured the melting temperatures of the kinase domain, kinase domain with regulatory segment, hub domain, holoenzyme with no linker and holoenzyme with a 30-residue linker (CaMKII\textalpha(14,18)). The nomenclature (CaMKII\textalpha(14,18)) designates that this variant of CaMKII\textalpha includes exons 14 and 18, which results in a 30-residue linker. For simplicity, since
we are only using two variants of CaMKIIα, we will refer to these as CaMKIIα-0 (no linker version) and CaMKIIα-30 (30-residue linker. The transcripts of both of these variants are found in human hippocampus (Sloutsky et al., 2020).

We first measured the kinase domain alone, and the maximum temperature of unfolding was 36 °C (Fig. 3.2A). Adding the regulatory segment to the kinase domain stabilized it by a remarkable 24 degrees (apparent $T_m = 60$ °C). The hub domain alone was extremely stable, with the majority of the sample unfolding at 90 °C. There is an additional transition that is stabilized by ~8 degrees (apparent $T_m = 98$). Fusing the kinase domain to the hub domain with no variable linker region (CaMKIIα-0) results in a transition very similar to that of the kinase domain plus regulatory segment (apparent $T_m = 57$ °C) (Fig. 3.2B). In the presence of a 30-residue disordered variable linker region (CaMKIIα-30), there is a small stabilization by 2 degrees (apparent $T_m = 59$ °C).

Both CaMKIIα-0 and CaMKIIα-30 had additional transitions that are approximately the same maximum temperature of unfolding as the hub domain alone (Fig. 3.2C). These transitions had significantly lower enthalpy ($\Delta H$) compared to the major transition around 56-58 °C. CaMKIIα-30 has an additional transition ($T_m = 81$ °C) with a similar $\Delta H$ as the most stable transition ($T_m = 93$ °C) (Fig. 3.2B).

### 3.4 Crystal structure of the CaMKII kinase domain

We crystallized the CaMKII kinase domain with an inactivating mutation (D135N) and lacking the regulatory segment (Figure 3.3, PDB: 6VZK). In the structure, the ATP binding site is occupied with $p$-coumaric acid, which was present in the silver bullet additive in our screen. ATP binding stabilizes the kinase domain by 4 °C ($T_m = 40$ °C) (Fig. 3.3B). The benzene ring of $p$-coumaric acid mimics where the adenine base of ATP would be oriented. The carboxylic acid tail of $p$-coumaric acid is in close contact with K42 (2.87 Å) and
D156 (3.34 Å). This lysine (K42 in CaMKIIα) is highly conserved among protein kinases to facilitate ATP binding, specifically phosphate group orientation (Gibbs & Zoller, 1991; Knighton et al., 1991). The hydroxyl group of p-coumaric acid is in contact with the backbone of V92 (3.27 Å and 2.85 Å).

In its auto inhibited state, the helical CaMKII regulatory segment occupies the substrate binding site (PDB: 2VZ6) (Rellos et al., 2010). This binding pocket on the kinase domain contains several hydrophobic residues, which interact with L290 on regulatory segment, protecting these residues from the solvent (Rellos et al., 2010) (Fig. 3.3C). While F98, I101 and I205 residues are completely buried, V102 is only partially buried as one gamma carbon atom is still exposed. In the absence of the regulatory segment, these hydrophobic residues are solvent exposed, shown in our structure (Fig. 3.3D). Similar to the active conformation (PDB: 2WEL (Rellos & al., 2010)), the αD helix reorients toward where the regulatory segment was bound (Fig. 3.6). This movement of the αD helix leaves the hydrophobic amino acid patch unoccupied, resulting in complete exposure to the solvent (Fig. 3.3D).

3.5 Analysis of CaMKII dissociation using mass photometry

We employed mass photometry (MP) to analyze the dissociation of CaMKII oligomers (Young et al., 2018). We measured the molecular weight (MW) of single molecules of CaMKII in solution over a concentration range to determine the concentration at which CaMKII oligomers dissociate. We used MP to compare the CaMKIIα hub domain alone to the full-length holoenzyme with and without a variable linker region (CaMKIIα-30 and CaMKIIα-0, respectively) over a concentration range of 100 nM to 45 nM (subunit concentration) (Fig. 3.4, for raw count profiles see Fig. 3.7-3.9). For all replicates, we observed populations of
molecules at high MW values (200-600 kDa). For most samples, we also observed the clear emergence of a population of molecules at low MW values (50-200 kDa).

The hub domain was the best-behaved sample across all concentrations tested (Fig. 3.4A, 3.7). At 100 nM subunit concentrations, there was a clear major population of molecules with an average MW of 239±2 kDa, which corresponds roughly to a 16mer (hub subunit = 15.3 kDa). This large oligomeric species was still intact even at 45 nM. The size distribution of large hub oligomers is centered on 16-18 subunits per molecule at these concentrations (Fig. 3.10A). At all concentrations except 90 nM, there was an additional population of molecules at a lower MW (~80-100 kDa). The ratio of molecules in the high MW population compared to low MW population remained high (22-fold) across all samples.

Both versions of the CaMKII holoenzyme tested were less stable than the hub domain alone (Fig. 3.4B and 3.4C). At concentrations of 90-100 nM, CaMKIIα-30 had a major population of molecules at a high MW: 519±23 kDa, which roughly corresponds to a 10mer (CaMKIIα-30 subunit = 53.1 kDa) (Fig. 3.10B). At these same concentrations, CaMKIIα-0 also had a major population of molecules at a high MW: 606±50 kDa, which roughly corresponds to a 12mer (CaMKIIα-0 subunit = 50.4 kDa) (Fig. 3.4C, 3.10C).

CaMKIIα-30 and CaMKIIα-0 showed an increase in the number of low MW molecules across the dilution series. For CaMKIIα-30, the low MW population was evident at 100 nM, and increased relative to the high MW population at the lowest concentrations tested (45 and 60 nM). The majority of the low MW species were roughly 95 - 245 kDa, corresponding to a dimer - pentamer. Similarly for CaMKIIα-0, the low MW population increased at 75 nM and continued to increase at the lowest
concentrations tested (60 and 45 nM). The majority of the low MW CaMKIIα-0 species were 77 - 151 kDa, corresponding to a monomer/dimer – trimer.

From our MP measurements, we summed the total number of molecules within specific MW ranges consistent with the size of larger oligomers (Fig.3.11, see Materials and Methods for calculation details). For the hub domain samples, the percentage of high MW molecules does not change over the concentration range we tested. This indicates that the K_d for the hub domain alone must be lower than that of the holoenzyme. For CaMKIIα-30 and CaMKIIα-0, there is a clear increase in the percentage of high MW molecules over this concentration range, indicating that we are sampling near the K_d.

3.6 Conclusions

It has been known that CaMKII is a large protein assembly since 1983, when it was determined to be a dodecamer (Bennett et al., 1983). However, there is still little known about the assembly and disassembly of CaMKII, including estimates for K_d. This has been a difficult question to tackle due to technical limitations in addition to indirect evidence suggesting CaMKII oligomerization must have a very low K_d. Our stability measurements combined with mass photometry shed new light on this old problem.

Our DSC data show that the hub domain alone is extremely stable, with the majority unfolding around 90 ºC (Fig. 3.2). The second transition has a 2.5 fold lower ΔH and is stabilized to 100 ºC. This transition is reproducible between all samples, which may represent a final unfolding transition such as monomer unfolding. Further experiments are needed to fully elucidate this. Conversely, the kinase domain is thermally unstable, with the majority unfolding around 36 ºC. This is similar to that measured for Mitogen Activated Protein kinase (Krishna et al., 2013).
Addition of the regulatory segment to the CaMKII kinase domain results in a remarkable 26 °C stabilization. Our crystal structure of the CaMKII kinase domain shows several hydrophobic residues (F98, I101, V102, I205) that are solvent exposed in the absence of the regulatory segment (Fig. 3.3). This is likely an entropic cost that accounts for the stability difference we observe. This hydrophobic pocket is largely responsible for substrate binding; further experiments would be needed to test whether substrate binding (or inhibitors such as CaMKIIIn tide) results in a similar stability shift. The crystals were obtained without any nucleotide added, however, an additive (p-coumaric acid) was resolved in the ATP-binding site. As we suspected, and is true for other kinases (Bauer et al., 2019), ATP/MgCl₂ binding stabilizes the kinase domain by 4 °C (Fig. 3.3B). Adding MgCl₂ without ATP may also have an effect on stability, which will be tested in future studies.

CaMKII holoenzyme stability falls in the middle of these two domains, indicating significant interactions and influence between domains within the oligomer. For CaMKIIα-30 and CaMKIIα-0, the first large transition around 56 °C (~40-60 kJ/mol) is likely the kinase domains unfolding. Included in this transition may also be dissociation of the hub domain into dimers. The second and third unfolding transitions, which produced a smaller heat release (~10-15 kJ/mol), may indicate the unfolding of hub dimers, or intact hub domains with unfolded kinase tails. Interestingly, the first transition is comparable between CaMKIIα-30 and CaMKIIα-0 whereas the right-shifted transitions are not exactly overlapping (Fig. 3.2C). This may indicate that the 30-residue variable linker region additionally destabilizes the hub domain to induce unfolding of some hub domains at a lower temperature (transition 2, 81 °C).
Structures of the human hub domains have revealed both 6-fold and 7-fold symmetries, forming dodecamers or tetradecamers (Bhattacharyya et al., 2016; Rellos et al., 2010; Rosenberg et al., 2006). More recently, CaMKII hub-like domains from green algae have been shown to assemble into 16-20mers (McSpadden et al., 2019). In our MP measurements with human CaMKIIα hub, the high MW population roughly corresponds to a 16mer. It is clear that this oligomeric complex is very stable since it does not dissociate significantly at 45 nM (Fig. 4A, S7). Though, it is clear that at 45 nM that additional populations are increasing in the sample, suggesting that we are approaching the $K_d$. Further experiments are needed to determine whether these hubs are actually forming 16mers at these concentrations and also to determine the $K_d$.

Structures of human CaMKII holoenzymes have also revealed both 6-fold and 7-fold symmetries (Bhattacharyya et al., 2016; Chao et al., 2011; Myers et al., 2017; Sloutsky et al., 2020). The crystal structure of CaMKIIα-0 showed a dodecamer with all the kinases docked onto the hub domain (Chao et al., 2011). Cryo-EM studies of CaMKIIα-30 showed that both stoichiometries are adopted, in addition to populations where the kinase is docked onto the hub domain in different orientations (Sloutsky et al., 2020). In our MP measurements of CaMKIIα-0, the high MW population corresponds to 12mer/14mer, which is consistent with existing data. For CaMKIIα-30, we observe a high MW population corresponding to 10mer/12mer. Decameric CaMKII assemblies have been observed in solution-based anisotropy measurements (Sarkar et al., 2017) and negative stain EM (Kanaseki T, Ikeuchi Y, Sugiura H, 1991). Since CaMKIIα-30 has a disordered region and likely leads to a myriad of possible conformations for all kinase domains, this flexibility may affect the contrast used to calculate MW in the
interferometric scattering microscopy (iSCAT) method. It is also unknown how dilution may affect oligomerization, since all structural studies are done at significantly higher concentrations by necessity. Regulation and implication of CaMKII stoichiometry is still largely not understood and will be the focus of future studies.

As CaMKII dissociates into smaller oligomers, interfaces that are normally buried must become solvent exposed. The solubility of these species is likely low. We suspect that the low MW molecules we measured are underrepresenting what is present in the solution due to adsorption to other surfaces, or aggregation. In order to calculate the $K_d$, it will be necessary to screen for conditions that stabilize these dissociated species but do not interfere with the contrast optics of the MP setup. In this way, an accurate number of low and high MW molecules can be calculated at equilibrium. However, our measurements indicate that CaMKII dissociation may be cooperative. This is most evident in the CaMKIIα-0 dilution series where the high MW population clearly starts to shift to 1-2 low MW populations (Fig. 3.4C). Perhaps as CaMKII dissociates it preferentially forms dimers and tetramers, as has been predicted from subunit exchange experiments, discussed below. The MP data are not as clear for CaMKIIα-30, which may either be because the dissociation kinetics are different, or because the variable linker region further destabilizes the released subunits, resulting in noisy measurements. As mentioned above, there is a hint of hub destabilization seen in the DSC measurement of CaMKIIα-30, where there is a transition at a lower $T_m$ ($81 \, ^\circ C$) compared to CaMKIIα-0. Stabilizing these species will help us clarify this.

Based on our measurements, we have roughly estimated $K_d$ values of $\sim 10-20 \, nM$ for CaMKII oligomerization. These new data have clear implications on
understanding the mechanism of CaMKII subunit exchange (Bhattacharyya et al., 2016; Singh & Bhalla, 2018; M. Stratton et al., 2014). The concentration of holoenzymes in dendritic spines is estimated to be 100 µM (Otmakhov & Lisman, 2012), indicating that complete dissociation of oligomers on a reasonable timescale in spines is unlikely. As previously discussed, the proposed mechanism for subunit exchange relies on holoenzyme activation by Ca²⁺/CaM followed by induced release of a vertical dimer by interaction between the regulatory segment and hub domain. Future MP experiments measuring active CaMKIIα will indicate whether activation also affects dissociation, and thereby potentially facilitates subunit exchange.

Importantly, subunit exchange measurements were largely performed using single-molecule fluorescence measurements (M. Stratton et al., 2014). The concentration of CaMKII used during the exchange experiments was ~5 µM. In order to make the single-molecule measurement, the sample was diluted to ~2 nM, affixed to a glass surface, and imaged. Given that this dilution is slightly below the apparent K₆, the total number of exchange events may have been underestimated due to some dissociation under these conditions. However, in order to further dissect this, future MP experiments will be geared towards taking time points under equilibrium conditions to determine the rate of dissociation.

Both the DSC and MP measurements suggest that the kinase and hub domain influence one another and do not behave like beads on a string. This is in line with structural studies showing interactions between the kinases and the hub domain (Chao et al., 2011; Sloutsky et al., 2020). Additionally, when comparing kinase activity between CaMKIIα and CaMKIIβ with no linker, there are significant differences in EC₅₀ value for
Ca\(^{2+}\)/CaM, indicating that different structures are being adopted (Sloutsky et al., 2020). It is logical that these two covalently linked domains have evolved ways to interact (Kuriyan & Eisenberg, 2007).

It will be interesting to test the remaining three CaMKII variants in terms of temperature stability and dissociation parameters. There are sufficient differences between the hub domain sequences (>80% identical) that may provide changes in oligomerization kinetics and stability. This may have further implication on understanding mixed oligomers, which are known to exist in the brain (Shen et al., 1998), and likely exist in other tissues as well.

3.7 Methods

**Molecular biology**

The WT CaMKII\(\alpha\) kinase, CaMKII\(\alpha\) kinase + regulatory segment and both CaMKII\(\alpha\) holoenzymes (with and without linker) were cloned into a vector containing an N-terminal His-SUMO tag. The CaMKII\(\alpha\) hub was cloned into a pET-28 vector with an N-terminal 6xHis-precission tag using Gibson assembly.

**Protein purification**

WT CaMKII\(\alpha\) kinase domain (residues 7-274, with and without D135N) and CaMKII\(\alpha\) kinase + regulatory segment (residues 7-314) were grown and co-expressed in *E. coli* BL21 with \(\lambda\) phosphatase. The cells were induced at 18 °C with 1 mM Isopropyl \(\beta\)-d-1-thiogalactopyranoside (IPTG) and grown overnight. The cells were then suspended using Buffer A (25 mM Tris, pH 8.5, 150 mM KCl, 40 mM imidazole, 10% glycerol), commercially available protease inhibitors (0.5 mM Benzamidine, 0.2 mM AEBSF, 0.1 mg/mL trypsin inhibitor, 0.005
mM Leupeptin, 1 μg/mL aproptinin, 1 μg/mL pepstatin) plus 1 μg/mL DNase. Purification was done at 4 °C using utilizing a ÄKTA Pure system (GE). Clarified lysate was loaded onto a Ni-NTA column and eluted with an imidazole gradient, desalted into Buffer C (25 mM Tris pH 8.8, 50 mM KCl, 40 mM imidazole, 2 mM TCEP, 10% glycerol) and cleaved overnight with Ulp1 at 4 °C. Following a subtractive step, the sample was then bound to a HiTrap Q FF column (GE) and eluted with a KCl gradient. Samples were concentrated and further purified and desalted using a Superdex 75 column into DSC buffer (25 mM Tris pH 8, 150 mM KCl, 1 mM TCEP, and 10% glycerol). The pure protein was then frozen and stored at -80 °C.

CaMKIIα-0 (7-475, Δ324-354) and CaMKIIα-30 (7-475) were co-expressed with λ phosphatase using Rosetta (DE-3)pLysS cells. They were purified as previously described. Holoenzymes were purified using a Superose 6 column (GE). All purified holoenzymes eluted consistently (retention volume 12 mL). Retention volumes were consistent with standards as well as other samples, which have been verified as holoenzymes using cryo-electron microscopy.

The CaMKIIα hub domain (residues 315-475) was expressed in BL21 cells and purified as described except a different Buffer C (25 mM Tris pH 8.8, 150 mM KCl, 1 mM EDTA, 2 mM DTT, 10% glycerol) to facilitate Prescission protease cleavage overnight at 4 °C. Samples were eluted from the Superose 6 column into DSC buffer. All purified hubs eluted consistently (retention volume 14 mL). Pure protein samples were frozen and stored at -80 °C.

Differential Scanning Calorimetry
All protein samples were diluted to 0.5 mg/mL in DSC buffer (25 mM Tris pH 8, 150 mM KCl, 1 mM TCEP, 10% glycerol). DSC measurements were performed on a MicroCal Automated PEAQ-DSC instrument (Malvern Panalytical, Westborough, MA). For the CaMKIIα kinase + ATP/MgCl₂, ATP (1 mM) and MgCl₂ (10 mM) were used and added to the buffer blank as well. Unless otherwise indicated, after a 5 min pre-scan equilibration step, samples were scanned from 10-120 °C at a scan rate of 90 °C/hr with no feedback. Data were analyzed using MicroCal PEAQ-DSC software, and baseline-subtracted data were fit to a non-two-state fitting model to obtain apparent Tₘ values.

Crystallization, data collection and structure determination

Crystals of CaMKII kinase domain (residues 7-274) with an inactivating mutation (D135N) were grown at 4 °C using hanging drop vapor diffusion. An additional mutation in this structure is Q223K, which was acquired during the cloning process. This residue is solvent-exposed on the αG helix, and comparing this structure to previous structures with Q223 shows no effect on the fold or orientation. Crystals were obtained in the following condition: 8% PEG 6000, 50 mM HEPES, pH 7.0 and H10 condition of the Silver Bullets screen (1:10 ratio) containing: 0.16% w/v 3-Aminobenzene-sulfonic acid; 0.16% 5-Sulfosalicylic acid dihydrate; 0.16% w/v p-Coumaric acid; 0.16% PIPES; 0.16% Terephthalic acid; 0.16% Vanillic acid; Buffer 0.02 M HEPES pH 6.8. The crystal was cryo-protected in 20% (v/v) ethylene glycol prior to being frozen in liquid nitrogen. Diffraction data were collected at a wavelength of 1.5418 Å using a Rigaku MicroMax-007 HF X-ray source, which was coupled to a Rigaku VariMax HF optic system (UMass Amherst).
The X-ray data were collected at 100 K. Data sets were integrated, merged and scaled using HKL-2000 (Otwinowski & Minor, 1997). The structure was solved by molecular replacement (MR) with Phaser (McCoy et al., 2005) using the coordinates of the kinase domain as an initial search model (PDB ID: 3SOA (Chao et al., 2011)). Model building was performed using (Emsley & Cowtan, 2004) Coot and refinement was performed with REFMAC (Winn et al., 2003).

**Mass Photometry**

All MP experiments were carried out using an OneMP mass photometer (Refeyn LTD, Oxford, UK) at room temperature and used Acquire MP software for data collection. Coverslips (cat #630-2105) were cleaned by rinsing with a series of H2O, ethanol, and isopropanol (HPLC grade), and then dried under a clean stream of nitrogen. Gaskets (catalog #CW-50R-1.0) were cleaned using H2O and ethanol, then dried under nitrogen. The gasket was then sealed onto the center of the glass coverslip. The coverslips were stored in a sterile container until used. 10 µl of buffer (25 mM Tris, 150 mM KCl and 1 mM TCEP at pH 8.0) was added to the coverslip and focused. Images were collected in an area of 3 x 10 µm at a frame rate of 1 kHz. Protein stock solutions (2 µM) were diluted to 200 nM (25 mM Tris, 150 mM KCl and 1 mM TCEP at pH 8.0) right before the experiment. Proteins were further diluted to achieve the following concentrations in the measurements: 100 nM, 90 nM, 75 nM, 60 nM and 45 nM. Three replicates were performed at each concentration. Collection time was varied to acquire the same number of binding events over the dilution series. Since the technical limit of the instrument at low MW
is 50 kDa, we will not be able to fully resolve monomeric hub subunits (15.3 kDa) (Wu & Piszczek, 2020)

MP Data Analysis

DiscoverMP software was used for all analyses using standard settings. Each replicate was analyzed individually. Only molecules that bound to the glass surface were counted. Populations were automatically fit with a Gaussian distribution (Young et al., 2018). For these populations, the mean Gaussian values from replicates were averaged to calculate an average MW, these values are reported ± the standard deviation between three replicates. Some low MW populations could not be fitted for a Gaussian distribution due to insufficient sampling of multiple populations. In order to quantify the relative number of low vs. high MW species in the total number of molecules, we established MW cutoffs in line with replicates where low and high MW populations were Gaussian distributed.

The MW ranges for low and high MW species used are as follows. CaMKIIα-hub: low = 14-161 kDa; CaMKIIα-0: low = 50-400 kDa, high = 400-800 kDa; CaMKIIα-30: low = 50-300 kDa, high = 400-800 kDa. These ranges were applied to the following samples. CaMKIIα-hub: low = 90, 75, 60 45 nM; CaMKIIα-0: low = 100, 90, 60 nM, and one replicate of 45 nM, high = 45 nM; CaMKIIα-30: low = 90, 75, 60 nM, 45 nM, high = 45 nM. For all other samples, the number of molecules was defined by the Gaussian fit.

3.8 Acknowledgements
We would like to thank Dr. Matthias Langhorst and Dr. Sofia Ferreira for their technical assistance and helpful discussion of mass photometry data. Thanks to Dr. Roman Sloutsky for helpful discussions and manuscript editing.
Figure 3.1 CaMKII architecture. (a) Linear depiction of the CaMKII subunit organization. Domain colors are consistent throughout the figure. (b) Ca^{2+}/calmodulin competitively binds the regulatory segment to expose the substrate-binding pocket and activate the enzyme. This allows for Thr286 to be trans-phosphorylated. (c) Cartoon depictions of the hub domain alone, CaMKIIα-0 (compact conformation), and CaMKIIα-30 (extended conformation). CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II.
Figure 3.2 Differential scanning calorimetry of CaMKII domains. Representative traces are shown as well as the average apparent $T_m$ rounded to the nearest integer calculated from all replicates (see replicate datasets in Figure 3.5). (a) From left to right: overlays of CaMKIIα kinase domain without (gray) and with (purple) regulatory segment and the CaMKIIα hub only (burgundy). (b) Overlays of CaMKIIα-0 (blue) and CaMKIIα-30 (green). (c) Overlays of all traces for comparison. CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II.
Figure 3.3 Crystal structure of the CaMKII kinase domain. (a) Kinase domain alone crystallized bound to p-coumaric acid. Inset (bottom) highlights residues coordinating p-coumaric acid. (b) DSC comparing kinase alone with no ATP or MgCl₂ (gray, shown in Figure 2a) to kinase domain plus 1 mM ATP/10 mM MgCl₂ (orange). (c) Crystal structure of kinase domain plus regulatory segment (PDB: 2VZ6). Hydrophobic residues on the kinase domain (dark blue, labeled in 3D) are buried by the regulatory segment, specifically interacting with L290 (cyan). Surface representation is shown below at a 90° rotation. (d) Hydrophobic residues on the kinase domain (dark blue) are solvent exposed. Surface representation is shown below at a 90° rotation. CaMKII, Ca²⁺/calmodulin-dependent protein kinase II; DSC, differential scanning calorimetry.
Figure 3.4 Mass photometry analysis of CaMKII variants. Overlays of three replicates for each concentration measured (100, 90, 75, 60, and 45 nM) are shown for CaMKIIα-hub (a), CaMKIIα-30 (b), and CaMKIIα-0 (c). Density is plotted against mass (kDa). Raw counts were converted to kernel density, which is used to better depict multistep processes. CaMKII, Ca$^{2+}$/calmodulin-dependent protein kinase II.
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Figure 3.5. DSC replicates

The raw data and corresponding fits for all DSC replicates are shown. Apparent $T_m$ values are listed for all observed transitions. A) kinase domain alone, B) hub domain alone, C) CaMKIIα-0, D)CaMKII-30, E) kinase + regulatory segment, F) kinase + ATP/MgCl$_2$.

Figure 3.6. Reorientation of the αD helix

Overlay of the crystal structures of kinase domain alone (magenta) and kinase domain plus regulatory segment (gray) where the regulatory segment has been removed for clarity. The αD helix shifts outward by ~18°, indicated by the arrow.
Figure 3.7. Raw MP data: CaMKIIα hub

Replicates for CaMKIIα-hub at each concentration: 100 nM, 90 nM, 75 nM, 60 nM and 45 nM. Total counts (# molecules) are plotted against mass (kDa).
Figure 3.8. Raw MP data: CaMKIIα-30
Replicates for CaMKIIα-30 at each concentration: 100 nM, 90 nM, 75 nM, 60 nM and 45 nM. Total counts (# molecules) is plotted against mass (kDa).

Figure 3.9. Raw MP data: CaMKIIα-0
Replicates for CaMKIIα-30 at each concentration: 100 nM, 90 nM, 75 nM, 60 nM and 45 nM. Total counts (# molecules) is plotted against mass (kDa).

Figure 3.10. Size estimation of CaMKII variants from Mass Photometry

The X-axis from the raw data sets (Fig. S3-S5) was converted to number of subunits.

We set mass ranges for the size of a CaMKII dimeric unit (110 kDa for holoenzymes,
30 kDa for the hub domain) and then calculated the number of molecules within these ranges (plotted on the Y-axis as counts).

**Figure 3.11. CaMKIIα oligomer dissociation.** The percentage of high MW molecules (large oligomer species) is shown over a concentration range for the hub alone, CaMKIIα-30 and CaMKIIα-0.
CHAPTER 4

Studies of CaMKII oligomerization domain and holoenzymes in solution

Torres-Ocampo Ana P. Özden Can, Samkutty Alfred, Flaherty Daniel, Esposito Edward and Stratton Margaret M.

I performed all mass photometry experiments, made all the figures, analyzed data, conceptualized and wrote manuscript.

4.1 Abstract

Ca²⁺/calmodulin dependent protein kinase II (CaMKII) is an oligomeric complex required for memory formation and other cellular cascades in the body. The CaMKII holoenzyme has a kinase domain, regulatory segment, and hub domain that allows it to oligomerize into a 12-mer or 14-mer. The hub domain alone has been structurally characterized as a 12-mer, 14-mer, and 16-mer. In comparison, the CaMKII holoenzyme is a 12-mer and 14-mer. Using mass photometry (MP) and differential scanning calorimetry (DSC), the hub domain dissociation pattern was correlated to its thermal stability. It was observed that with a lower melting temperature, there was higher dissociation of the hub domain. The CaMKII holoenzyme size was correlated to its degree of Ca²⁺ sensitivity at the same concentration. CaMKII holoenzymes that were 14-mer were shown to have a lower Ca²⁺ sensitivity while 12-mer had a higher Ca²⁺ sensitivity. Surprisingly, the CaMKII chimeras holoenzymes also confirmed that the hub domain determines the size of the holoenzyme and predicts Ca²⁺ sensitivity. This study is the first characterization of CaMKII oligomerization in solution and how this may regulate dissociation and Ca²⁺ sensitivity.
4.2 Introduction

Ca\(^{2+}\)/calmodulin dependent protein kinase II (CaMKII) is a Ser/Thr oligomeric kinase. CaMKII has a kinase domain, regulatory segment and hub or oligomerization domain (Fig 4.1A) CaMKII has four genes in the human body: CaMKII\(\alpha\), CaMKII\(\beta\), CaMKII\(\gamma\) and CaMKII\(\delta\). CaMKII is crucial to several organs in the body, including brain, eggs, and heart muscle (Backs et al., 2009; Herring & Nicoll, 2016a; Yoon et al., 2008). Historically, CaMKII\(\alpha\) and CaMKII\(\beta\) are implicated in memory formation (Herring & Nicoll, 2016a). However, there are mRNA expression profiles in hippocampal neurons derived from all four variants (Sloutsky et al., 2020). There is evidence that CaMKII\(\gamma\) and CaMKII\(\delta\) are involved in neuronal signaling pathways (Ma et al., 2014; Zalcman et al., 2019). Moreover, mutations in neuronal CaMKII\(\delta\) show severe intellectual disabilities phenotypes in humans (unpublished data, Geeske M.van Woerden). This opens up the question about the specific roles of each CaMKII in neurons and the calcium driven cellular cascades there.

If all four CaMKII are present in neurons, how are they regulated, and how can it be teased out which CaMKII is participating in what pathway? One possibility is by looking at their EC\(_{50}\) for Ca\(^{2+}\)/CaM. The EC\(_{50}\) is a measurement of required Ca\(^{2+}\)/CaM for half-maximal activation for CaMKII. CaMKII holoenzyme requires Ca\(^{2+}\)/CaM for activation so that Thr286 can be auto-phosphorylated and turn one kinase activity. Consequently, in a previous publication, the EC\(_{50}\) for Ca\(^{2+}\)/CaM for each CaMKII\(\alpha\) and CaMKII\(\beta\) and their corresponding splicing variants were elucidated (Sloutsky et al., 2020). The EC\(_{50}\) of CaMKII\(\gamma\) and CaMKII\(\delta\) with no linker were also reported. Surprisingly, it was observed that EC\(_{50}\), or Ca\(^{2+}\) sensitivity, tracked with the hub evolutionary divergence (Sloutsky et al., 2020). CaMKII\(\alpha\) (EC\(_{50}\) = 313 nM) and CaMKII\(\delta\) (EC\(_{50}\) = 167 nM) are closely related and showed a similar EC\(_{50}\) (these measurements were done with a no linker variant). CaMKII\(\beta\) (EC\(_{50}\) = 21 nM) and CaMKII\(\delta\) (EC\(_{50}\) = 28) are closely as well and also showed a similar EC\(_{50}\). CaMKII chimeras, \(\alpha\) kinase \(\beta\)
hub, and β kinase α hub were also tested, and their $EC_{50}$ measurement helped reveal an allosteric role for the hub for kinase activity. CaMKII α kinase β hub showed an $EC_{50} = 24$ nM and CaMKII β kinase α hub showed an $EC_{50} = 102$ nM. This means that despite the CaMKII kinase domain that the CaMKII hub is attached to, the CaMKII hub regulates $EC_{50}$.

This finding was quite surprising because the hub is known for being the oligomerization domain of the CaMKII holoenzyme but somehow its also regulating kinase activity. This led us to study the oligomerization state of the CaMKII hub and CaMKII holoenzyme and interrogate if its also involved in regulating kinase activity. The oligomerization states of CaMKIIα holoenzymes and the hub domain alone have been measured using mass photometry (Torres-Ocampo et al., 2020). Mass photometry is a technique that allows measuring a macromolecule molecular weight with high accuracy in solution (Young et al., 2018). The CaMKIIα hub domain in solution was more stable in comparison with the CaMKIIα holoenzymes, which dissociated into dimers at lower concentrations (Torres-Ocampo et al., 2020). Thus, we took advantage of mass photometry and studied the stoichiometry all four CaMKII hubs and their corresponding holoenzymes (no linker variants were used).

So far, all the studies analyzing the stoichiometries of CaMKII hubs and CaMKII holoenzymes are not in solution but instead are from crystal structures, cryo-EM, and negative stain EM (Bhattacharyya et al., 2016; Buonarati et al., 2021; Chao et al., 2011; Myers et al., 2017) Here, we present stoichiometry data of all four CaMKII hubs and holoenzymes in solution. Differential scanning calorimetry (DSC) was used to compare thermal stability to stoichiometry. Two CaMKIIβ hub crystal structures are presented, showing 14 and 16-mer architectures. Our findings expand our understanding of CaMKII oligomerization in solution and how it relates to kinase activity, which might shed light on how CaMKII is regulated in cells and their Ca$^{2+}$-regulated pathways.
4.3 **CaMKII hub oligomerization state and thermal stability**

The stoichiometries of all four CaMKII hubs were tested at 100 nM (Fig 4.1B). To estimate the size of each hub oligomer, the molecular weight of a CaMKII hub monomer was used, which is \(~15\) kDa. Consequently, a 14-mer falls within the range of \(~210\) kDa, and a 16-mer falls under \(~240\) kDa. Notably, it was found that CaMKIIα hub (218 kDa ± 30 kDa) and CaMKIIγ hub (226 ± 43.2 kDa)) hubs are mainly 14-mers whereas CaMKIIβ hub (251 ± 19.4 kDa) and CaMKIIδ hub (251 ± 52.5 kDa) are 16-mers. (Figure 4.2A). This in contrast with a CaMKIIα hub crystal structure, which showed a stoichiometry of a 12-mer (Bhattacharyya et al., 2016). CaMKIIγ hub crystal structure showed the stoichiometry of a 12-mer and CaMKIIδ hub crystal structure showed a stoichiometry of a 14-mer (Rellos et al., 2010). There is no previous CaMKIIβ hub crystal structure reported.

When the mass photometry measurements are acquired, peaks in different populations are observed. Curiously, for CaMKIIβ hub and CaMKIIγ hub showed fewer smaller species compared to CaMKIIα hub and CaMKIIδ hub at the same concentration (Figure 4.2B). Big species are referred as the 14-mer or 16-mer detected and, small species are referred to as species 150 kDa or below. In order to examine if this was a trend, all four hubs were measured at 90 nM, 80 nM, and 70 nM (Figure 4.3-4.6). Then, for each concentration, the number of small species were divided by the big species observed that indeed, over the range of concentration tested, CaMKIIβ hub and CaMKIIγ has a fewer smaller species versus CaMKIIα hub and CaMKIIδ hub has more big species (Figure 4.2B).

Driven by this observation, we compared the melting temperatures (\(T_m\)) of the hubs using DSC. We observed that CaMKIIβ hub (\(T_m = 110 \text{ °C}\)) and CaMKIIγ hub (\(T_m = 102 \text{ °C}\)) had significantly higher stabilities compared to CaMKIIα hub (\(T_m = 90 \text{ °C}\)) and CaMKIIδ hub (\(T_m = 82 \text{ °C}\)) (Figure 4.7A).
Lastly, we solved two crystal structures of the CaMKIIβ hub showing two stoichiometries: 14-mer and 16-mer. This also highlights the importance of mass photometry as it allows us to study CaMKII molecules in solution, which may behave differently compared to crystallography conditions where the protein is highly concentrated (Figure 4.8).

4.4 CaMKII hub stoichiometry may predict calcium sensitivity

After looking at the CaMKII hubs, looking at the CaMKII holoenzymes became a point of interest. To estimate size for the CaMKII holoenzyme, the size of the CaMKII holoenzyme monomer was used, which is ~50 kDa. Thus, a 12-mer is ~600 kDa and a 14-mer is ~700 kDa. All the holoenzymes tested had no linker between the regulatory segment and hub. CaMKIIα holoenzyme was (620 ± 69.7 kDa), making it a 12-mer. For the rest of the CaMKII holoenzymes, we observed the following: CaMKIIβ (703.3 ± 44 kDa), CaMKIIδ (664 kDa ± 64 kDa) and CaMKIIγ (701 kDa ± 50 kDa). This means these are 14-mers (Figure 4.9-4.10).

Interestingly, a very striking observation was made. As previously mentioned, the EC$_{50}$ for Ca$^{2+}$/CaM of the CaMKII holoenzymes (with no linker) had already been reported (Sloutsky et al., 2020). When comparing those values, it seems that the 12-mer (CaMKIIα) needs more Ca$^{2+}$/CaM (EC$_{50}$ = 313 nM). Meanwhile, the 14-mers (CaMKIIβ, CaMKIIδ and CaMKIIγ) need less Ca$^{2+}$/CaM. CaMKIIβ EC$_{50}$ = 21 nM, CaMKIIδ EC$_{50}$ = 167 nM and CaMKIIγ EC$_{50}$ = 28 nM (Figure 4.12A). This is also correlated with their evolutionary divergence (Sloutsky et al., 2020).

4.5 CaMKII chimeras stoichiometry support an allosteric role for the hub regulating Ca$^{2+}$ sensitivity for the CaMKII holoenzyme.
Previously, CaMKII chimeras were used to support the allosteric effect of the CaMKII hub on kinase activity (Sloutsky et al., 2020). CaMKII α kinase β hub showed an EC\textsubscript{50} = 24 nM and CaMKII β kinase α hub showed an EC\textsubscript{50} = 102 nM. In section 4.4, it was shown that CaMKII holoenzyme stoichiometry predicted Ca\textsuperscript{2+} sensitivity. Therefore, the CaMKII chimeras stoichiometry was also measured to analyze if this trend was also observed. First, CaMKII α kinase β hub was measured at (660 kDa ± 64.4) and CaMKII β kinase α hub was measured at (589.7 ± 82). This makes the CaMKII chimeras a 14- mer and 12-mer, respectively (Figure 4.13). Thus, supporting a hypothesis where CaMKII holoenzyme stoichiometry, apart from the CaMKII hub, may also allosterically regulate Ca\textsuperscript{2+} sensitivity.

4.6 Conclusions

The four human CaMKII hub domains are 90% identical (Sloutsky et al., 2020). Therefore, it was expected that they should have similar if not identical oligomerization states in solution. However, two stoichiometries were observed: 14-mer (CaMKIIα and CaMKIIγ) and 16-mer (CaMKIIβ and CaMKIIδ) (Fig. 4.2A) From these data, two observations were made. First, the stoichiometries are related to the CaMKII hub evolutionary divergence. CaMKIIα and CaMKIIγ are closely related, as well CaMKIIβ and CaMKIIδ. Therefore, it was interesting to see that their stoichiometries were the same. Second, when looking at the population of stoichiometries when acquiring the MP measurements for the hubs, CaMKIIβ and CaMKIIδ hubs had fewer smaller species compared to CaMKIIα and CaMKIIγ hubs. In order to understand this better, we measured their thermal stability with DSC. This led us to detect that CaMKIIα (T\textsubscript{m} = 90 °C) and CaMKIIδ (T\textsubscript{m} = 82 °C) had lower melting temperatures. CaMKIIβ and CaMKIIγ had higher melting temperatures of (T\textsubscript{m} = 102 °C) and (T\textsubscript{m} = 110 °C) respectively. With this information, it was concluded that the lower the thermal stability, dissociation occurs more readily. Lastly, the hub stoichiometries detected in solution were different than the crystal structures stoichiometries previously reported (Rellos et al.,
Our CaMKIIβ hub crystal structure stoichiometries show a 14-mer and a 16-mer, which the latter corresponds to our MP measurement. Further studies need to be done to understand better what specifically causes these different stoichiometries.

In order to have a better understanding of how stoichiometry might affect kinase activity, the stoichiometries of the CaMKII holoenzymes were investigated. Since the CaMKII hub is the oligomerization domain, we expected their corresponding holoenzymes would have similar stoichiometries. To our surprise, different patterns were observed. CaMKIIα holoenzyme is a 12-mer and CaMKIIβ, CaMKIIγ and CaMKIIδ holoenzymes are 14-mers! Excitingly, this correlated to the EC\textsubscript{50} values because CaMKIIα shows an EC\textsubscript{50} of 313 nM while the bigger holoenzymes need significantly less. CaMKIIβ EC\textsubscript{50} = 21 nM, CaMKIIδ EC\textsubscript{50} = 167 nM and CaMKIIγ EC\textsubscript{50} = 28 nM. CaMKII chimeras stoichiometries were also measured, and they showed a 12-mer for CaMKII β kinase α hub and a 14-mer for CaMKII α kinase β hub. Their corresponding are EC\textsubscript{50} = 102 nM and EC\textsubscript{50} = 24 nM. This supports that the hub, when connected to a kinase, determines the stoichiometry of the CaMKII holoenzyme and modulates Ca\textsuperscript{2+} sensitivity.

Our study is the first one to characterize CaMKII hub and CaMKII holoenzymes stoichiometry in solution. The correlation between Ca\textsuperscript{2+} sensitivity and stoichiometry of the holoenzyme is clear and uncovers a new role for the hub. Additional studies need to be done to inquire if this behavior might also be observed in cells. And if CaMKII holoenzyme oligomer’s size might be another way how CaMKII regulates its activity in the different cellular cascades involved in.

4.7 Methods

Molecular biology
The WT CaMKIIα, CaMKIIβ, CaMKIIδ and CaMKIIγ holoenzymes with no linker were cloned into a vector containing an N-terminal His-SUMO tag. The CaMKII hubs were cloned from these constructs by inverse PCR to eliminate the kinases and regulatory domain. The CaMKIIα hub was cloned into a pET-28 vector with an N-terminal 6xHis-precision tag using Gibson assembly.

**Protein purification**

All four CaMKII holoenzymes were co-expressed with λ phosphatase using Rosetta (DE-3)pLysS cells. The cells were induced at 18 °C with 1 mM Isopropyl β-d-1-thiogalactopyranoside (IPTG) and grown overnight. The cells were then suspended using Buffer A (25 mM Tris, pH 8.5, 150 mM KCl, 40 mM imidazole, 10% glycerol), commercially available protease inhibitors (0.5 mM Benzamidine, 0.2 mM AEBSF, 0.1 mg/mL trypsin inhibitor, 0.005 mM Leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatin) plus 1 μg/mL DNAse. Purification was done at 4 °C using utilizing a ÄKTA Pure system (GE). Clarified lysate was loaded onto a Ni-NTA column and eluted with an imidazole gradient, desalted into Buffer C (25 mM Tris pH 8.8, 50 mM KCl, 40 mM imidazole, 2 mM TCEP, 10% glycerol) and cleaved overnight with Ulp1 at 4 °C. Following a subtractive step, the sample was then bound to a HiTrap Q FF column (GE) and eluded with a KCl gradient. Holoenzymes were purified using a Superose 6 column (GE) into DSC buffer (25 mM Tris pH 8, 150 mM KCl, 1 mM TCEP, and 10% glycerol). All purified holoenzymes eluted consistently (retention volume12 mL). The pure protein was then frozen and stored at -80 °C.

The CaMKIIβ, CaMKIIγ, CaMKIIδ hubs (335-475) were expressed separately using BL21 cells and purified the same as the holoenzymes. The CaMKIIα hub
domain (residues 315-475) was expressed in BL21 cells and purified as described except a different Buffer C (25 mM Tris pH 8.8, 150 mM KCl, 1 mM EDTA, 2 mM DTT, 10 % glycerol) to facilitate Prescision protease cleavage overnight at 4 °C. Samples were eluted from the Superose 6 column into DSC buffer. All purified hubs eluted consistently (retention volume 14 mL). Pure protein samples were frozen and stored at -80 °C.

**Mass photometer measurements**

All MP experiments were carried out using an OneMP mass photometer (Refeyn LTD, Oxford, UK) at room temperature and used Acquire MP software for data collection. Coverslips (cat #630-2105) were cleaned by rinsing with a series of H2O, ethanol, and isopropanol (HPLC grade), and then dried under a clean stream of nitrogen. Gaskets (catalog #CW-50R-1.0) were cleaned using H2O and ethanol, then dried under nitrogen. The gasket was then sealed onto the center of the glass coverslip. The coverslips were stored in a sterile container until used. 10 µl of buffer (25 mM Tris, 150 mM KCl and 1 mM TCEP at pH 8.0) was added to the coverslip and focused. Images were collected in an area of 3 x 10 µm at a frame rate of 1 kHz. Protein stock solutions (2 µM) were diluted to 200 nM (25 mM Tris, 150 mM KCl and 1 mM TCEP at pH 8.0) right before the experiment. Proteins diluted to achieve the following concentrations in the measurements: 100 nM, 90 nM, 80 nM, and 70 nM and 45 nM for all four hubs. And only 100 nM four holoenzymes. Three replicates were performed at each concentration. Since the technical limit of the instrument at low MW is 50 kDa, we will not be able to fully resolve monomeric hub subunits (15.3 kDa) (Wu & Piszczek, 2020)
**Differential Scanning Calorimetry**

All protein samples were diluted to 0.5 mg/mL in DSC buffer (25 mM Tris pH 8, 150 mM KCl, 1 mM TCEP, 10% glycerol). DSC measurements were performed on a MicroCal Automated PEAQ-DSC instrument (Malvern Panalytical, Westborough, MA). Unless otherwise indicated, after a 5 min pre-scan equilibration step, samples were scanned from 10-120 °C at a scan rate of 90 °C/hr with no feedback. Data were analyzed using MicroCal PEAQ-DSC software, and baseline-subtracted data were fit to a non-two-state fitting model to obtain apparent T_m values.

**CaMKIIβ hub crystal structure**

In order to find suitable crystallization conditions, PEGrx HT (Catalog #: HR2-086, Hampton Research) and Index HT (Catalog #: HR2-134, Hampton Research) screening trays (3 Well Midi UVP, Catalog #: HR3-123, Hampton Research) were set up for β hub using a Formulatrix brand NT8-Drop Setter. Crystallization for the screening trays was approached using sitting drop vapor diffusion. The total drop size was 400 μL, consisting of 200 μL reservoir solution and 200 μL protein sample at a concentration of 30 mg/mL. All crystals were allowed to grow at room temperature (25 °C). These trays were then screened for hits based on crystal size, shape, and quantity. Condition E12 on the PEGrx screening tray (0.15 M DL - malic acid pH 7.0, 22% PEG monomethyl ether 550, 0.1 M imidazole pH 7.0) produced the best crystals and the most promising diffraction when shot at room temperature. A 24 well crystal tray was then manually set up around this condition, so that the concentration of DL-malic acid varied from 0.1 M to 0.2 M and the concentration of PEG monomethyl ether 550 varied from 20% to 22%. Protein crystallization for the manual tray (VDX Plate, Catalog #: HR3-140, Hampton Research) (22 mm Siliconized Glass Cover Slides, Catalog #: HR3-233, Hampton Research) was approached via hanging drop vapor diffusion.
Drops consisted of 1 μL reservoir solution and 1 μL protein at 30 mg/mL. Drops were allowed to equilibrate over 500 μL of reservoir solution.

All crystals were grown at room temperature (25 °C). Crystals were observed using a Volpi NCL-150 Fiber Optic Cold Light Source Illuminator after 24 hours. Crystals from the C3 well (0.15 M DL - malic acid pH 7.0, 22% PEG monomethyl ether 550, 0.1 M imidazole pH 7.0) were shot under cryo-conditions using a Rigaku MicroMax-007 HF X-ray source, which was coupled to a Rigaku VariMax HF optic system (UMass Amherst). No cryoprotectant solutions were used, as PEG monomethyl ether 550 acts as a cryoprotectant at high enough percentages (however some freezing was observed at 20% PEG monomethyl ether 550). Several days after looping, the formation of crystals with different geometry was observed in the same well. The aforementioned process was repeated for these crystals as well.

Data Collection and Analysis

Diffraction data were collected at a wavelength of 1.5418 Å using a Rigaku MicroMax-007 HF X-ray source, which was coupled to a Rigaku VariMax HF optic system (UMass Amherst). The X-ray data were collected at 100 K. Data sets were integrated, merged, and scaled using HKL-2000. For data collection of the first crystal, 180 frames were collected with a 4-minute exposure per frame. The space group was determined to be C2221 (a, b, c = 104.245, 183.204, 108.369; α, β, γ = 90.00, 90.00, 90.00) and the asymmetric unit consisted of 7 monomers. For data collection of the second crystal, 180 frames were collected with a 15-minute exposure per frame. Frames 105-145 were cut out due to poor quality. The space group was determined to be P42212 (a, b, c = 81.790, 81.790, 180.184; α, β, γ = 90.00, 90.00, 90.00) and the asymmetric unit consisted of 4 monomers. Molecular placement for both cases was accomplished with Phaser using coordinates for the hub
domain of CaMKIIγ (PDB identification code = 2UX0) as an initial search model. The structures were built using the software Coot and refinement was performed with REFMAC.

*MP Data Analysis*

DiscoverMP software was used for all analyses using standard settings. Each replicate was analyzed individually. Only molecules that bound to the glass surface were counted. Populations were automatically fit with a Gaussian distribution ([Young et al., 2018](#)). For these populations, the mean Gaussian values from replicates were averaged to calculate an average MW, these values are reported ± the standard deviation between three replicates.

4.8 Acknowledgements

We would like to thank Dr. Brenda Watts and Dr. Adam Perry for all their help with the use of the mass photometry machine. Also, Dr. Peter Chien, Dr. Scott Garman and Dr. Luke Chao for helpful discussion about project conceptualization and feedback on experiment design.
Figure 4.1 CaMKII protein domains and the four hub domains for the four CaMKII genes. A) CaMKII subunit domain architecture B) CaMKII hub domains of all four genes. CaMKIIα (blue), CaMKIIβ (pink), CaMKIIδ (green) and CaMKIIγ (purple).
Figure 4.2 CaMKII hubs in solutions show different stoichiometry and different patterns of dissociation at the same concentration (100 nM) with mass photometry.
A) Mass photometry of all four CaMKII hubs: CaMKIIα (blue), CaMKIIβ (pink), CaMKIIδ (green) and CaMKIIγ (purple). B) Small species/big species plotted for all 4 hubs from 100 nM, 90 nM, 80 nM and 70 nM.

**Figure 4.3. CaMKIIα hub dilution from 100 to 70 nM using mass photometry.** Each color: green, blue and yellow is a replicate. Therefore, each protein concentration was done in triplicates.
Figure 4.4. CaMKIIβ hub dilution from 100 to 70 nM using mass photometry. Each color: green, blue and yellow is a replicate. Therefore, each protein concentration was done in triplicates.
Figure 4.5. CaMKIIδ hub dilution from 100 to 70 nM using mass photometry. Each color: green, blue and yellow is a replicate. Therefore, each protein concentration was done in triplicates.
Figure 4.6. CaMKIIγ hub dilution from 100 to 70 nM using mass photometry. Each color: green, blue and yellow is a replicate. Therefore, each protein concentration was done in triplicates.
**Figure 4.7 Differential Scanning Calorimetry for all four CaMKII hubs.** Representative traces are shown as well as the average apparent $T_m$, rounded to the nearest integer calculated from all replicates (replicates are in 4.8). A) CaMKIIβ (burgundy) and CaMKIIγ hub (blue). B) CaMKIIα hub (blue) and CaMKIIδ (burgundy). C) All four CaMKII hubs.
Figure 4.8 The replicates for DSC CaMKII hubs. The raw data and corresponding fits for all DSC replicates are shown. Apparent T_m values are listed for all observed transitions. A) CaMKIIβ hub, B) CaMKIIδ hub and C) CaMKIIγ hub.
Figure 4.9 CaMKIIβ hubs crystal structure shows two different stoichiometries. A) CaMKIIβ14-mer (light green). B) CaMKIIβ 16-mer (gray).
Figure 4.10 CaMKII holoenzyme stoichiometries using mass photometry. CaMKIIα holoenzyme (blue), CaMKIIβ (dark pink), CaMKIIδ (aqua) and CaMKIIγ (purple). Each color: green, blue and yellow is a replicate. Therefore, each protein concentration was done in triplicates.
Figure 4.11 CaMKII holoenzymes stoichiometries using mass photometry replicates.

CaMKIIα holoenzyme (blue), CaMKIIβ (dark pink), CaMKIIδ (aqua) and CaMKIIγ (purple).

Each color: green, blue and yellow is a replicate. Therefore, each protein concentration was done in triplicates.
Figure 4.12 CaMKII holoenzymes and CaMKII chimeras stoichiometries using mass photometry are related to calcium sensitivity. A) CaMKII holoenzymes with their calculated oligomer size using mass photometry and their corresponding EC_{50} value from (Sloutsky et al., 2020). CaMKIIα holoenzyme (blue), CaMKIIβ (dark pink), CaMKIIδ (aqua) and CaMKIIγ (purple). B) CaMKII chimeras: CaMKII α kinase β hub (blue kinase, purple hub) and CaMKIIβ kinase α hub (purple kinase, blue hub) with their calculated oligomer size using mass photometry and their corresponding EC_{50} value from (Sloutsky et al., 2020).
Figure 4.13 CaMKII chimeras stoichiometries using mass photometry and replicates

A) CaMKII chimeras: CaMKII \( \alpha \) kinase \( \beta \) hub (blue kinase, purple hub) and CaMKII \( \beta \) kinase \( \alpha \) hub (purple kinase, blue hub) B) Each color: green, blue and yellow is a replicate. Therefore, each protein concentration was done in triplicates.
CHAPTER 5
LIVE-CELL FLIM-FRET USING A COMMERCIALLY AVAILABLE SYSTEM

As written by Castellani Colleen M, Torres-Ocampo Ana P., Breffke Jens, White Adam B., Chambers James J., Stratton Margaret M. and Maresca Thomas J. Published in Methods in Cell Biology in March 2020

I imaged Camui FRET biosensor in HEK cells and wrote part 5.6. I also helped with the edits for the final manuscript.

5.1 Abstract

Förster resonance energy transfer (FRET)-based sensors have been powerful tools in cell biologists’ toolkit for decades. Informed by fundamental understanding of fluorescent proteins, protein-protein interactions, and the structural biology of reporter components, researchers have been able to employ creative design approaches to build sensors that are uniquely capable of probing a wide range of phenomena in living cells including visualization of localized calcium signaling, sub-cellular activity gradients, and tension generation to name but a few. While FRET sensors have significantly impacted many fields, one must also be cognizant of the limitations to conventional, intensity-based FRET measurements stemming from variation in probe concentration, sensitivity to photobleaching, and bleed-through between the FRET fluorophores. Fluorescence lifetime imaging microscopy (FLIM) largely overcomes the limitations of intensity-based FRET measurements. In general terms, FLIM measures the time, which for the reporters described in this chapter is nanoseconds (ns), between photon absorption and emission by a fluorophore. When FLIM is applied to FRET sensors (FLIM-FRET), measurement of the donor fluorophore lifetime provides valuable information such as FRET efficiency and the percentage of reporters engaged in FRET. This chapter introduces fundamental principles of FLIM-FRET toward informing the practical application of the technique and, using two
established FRET reporters as proofs of concept, outlines how to use a commercially available FLIM system.

5.2 Introduction

For years the application of fluorescence lifetime imaging microscopy (FLIM) techniques has largely been the purview of specialists; the objective of this methods chapter is to make FLIM-FRET accessible to generalists—specifically cell biologists who are already familiar with live-cell fluorescence microscopy approaches. Many FLIM systems in use today are custom-built and housed in labs with expertise in developing and maintaining advanced microscope systems. The microscope described in this chapter is a commercially available “turn-key” system that is stable enough to be housed in a microscopy core facility. For most, the core facility will be the best economic model for incorporating FLIM-FRET into their research program as the hardware requirements include a laser scanning confocal microscope, high-frequency pulsed lasers, and time-correlated single photon counting (TCSPC) detectors. The protocol will also include standard operating procedures for acquiring and analyzing FLIM-FRET data using well-established software from Nikon (NIS-Elements) and Becker & Hickl.

FLIM measures how long a fluorophore is in its excited state to determine its fluorescent lifetime (Elangovan et al., 2002). There are two major modalities of acquiring FLIM data referred to generally as “frequency domain” FLIM and “time domain” FLIM. This chapter will deal exclusively with time domain FLIM using time-correlated single photon counting (TCSPC). TCSPC uses a pulsed laser with one or more single photon detectors. Photons are sorted based on arrival time to the detector in relation to the excitation signal. A decay curve is constructed by plotting the time of photon arrivals following an excitation pulse versus intensity, which is the number of photons detected at a given arrival time (Fig.
5.1. The lifetime of a fluorophore ($\tau$) is defined as the arrival time where the population of photons has decayed to $1/e$ of the original population or intensity. Using TCSPC with a scanning confocal head, high spatial resolution can be attained, but depending on the lasers, detectors, and reporter brightness, long acquisition times (minutes) may be required to attain a sufficient number of photons for robust data analysis (Padilla-Parra et al., 2008). This methods chapter will begin by outlining basic principles of FRET and FLIM before shifting focus to practical considerations such as hardware requirements (with associated cost estimates) and appropriate donor selection for FLIM-FRET sensors. A step-by-step protocol for acquiring FLIM images on a Nikon scanning confocal system using NIS-Elements in sync with SPCM64 (Becker & Hickl) will be detailed using the real world example of a previously characterized FRET biosensor.

Step-by-step protocols are presented for conducting FLIM analyses in SPCIImage (Becker & Hickl) to determine: (1) the donor lifetimes in the presence and absence of FRET, (2) the percentage of reporter engaged in FRET in your sample, and (3) FRET efficiency. The principles of the Phasor Plot—an approach for displaying and analyzing FLIM images will be introduced and a protocol for Phasor Plot analysis in SPCIImage is presented. A second proof-of-concept example of FLIM-FRET using the system described here is presented followed by the introduction of a FLIM-FRET acquisition and analysis platform that will soon be released by Nikon to interface with their NIS-Elements software. In summary, this chapter describes how to visualize and analyze FRET sensors using a commercially available FLIM system that has proven robust enough to become a workhorse microscope in a core facility.

5.3 Principles of FLIM-FRET
FRET is a non-radiative energy transfer from one fluorophore in the excited state (the donor) to another molecule in the ground state (the acceptor), which is typically located less than 10 nm away. In order for FRET to occur three conditions must be fulfilled:

1. The donor emission spectrum must overlap with the absorption spectrum of the acceptor
2. Both molecules must be within angstroms to nanometers distance to each other
3. Both molecules must have a matching dipolar orientation

Since the energy transfer efficiency changes as the inverse sixth power ($r^{-6}$) of the distance $r$ between the donor and acceptor, selection of the proper FRET pairs allows the researcher to monitor distance changes in the angstrom and nanometer range, which makes the approach relevant for probing macromolecular structural dynamics at the scale of proteins and nucleic acids within living samples. A common measurement technique is intensity-based FRET, which can be performed with confocal or wide field microscopy approaches. Intensity-based FRET can be measured either through the donor (acceptor-photo bleaching) or the acceptor (sensitized emission). Unfortunately, both techniques generate experimental challenges due to spectral cross-talk, photo bleaching sensitivity, and variable reporter concentration.

A better way to determine FRET efficiencies has been the use of measuring the fluorescence lifetime of the donor. The lifetime is the half time a molecule spends in the first excited state and is on the nanosecond time scale ($10^{-9}$ seconds) for common FRET donors. In a FRET experiment a fluorescent molecule (the donor) in the excited state can return to the ground state either by emitting a photon (fluorescence) or in a non-radiative way by transferring energy to the acceptor, as discussed above. This transfer is associated with a probability over time meaning the longer the donor is in the excited state the higher the
likelihood it will engage in FRET. Since there are two competing kinetic pathways (fluorescence and non-radiative) under FRET conditions, the lifetime of the donor becomes shorter than its lifetime under non-FRET conditions. The lifetime (τ) can be described and mathematically fitted using an exponential function or a sum thereof of the fluorescence intensity as a function of time:

\[ I(t) = \sum a_i e^{-t/\tau_i} \]

Where \( \sum a_i = 1 \)

An ideal FRET donor should exhibit a single exponential decay (further details below). When engaged in FRET, the single exponential decay curve now exhibits a double-exponential behavior (Fig. 5.2). Since some fraction \( a_1 \) of donors will not have acceptor in their vicinity, these donors would fluoresce with the normal baseline lifetime \( \tau_1 \). However, a fraction of donor \( a_2 \) will be undergoing FRET, which introduces a second lifetime component \( \tau_2 \) that is shorter (or faster) than the native donor. The result is an average fluorescence lifetime (\( \tau_{FRET} \)) where:

\[ \tau_{FRET} = a_1 \tau_1 + a_2 \tau_2 \]

For the FLIM-FRET approaches outlined in this chapter you will need two measurements: (1) cells with only the donor expressed to determine the lifetime (\( \tau \)) of the donor in your model system and (2) cells expressing the FRET reporter that includes both the donor and the acceptor in the same sample system. Once the baseline lifetime of the donor under non-FRET conditions in your cell line is known (\( \tau_D \)) and the lifetime of the donor under possible FRET conditions (in the presence of an acceptor) has been measured (\( \tau_{FRET} \)), the FRET efficiency (E) can also be calculated by:
\[ E = \left(1 - \frac{\tau_{\text{FRET}}}{\tau_D}\right) \times 100\% \]

Unlike intensity-based FRET experiments, the acceptor can be “dark” or nonfluorescent (Ganesan et al., 2006; Murakoshi et al., 2008, 2015) because only the lifetime of the donor needs to be measured. In addition, you can measure each experiment independent of excitation intensity and integration time as long as your data has sufficient signal-to-noise to reveal its decay characteristics. Consequently, when FLIM is applied to FRET sensors (FLIM-FRET), measurement of only the donor fluorophore lifetime, which negates bleed-through concerns, provides valuable information such as FRET efficiency and the percentage of reporter engaged in FRET in a manner that is independent of local probe concentration and less sensitive to photo bleaching than conventional FRET measurements.

## 5.4 Equipment and materials

A FLIM system can be set up on an existing scanning confocal microscope system such as the Nikon A1 confocal system described here, which is depicted in Fig. 5.3. In the method outlined in this chapter the system was set up to visualize the FRET donor mTurquoise2 (CFP variant) and FRET acceptor mVenus (YFP variant) although other FRET pairs can be used with the appropriate pulsed lasers, filters, and dichroic mirrors. A parts list and estimated costs for a system with four pulsed lasers (405, 445, 488, and 561 nm), accompanying filters and dichroic mirrors, and two TCSPC detectors is provided in Table 1. A system need not require this number of laser lines (plus appropriate filters and dichroic mirrors) and detectors and could function with as little as a single pulsed laser for your FRET donor of choice and a single detector to count the emitted photons. It is also important to note that the addition of FLIM capabilities does not negatively impact the other imaging modalities on the microscope to which it is added.
Live cell imaging

1. **Scanning confocal microscopy equipment** - Images and FLIM data were acquired on a Nikon Ti-E microscope body that was fitted with an A1 confocal scan head. The scan head has two laser input ports and three emission output ports. Four continuous-wave lasers (Nikon LUN-V; 405, 488, 561, and 640 nm) were combined and fiber-coupled into one input port and four pulsed lasers (Becker & Hickl; BDL-405-SMN, BDL-445-SMN, BDL-488-SMN, and BDS-561-SMY; 405, 445, 488, and 561 nm, respectively) were combined and fiber-coupled into the second input port. The first output port of the scan head was connected to the Nikon A1-DU4G that contains five PMT detectors, the second port connected to the Nikon 32-channel spectral detector A1-DUs, and the third port connected to a pair of Becker & Hickl HPM-100-40 detectors.

2. **Time-correlated single photon counting (TCSPC) detectors** - Two Becker & Hickl HPM-100-40 detectors were coupled to a changeable filter cube that consisted of an appropriate dichroic mirror and two band pass emission filters; for CFP/YFP 488/50BP, 520LP, 550/49BP (Semrock, FF01-488/50, FF520-Di02, FF01-550/49, respectively).

3. **Final comments on approaches to live-cell imaging** - Once cell lines expressing the FRET reporters have been made, sufficient time should be spent at the microscope familiarizing oneself with the cells prior to doing FLIM-FRET experiments. If it is difficult to readily assess the extent of chromosome alignment by phase contract or DIC microscopy, as is the case for *Drosophila S2* cells, then “scanning” for cells will require the use of epifluorescence and; therefore, limiting photo bleaching of the reporters is critical. If CFP-YFP variants are used as your FRET pairing (as is the case in this protocol) then co-transfecting the cells with an RFP-tagged construct such as mCherry-α-tubulin will aid in this process as you can scan on the RFP channel to avoid photobleaching of the donor or acceptor. If you will be scanning
cells with epifluorescence then aim for a level of proficiency in which the necessary cellular states (e.g., mitotic versus interphase) can be identified and centered in the field of view within ~3–5 seconds.

5.5 Proof of concept 1 – CyclinB1-Cdk1 activity reporter in Drosophila S2 cells

The first proof of concept will make use of a previously characterized reporter for CyclinB1- Cdk1 kinase activity (Gavet & Pines, 2010) in which the donor (mCerulean) has been replaced with mTurquoise2 (Goedhart et al., 2012), which exhibits a mono-exponential decay, and the acceptor (YPet) has been replaced with mVenus. The FRET pairs flank the autophosphorylation site of human CyclinB1, which is a substrate of active CyclinB1-Cdk1, and a phospho-binding domain comprised of the Polo Box Domain (PBD) of Polo-like kinase 1 (Plk1). Consequently, the sensor undergoes a conformational change when it is phosphorylated by Cdk1 that results in an increase in intramolecular FRET (Figure 5.4). Prior work demonstrated that this reporter specifically reports on CyclinB1-Cdk1 activity and exhibits increased FRET in mitosis and decreased FRET in interphase. In the case of the CyclinB1-Cdk1 reporter used in this proof of principle, a non-phosphorylatable control was also used to confirm that any measured changes in donor lifetime and FRET efficiency were indeed due to its phosphorylation rather than non-specific changes in its conformation between interphase and mitosis. It is also important that the lifetime of the donor in the absence of an acceptor (no FRET condition) be measured in the cell line of interest to accurately analyze FLIM-FRET data under possible FRET conditions. The Cdk1 activity reporter, control non-phosphorylatable reporter, and mTurquoise2 (donor alone) were cloned into the Drosophila cell expression vector pMT-V5-HisB (Thermo Fisher) in which their expression was under the control of a promoter sequence that drives constitutive, but moderate levels of expression. The chapter will not
detail the transfection protocol or general culturing of *Drosophila S2* cells as we have
detailed this elsewhere (Ye & Maresca, 2016).

A. Measuring donor lifetime

Ideally, the donors used for FLIM should exhibit single exponential decay (Martin et
al., 2018). Unfortunately, this property excludes many commonly used fluorophores. Since
analysis of donors that exhibit multi-exponential decay is technically challenging and not as
clean or reliable as the analysis of mono-exponential decay data we strongly encourage
researchers to invest time up front in engineering their FRET sensors to contain an
appropriate mono-exponential donor. There are now multiple fluorophore options that are
well-suited for use with FLIM-FRET since newly innovated fluorophores are increasingly
becoming available (Goedhart et al., 2010). These include the modification of multi-
exponential decay fluorophores to exhibit single exponential decay (Goedhart et al., 2010)
as well as modifications of single exponential decay fluorophores to increase their
fluorescence intensity and/or increase fluorescence lifetime (Goedhart et al., 2010; Kremers
et al., 2006; Markwardt et al., 2011). Table II shows fluorophores that exhibit single
exponential decay, and thus are best suited for use with FLIM-FRET. The reporters
highlighted in this chapter as proof of principles all involve intramolecular FRET using
mTurquoise2 as the donor and mVenus as the acceptor and the protocols/hardware are
outlined as such. If you are not using a CFP/YFP pairing then pulsed lasers, filters, and
dichroic mirrors should be changed accordingly to match your FRET pair of choice.

1. Establishing donor fluorophore lifetime in the absence an acceptor/FRET

a. Procedure
1. Seed *Drosophila S2* cells expressing the donor (mTurquoise2) on a concanavalin A coated glass bottom 35 mm dish (Cellvis, Part No. D35-20-1.5-N) at ~70% confluency. After the cells adhere (~30 minutes), bring the volume up to 2 mls using fresh Schneider’s media supplemented with 10% heat inactivated FBS (Life Technologies). Allow the cells to flatten for an additional 30 minutes.

2. Once mounted on the microscope, quickly scan by eye under widefield fluorescence settings on the GFP channel (or the RFP channel if co-transfected with an RFP-tagged construct) and center cells of interest in the field of view.

3. Setup the confocal imaging parameters in the A1Plus Compact GUI. In our system the settings were set to image size 512×512 with a pixel dwell time set to 2.2 μs/pixel and a pinhole size set to 1.0 airy units (AU), which is based on the excitation wavelength and is automatically defined in the GUI. Note: these settings matched the FLIM acquisition parameters allowing a direct comparison of the fields between the confocal image and the FLIM image.

4. In the A1Plus Compact GUI, click on “Scan” to obtain the best focal plane of your cell of interest. If you expect to present confocal images alongside your FLIM images then define parameters such as laser power and gain, avoiding pixel saturation, to acquire a quality confocal image of your sample. Save the image to the appropriate folder.

5. In the A1plus Compact GUI click on “AUX”. This changes the output from the scan head so that emitted photons will be sent to the TCSPC detectors.

6. In the A1plus Settings GUI in the “Filter and Dye” tab click on “AUX”. The “AUX Settings” GUI will appear to allow you to define the system settings such that the input into the scan head will be the pulsed laser and the proper dichroic mirror will be positioned in the light path to allow the appropriate emitted photons to reach the TCSPC detectors. On our system, under “Filter and
Dye” 400–457/514 was selected in the dropdown next to “1st DM” and the laser was set to “Port 2” in the dropdown under “Detector External”. All boxes under “Acquisition” in the AUX Settings GUI were unchecked.

7. Click on “START” at the top of the AUX SETTINGS interface. You will hear the scan head turn on although no laser light will be hitting the sample yet. At this point, be sure that the appropriate pulsed laser (ours was 445 nm set to 50 mHz) is turned on.

8. Open SPCM64 and load settings from a template file supplied by the system installer or, if a return user, by loading a file from a prior experiment. To load a file, select the “MAIN” tab at the top of the window and click on “LOAD” in the dropdown. In the “SPC-150 Load SPC-file” pop-up window select “file name” to open a file from a destination folder. After selecting the file name be sure to complete the action by clicking on “LOAD (F10)”. Once completed, the appropriate hardware settings will be loaded and appear in the “M1/M2 DCC-100” pop-up window.

9. Configuration settings will vary depending on the hardware you have and its installment. In the hardware configuration for our system, Module 1 controls the TCSPC detectors and a shutter that allows light into the detectors, Module 2 controls the 405 and 445 nm pulsed lasers, and Module 3 controls the 488 and 561 nm pulsed lasers. Thus the configuration was as follows:
10. To begin acquiring an image toggle to Module 1 in the lower left hand corner of M2 DCC-100 pop-up window to control the two detectors (Connectors 1 and 3 – with the +12V, +5V, and −5V boxes highlighted and “Gain /HV set to 84.00) and the shutter (Connector 2 – only “b0” highlighted). Click on “Enable outputs” to provide power to the detectors and shutter in module 1. After doing this step you will see baseline signal in the detectors in the lower left of the SPCM64 screen above columns labeled “CFD” and “TAC”.

11. Since the 445 nm laser is being used, toggle to Module 2 in the pop-up window. The interface will now allow you to power the appropriate laser and set its power. To do so highlight +5v under Connector 3 (445 nm pulsed laser) and adjust laser to desired power. For our experiments, we found that up to 50% laser power worked well. Click on “Enable Outputs” to provide power to the laser. After doing this step you will see laser light through the objective and increased signal will be evident in the lower left of the SPCM64 screen above all four columns labeled “SYNC”, “CFD”, “TAC”, and “ADC”.

12. Click on the “Start!” tab at the top of the window to acquire data. Images of the FRET donor and acceptor will appear in two separate windows. In our system, the “W1-FLIM1” window on the left shows the mVenus image while the “W2-FLIM2” window on the right shows the mTurquoise2 image – both of which will appear brighter over time as more photons are collected.
Aim to acquire 30–50 million photons per scan, which will be tallied (as well as the elapsed time) in a blue highlighted window in SPCImage next to “Collecting data”.

13. Once enough photons have been collected click on the “Stop!” tab at the top of the window to stop acquiring photons.

14. In the M1/M2 DCC-100 pop-up window click on “Disable all modules” to turn off the detectors, shutter, and pulsed laser.

15. Save the data by selecting the MAIN tab and clicking on “SAVE” in the drop down. In the “SPC-150 Save SPC file” pop-up click on “Select File (F9)” and select or create a file folder in which to save the FLIM images and to name the file. After selecting the destination folder and creating the file name, which should have a similar naming convention as the confocal image file saved in step 4, be sure to complete the saving action by clicking on “Save (F10)”.

16. Return to NIS-Elements and click on “STOP” in the AUX SETTINGS GUI and then click on “Close” at the bottom of the AUX SETTINGS window. This will close the window and allow you to search for new cells to image.

17. Repeat steps 2–16 for as long as your sample is viable or the duration of your microscope time – whichever comes first.

b. Donor lifetime measurements using SPCImage in donor alone conditions

1. Open your FLIM data in the SPCImage software by selecting the “File” tab at the top of the SPCImage window and clicking on “Import”. In the “Open” pop-up window select your file of interest and click on “Open”.

2. A pop-up window entitled “Options for YOUR FILE NAME.sdt” will appear. Under “Select data” in the lower right hand corner of the pop-up, identify the “Module Number” to open the data from the detector used to collect photons from the donor. Since our data was collected on
detector 2, we selected Module Number “2” for both column options. After clicking “OK” in the pop-up window, the FLIM data will appear in the left window in SPCImage (Figure 5.5).

3. In the “Multiexponential Decay” interface (bottom of screen, right of the decay curve) select “1” for “Components”. None of the values in the Multiexponential Decay interface should be fixed except “Scatter”, which should be fixed at “0” by checking the “Fix” box. (Figure 5.5 – Inset 1).

4. Select the “Options” tab at the top of the SPCImage window and click on “Color” in the drop-down. In the pop-up window entitled “Color”, click on “Continuous” and set the appropriate lifetime “Range” to encompass the expected lifetime ranges – for the mTurquoise2 donor this was set from 600 (ps) to 3800 (ps). The lifetimes will be displayed in a color scale depending on whether the “Direction” is set to “R-G-B” or “B-G-R”. For example, if “R-G-B” is selected then the longest lifetimes (3800 ps) will be displayed as blue and the shortest lifetimes (600 ps) will be displayed as red, which would be inversed if “B-G-R” is selected. Since longer average lifetimes indicate lower FRET and shorter average lifetimes indicate higher FRET, we chose to display as R-G-B. Under “Coding of” at the bottom of the pop-up select “tm (ps)” in the dropdown for the “Value”. Click on “OK”.

5. Blue crosshairs will automatically center upon the brightest pixel when you import an image. Move the cursor to the dimmest area of the cell you wish to analyze. In the decay curve window below the image, adjust the binning such that the peak intensity of the decay function (bottom of screen, below FLIM image window) is at least 100 A.U. (y-axis) (Figure 5.5 – Inset 2).

6. At this point, if you plan on analyzing multiple images using the same analysis parameters then it is helpful to “Store the fit conditions” by clicking on the camera icon in the tab to the left of the FLIM image window (Figure 5.5 – Inset 3).
7. Select the “Calculate” tab at the top of the SPCImage window and click on “Decay Matrix” in the drop-down, which will generate a color-coded FLIM lifetime image in the right window in SPCImage.

8. The blue crosshairs can be positioned in either image window to assess local lifetimes. When the crosshairs are moved the lifetime value displayed above the decay curve as “tm = X ps” (Figure 5.5 – Inset 4) will change. The values in the “Multiexponential Decay” interface will also change. In the case of the mono-exponential fit being done in this section, the t1[ps] value will match the lifetime value above the decay curve and the a1[%] will read “100”.

9. The pixels encompassing a region of interest (ROI) can be grouped together to measure the lifetime parameters for an entire cell or region of a cell. The “τm (shown above the decay curve) is generated by binning all the pixels in the ROI. To define an ROI, click on the “Define mask (move red crosshair)” icon in the tab to the left of the FLIM image (Figure 5.5 – Inset 5). Red crosshairs will appear in the FLIM image windows. Left click on the red crosshair to position it where you want to draw the ROI and then alternate between left clicking and moving the cross hair around your cell of interest in the left image window to draw the ROI.

10. Once the ROI is drawn, “Activate binning of all pixels inside mask” by clicking on the red lock icon in the left tab (Figure 5.5 – Inset 6). Once the mask is locked, the blue crosshairs will disappear and the ROI will become bold. At this point, all the lifetime value parameters will be for the area within the ROI.

11. When you are ready to generate a new ROI, “Deactivate binning of all pixels inside mask” by clicking on the red unlocked lock icon in the left tab (Figure 5.5 – Inset 7). The ROI will no longer be in bold and the blue crosshairs will reappear. Click on “Undefine the mask” in the left tab (Figure 5.5 – Inset 8) and the ROI will disappear.
12. Repeat steps 9 – 11 on all the cells or regions of cells you would like to analyze in a given image. Import a new file once you are done analyzing an image. If you are sequentially analyzing the same experimental conditions then “Load fit conditions” (Figure 5.5 – Inset 9) by clicking on the yellow arrow in the left tab – confirm parameters in the “Multiexponential Decay” interface are appropriate and repeat steps 7 – 12 as needed.

13. We analyzed and averaged data from 50 cells expressing mTurquoise2 to attain the lifetime (τD) of 3.57 ns +/- 0.07 ns (mean +/- S.D.) in Drosophila S2 cells. The value of 3573.2 ps (note SPCM64 software deals in ps rather than ns) was used for all future analyses (described below) to fit a double-exponential decay function under possible FRET conditions in which the acceptor mVenus was present.

14. Protocol note: FLIM analysis can be done on a smaller region of the field of view by dragging in the white crosshairs from the corners of the donor image in the left window. To “Zoom In” on a highlighted region, which will be outlined by white lines, click on the “+” magnifying glass icon in the left tab (Figure 5.5 – Inset 10). Zooming in will speed up the calculation of the decay matrix. You can “Zoom Out” to the original field of view by clicking on the “-” magnifying glass icon in the left tab (Figure 5.5 – Inset 11).

2. Measuring donor lifetime and FRET efficiency in the presence of an acceptor

a. Procedure

1. Repeat the procedure from the “Establishing donor fluorophore lifetime in the absence an acceptor/FRET” on cells expressing the FRET reporter in which both the donor (mTurquoise2) and acceptor (mVenus) are present.
b. Donor lifetime measurements via double exponential fitting of FLIM-FRET data using SPCImage

1. Repeat steps 1 and 2 from Donor lifetime measurements using SPCImage in donor alone conditions

2. In the “Multiexpontial Decay” interface (bottom of screen, right of the decay curve) select “2” for “Components”. Fix the long (no FRET) donor lifetime for t2[ps] by entering the lifetime measured for the donor alone in your sample of interest and checking on the “Fix” box. In our case the t2 [ps] was fixed at “3573.2” (measured above). Fix the “Scatter” at “0”. No other parameters should be fixed.

3. Repeat steps 4 – 7 from Donor lifetime measurements using SPCImage in donor alone conditions

4. The blue crosshairs can be positioned in either image window to assess local lifetimes. When the crosshairs are moved the lifetime value displayed above the decay curve as “tm = X ps” will change. This value is the lifetime calculated from the double exponential fit values shown in the “Multiexpontial Decay” interface. In the case of the 2- exponential fit being done in this section, the interface will display the percentage of the reporter engaged in FRET as a1[%], the short lifetime of the donor (due to FRET) as t1[ps], and the percentage of reporter not engaged in FRET as a2[%]. The long lifetime t2[ps] will read the value at which it was fixed (for us this was 3573.2).

5. Repeat steps 9 – 12 from Donor lifetime measurements using SPCImage in donor alone conditions as necessary to acquire a sufficient amount of data for your needs. FLIM-FRET lifetime measurements for the cylinB1-Cdk1 reporters in this proof of principle are reported in Table III.

c. Measuring FRET efficiency from FLIM-FRET data using SPCImage
FRET efficiency can be calculated given the fluorescent lifetime of the donor fluorophore under both FRET and non-FRET conditions. The equation:

\[ E = \left( 1 - \frac{\tau_{\text{FRET}}}{\tau_{\text{D}}} \right) \times 100 \%
\]

calculates FRET efficiency (E), where \( \tau_{\text{FRET}} \) is the lifetime of the donor under FRET conditions, and \( \tau_{\text{D}} \) is the lifetime of the donor under non-FRET conditions (measured above). FRET efficiency can be calculated manually by recording the average lifetime of your ROI (displayed as “tm = X ps” above the decay curve) and plugging this value into the equation above for that you have measured. FRET efficiencies calculated from the values measured using the protocols described above for the cyclinB1-Cdk1 reporters in this proof of principle are reported in Table IV. SPCImage also has a useful function to display the decay matrix as a function of FRET efficiency using the long lifetime that you enter into the software in the double-exponential fitting interface. The following protocol discusses how to use SPCImage to convert lifetime data to FRET efficiency for visual representations.

1. Repeat steps 1 and 2 from *Donor lifetime measurements using SPCImage in donor alone conditions*

2. In the “Multiexpontial Decay” interface (bottom of screen, right of the decay curve) select “2” for “Components”. Fix the long (no FRET) donor lifetime for \( t_2[\text{ps}] \) by entering the lifetime measured for the donor alone in your sample of interest and checking on the “Fix” box. In our case the \( t_2 \ [\text{ps}] \) was fixed at “3573.2” (measured above). Fix the “Scatter” at “0”. No other parameters should be fixed.

3. Select the “Options” tab at the top of the SPCImage window and click on “Color” in the drop-down. In the pop-up window entitled “Color”, click on “Continuous” and set the appropriate lifetime “Range” to encompass the expected FRET efficiency range – for the CyclinB1-Cdk1
reporter this was set from 0 (%) to 55 (%). The FRET efficiencies will be displayed in a color scale depending on whether the “Direction” is set to “R-G-B” or “B-G-R”. For example, if “R-G-B” is selected then the highest FRET efficiency (55%) will be displayed as blue and the lowest FRET efficiency (0 %) will be displayed as red, which would be inversed if “B-G-R” is selected. We chose to display the FRET efficiencies as B-G-R as this is the convention for displaying FRET data. Under “Coding of” at the bottom of the pop-up select “$1 - \frac{\tau_m}{\tau^2}$” in the dropdown for the “Value”. Click on “OK”.

4. Blue crosshairs will automatically center upon the brightest pixel when you import an image. Move the cursor to the dimmest area of the cell you wish to analyze. In the decay curve window below the image, adjust the binning such that the peak intensity of the decay function (bottom of screen, below FLIM image window) is at least 100 A.U. (y-axis) (Figure 5.5 – Inset 2).

5. At this point, if you plan on analyzing multiple images using the same analysis parameters then it is helpful to “Store the fit conditions” by clicking on the camera icon in the tab to the left of the FLIM image window (Figure 5.5 – Inset 3).

6. Select the “Calculate” tab at the top of the SPCImage window and click on “Decay Matrix” in the drop-down, which will generate a color-coded image in the right window in SPCImage displaying FRET efficiencies with the lowest FRET in blue and the highest FRET in red.

7. The FRET efficiencies can be analyzed with ROIs by repeating steps 9 – 12 from *Donor lifetime measurements using SPCImage in donor alone conditions*; however, the value reported above the decay curve will now display the FRET efficiency of the binned pixels in the ROI rather than lifetime.

B. Phasor plots
The Phasor plot originates from measuring lifetime in the frequency domain and was developed by Enrico Gratton (Digman et al., 2008). In such, the lifetime data is illustrated in a polar system in which the data is presented in a semicircle. The vector pointer (also called “phasor”) from the left hand coordinate origin is drawn with the phase as its angle and the amplitude as its length (Figure 5.6). The lifetime is plotted based on these two numbers independent of the pixel location within the image. The advantages are:

1. The phasors of pixels with single-exponential decay profiles are located along the semicircle
2. Phasors of multiple decay components are linear combinations of the phasors of the components and will end up inside the semicircle
3. Pixels with similar phase and amplitude values form visible clusters. Therefore, pixels with similar lifetime signatures can be identified in the phasor plot.
4. Lifetime clusters in the dataset can be back annotated and identified in the image display using a selection tool (Figure 5.7).

Taking the time-domain lifetime data you get from TCSPC measurements we can obtain the phase and amplitude values by its first Fourier components.

**Phasor plot analysis to determine spatial distribution of fluorescent lifetimes in SPCImage**

1. Repeat steps 1–3 from *Donor lifetime measurements via double exponential fitting of FLIM-FRET data using SPCImage*
2. Select the “Tools” tab at the top of the SPCImage window and click on “Phasor Plot” in the drop-down.
3. In the “Phasor Plot” GUI that opens set the “Repetition Time (Laser):” to 20.0 ns and check on the box next to “Combine with FLIM analysis.” Click on the “Recalculate” button,
4. The crosshairs can be dragged and resized with the mouse to highlight clusters of pixels that are apparent in the phasor plot (Figure 5.7B, C). To selectively view pixels in the ROI from the phasor plot in the FLIM image, click on the “Select cluster” box. Pixels exhibiting the lifetime within the selected circle will be displayed in the FLIM image while pixels not exhibiting the lifetime within the selected circle will be displayed in the grayscale image on the left. The image displays will be update when you move and/or resize the ROI in the phasor plot.

5. Another useful feature of the phasor plot analysis interface is that when you move the blue crosshair in the donor/FLIM image windows, a corresponding blue crosshair will be positioned in the phasor plot so that you see if the FLIM ROI falls into a cluster in the phasor plot.

5.6 Proof of concept – Camui CaMKII sensor in human HEK293T cells

Ca²⁺-calmodulin dependent protein kinase II (CaMKII) is a Ser/Thr kinase that plays crucial roles in memory formation, cardiac signaling, and fertilization (H. Y. Chang et al., 2009; Eisner et al., 2017; Herring & Nicoll, 2016b) As suggested by its name, CaMKII is turned on by a Ca²⁺ elevation, leading to Ca²⁺ loaded calmodulin (Ca²⁺-CaM), which ultimately leads to the relief of autoinhibition in CaMKII (M. M. Stratton et al., 2013). Camui is a FRET biosensor
for CaMKII activity (Takao et al., 2005). When CaMKII binds Ca\(^{2+}\)-CaM, it undergoes significant conformational change, which is reported in Camui by placing a FRET pair at the N- and C-termini (Fig. 5.8).

**A. Expression of Camui in HEK293T cells**

1. Seed 150,000 cells in a round dish (35mm glass bottom dish with 20mm microwell #1 cover glass, D35-20-1-N) with 1mL of Dulbecco’s Modified Eagle’s Medium—high glucose (Sigma Aldrich) + 10% of Fetal Bovine Serum (Sigma Aldrich).

2. Once the cells are 60–70% confluent (typically after 2–3 days), transfec with 600–700 ng of Camui (mTurquoise2/mVenus) using Lipofectamine (Thermo Fischer) according to the manufacturers protocol.

3. Subject Camui expressing cells to FLIM imaging as described in the procedure from Measuring donor lifetime and FRET efficiency in the presence of an acceptor 48 h post-transfection.

**B. Establishing timing of Ca\(^{2+}\) release in HEK293T cells by addition of ionomycin**

Ca\(^{2+}\) entry into the cell leads to CaMKII activation. We use ionomycin to induce Ca\(^{2+}\) release in HEK293T cells. In order to determine the kinetics of Ca\(^{2+}\) release after ionomycin addition, we used Fluo-4 AM, a cell permeant Ca\(^{2+}\) indicator. Robust Ca\(^{2+}\) release occurred roughly 40 s after addition of 2.5μM ionomycin.

**C. Measuring CaMKII activity using Camui**

As described above, Camui is a biosensor for CaMKII activation where there is a FRET decrease coincident with CaMKII activation. Prior to Ca\(^{2+}\) release, CaMKII should be in the OFF conformation and this is read out as a shorter lifetime from
Camui. After ionomycin addition and subsequent Ca\(^{2+}\) release, CaMKII should be in the ON conformation and this is read out as a longer lifetime from Camui. To provide a direct comparison to the experiment described above, we cloned mTurquoise2 into Camui in place of Cerulean. Furthermore, this version of Camui is better suited to FLIM analysis since mTurquoise2 exhibits a mono-exponential decay in its lifetime while Cerulean does not. Data acquisition and analysis were performed as described above. We acquired 60 million photons before ionomycin addition, then ionomycin was added and after 40 s we began another acquisition for 60 million photons. The only change compared to the proof of concept 1 was in establishing the color display to a 600–4000 ps range.

5.7 Utilizing the Nikon A1-FLIM platform

Nikon and Becker & Hickl have collaborated to integrate the Becker & Hickl hardware into a seamless module that is now part of Nikon’s software, NIS-Elements. The A1-FLIM module combines advanced lifetime imaging technology with the software platform used to drive conventional scanning confocal systems. The result is the ability to use typical confocal system workflows to collect, visualize, and analyze lifetime data. The module allows for multiplexed FLIM acquisition, providing Z-stacks, time-lapses, multi-points, or any combination therein. All experiments are set up like normal confocal imaging on a Nikon system using NIS-Elements. Thus, anyone familiar with a Nikon A1 confocal system can begin to carry out lifetime experiments without specialized training. To begin an experiment, one must first consider confocal imaging parameters including pinhole, image speed and size and laser power. Once this is set, there are two settings necessary for lifetime imaging: the capture mode and the time channels. Capture mode can be either a set number of frames or can be dynamic based on the number of photons collected in a given pixel. Generally, better contrast can be achieved with more data; however, the cost of doing
so is time. Once these parameters are decided upon, one simply needs to press the “capture” button in order to acquire lifetime images.

5.8 Conclusions

The hardware and protocols outlined here are meant to serve as an entry point for researchers to begin thinking about how FLIM-FRET could benefit their research program and, if it is deemed a worthwhile pursuit, how to assemble/purchase a system in their lab or imaging facility. While we described Becker & Hickl TCSPC components connected to a Nikon scanning confocal, other commercially available FLIM systems are available from PicoQuant Photonics andas upgrade packages from major microscope companies such as Leica, Olympus, and Zeiss, and we hope this chapter will inform comparison shopping between the available options. It is also noteworthy that, in addition to FLIM-based applications, these systems provide fluorescence correlation spectroscopy (FCS) capabilities. In conclusion, FLIM-FRET offers significant improvements over conventional intensity-based FRET approaches. Furthermore, creative FLIM-FRET applications that would be impossible to accomplish with intensity-based FRET are and will continue to arise. For example, the development of large Stoke’s shift (LSS) and “dark” fluorescent proteins (Murakoshi et al., 2015; Shcherbakova et al., 2012) have facilitated the application of dual FLIM-FRET applications in which multiple FRET-based sensors can be subjected to FLIM simultaneously (Demeautis et al., 2017; Ringer et al., 2017) In this method, a single wavelength (440 nm) can be used to excite two separate mono-exponential donors such as the CFP variants mTurquoise2 or mTFP1 and LSSmOrange. The mTurquoise2 is paired with a “dark” acceptor such as the GFP variant ShadowG, which has a 120-fold lower quantum yield than EGFP and; therefore, emits very few photons when energy is transferred to it from a donor, while the LSSmOrange is paired with mKate2 as an acceptor. Since there is no overlap in the emission spectra of mTurquoise2/mTFP1 and LSSmOrange the lifetimes of
each donor can be measured simultaneously with two TCSPC detectors following excitation with the 445 nm pulsed laser. In the system described in this chapter this would simply require changing the dichroic mirror and bandpass (BP) filters in the TCSPC module and a dichroic mirror in the confocal scanning microscope. When acquiring dual FLIM data in a modified setup, one window in the SPCM64 interface will display the mTurquoise2 signal and the other window will display the LSSmOrange signal. Thus, analysis of each donor can be conducted in SPCImage as described above to measure lifetimes and FRET efficiencies of two FRET sensors in the same cell simultaneously. The fast pace at which TCSPC and scanning confocal hardware is improving combined with the continued development of new fluorescent proteins and dye-based technologies means that significant advancements in FLIM-FRET in the vein of dual FLIM imaging will continue for years to come. We hope this chapter will enable cell biologists to take advantage of these emerging technologies by incorporating FLIM-FRET into their research programs.

5.9 Acknowledgements

This work was supported by R01GM107026 to T.J.M., R01GM123157 to M.M.S., and a Commonwealth Honors College grant to C.M.C. Confocal and FLIM microscopy data collection was performed in the Light Microscopy Facility and Nikon Center of Excellence at the Institute for Applied Life Sciences, University of Massachusetts Amherst with support from the Massachusetts Life Science Center.
Figure 5.1 Schematic of how a decay curve is generated in the time-correlated single photon counting (TCSPC) FLIM technique.
Fig. 5.2 Schematic showing how a single exponential decay donor behaves in the absence of FRET and when engaged in FRET with an acceptor.
Fig. 5.3 Layout of the FLIM system outlined in this methods chapter. CW, continuous wavelength; PMT, photomultiplier tube. Note 1: the CW lasers are contained in the box to the right most edge of the photograph. Note 2: The “spectral detector” is part of the FLIM system in the local user facility but is not used in the FLIM imaging protocol.
Fig. 5.4. CyclinB1-Cdk1 FLIM-FRET reporter design. FRET increases when the sensor is phosphorylated during mitosis due to a conformational change triggered by the Polo Box Domain (PBD) of Polo-like kinase 1 (Plk1) binding to a phosphorylated recognition sequence from human CyclinB1.
Fig. 5.5. Lifetime analysis using SPCImage software (Becker & Hickl). This example highlights analysis of the donor mTurquoise2 in the absence of acceptor using a mono-exponential fit, which allowed us to measure the lifetime of the donor in our cell line.
Fig. 5.6. Layout and principles of a phasor plot.
Fig. 5.7. FLIM-FRET data analysis and visual display using the phasor plot. (A) Phasor plot with both populations displayed in the FLIM image. (B) Single exponential population (no FRET) in the phasor plot selected and displayed in the FLIM image. Note that the interphase cell is all that is displayed in the FLIM image. (C) Double-exponential population (FRET) in the phasor plot selected and displayed in the FLIM image. Note that the mitotic cell is all that is displayed in the FLIM image.
**Fig. 5.8. Camui FLIM-FRET sensor for CaMKII activation.** (A) The Camui sensor is comprised of CaMKII, the domains are highlighted in the figure. In this version of Camui, there is mVenus at the N-terminus and mTurquoise2 at the C-terminus of CaMKIIα. In the presence of Ca²⁺, Ca²⁺-bound calmodulin (CaM) binds to CaMKII and turns activity ON. This also results in a large conformational change where the kinase domain moves away from the hub domain, causing a decrease in FRET, or higher lifetime measurement. (B) Representative images of HEK293 cells expressing Camui are shown beneath the cartoon, corresponding to low lifetime (left image) and high lifetime (right image). FRET efficiencies and lifetimes are listed in the table given in this figure and numbers correspond to those
ROIs highlighted in the images.

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<th>Product number</th>
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<td>DCC-100</td>
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<td>HPM-100-40</td>
<td>TCSPC detector units</td>
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Total: $160,740

Table 1. Parts list and cost estimates for the compounds of TCSPC FLIM system.
Table 2. Properties of fluorescent proteins donors that exhibit mono-exponential decay.

(Ai et al., 2006; Betolngar et al., 2015; Demeautis et al., 2017; Goedhart et al., 2010, 2012; Heikal et al., 2000; Kremers et al., 2006; Markwardt et al., 2011; Padilla-Parra et al., 2008; Piatkevich & Verkhusha, 2011; Shaner et al., 2008, 2013; Shcherbakova et al., 2012)
Table 3. Lifetime measurements of CyclinB1-Cdk1 reporters in proof of concept 1.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Average lifetime (ns)</th>
<th>% Short lifetime</th>
<th>Short lifetime (ns)</th>
<th>% Long lifetime</th>
</tr>
</thead>
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<tr>
<td>Nonphospho-FRET interphase</td>
<td>3.44</td>
<td>32%</td>
<td>2.04</td>
<td>68%</td>
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<tr>
<td>Nonphospho-FRET mitosis</td>
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<td>33%</td>
<td>2.05</td>
<td>67%</td>
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<tr>
<td>Phospho-FRET interphase</td>
<td>3.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34%</td>
<td>1.89</td>
<td>66%</td>
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<tr>
<td>Phospho-FRET mitosis</td>
<td>2.53&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>46%&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1.48&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>54%&lt;sup&gt;a,b&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>a</sup>Indicates $P < 0.05$ relative to the control, non-phosphorylatable FRET reporter in interphase.

<sup>b</sup>Indicates $P < 0.05$ relative to the phosphorylatable FRET reporter in interphase. Two-tailed P-values from a Student’s t-test are reported.
Table 4. FRET efficiency measurements of soluble CyclinB1-Cdk1 reporters in proof of concept 1.

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>Mean E</th>
<th>SD</th>
<th>SEM</th>
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<tbody>
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<td>Phospho-FRET mitosis</td>
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<td>&lt;0.001</td>
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</table>

*P-values calculated using the PlotsOfDifferences web app (Postma & Goedhart, 2019). N-values reported in the table apply to Table 3.*
CHAPTER 6

FUTURE PERSPECTIVES ON CAMKII SUBUNIT EXCHANGE ROLE IN LONG-TERM MEMORY FORMATION

Written by Torres-Ocampo Ana P.

6.1 Overview

This section will be addressing the current state of each project discussed in this thesis work and its possible future directions for each. The work presented answered some questions about CaMKII subunit exchange, but it also opened the door for new questions about its mechanism, subunit exchange between CaMKII variants. It will also include the topic of CaMKII holoenzyme stability and oligomerization state for each CaMKII holoenzyme and hub domain.

6.2 CaMKII subunit exchange

So far, with data previously published (Bhattacharyya et al., 2016; M. Stratton et al., 2014) and Chapter 2 from this thesis, it strongly suggested that all four CaMKII variants can go through activation-triggered subunit exchange. For CaMKIIγ homo-oligomeric exchange, it would be interesting to find another way to confirm subunit exchange since the single-molecule TIRF approach is not feasible. By taking advantage of the already established setup with the fluorescent dyes labeling for the CaMKII holoenzyme, one possible idea
would be to show a mixed CaMKII, with red and green, i.e. an exchanged CaMKII, through mass spectrometry or any other method that would allow us to quantify a holoenzyme with both colors. It would also be interesting to look at hetero-oligomeric subunit exchange with the other CaMKII variants, CaMKIIβ and CaMKIIδ. Once these experiments are performed, it will be interesting to characterize their half-times and observe if the trend of slower the hetero-oligomeric exchange is observed for all CaMKII variants.

For CaMKIIδ, the role of the AviTag still needs to be studied more thoroughly as the FRET bulk assay with no AviTag for CaMKIIδ/CaMKIIδ did not show subunit exchange. Nevertheless, when it is mixed with CaMKIIα with an AviTag, we observe subunit exchange. Mass photometry could be used to study the effect of the AviTag in the holoenzyme stability since it seems that it is facilitating exchange it by destabilizing the holoenzyme in some way. The subsequent single-molecule TIRF experiments need to confirm that subunit exchange is also observed in CaMKIIδ. Also, with both CaMKIIδ and CaMKIIγ, we need to test whether they can also spread their phosphorylation state to previously unactivated CaMKIIs like with CaMKIIα. Briefly, a CaMKII that is constitutively active by a T286D mutation, labeled with one color, is mixed with an inactive CaMKII labeled with the other color. And then, pThr286 is added labeled with a third color. Therefore, for the analysis, three colors are co-localized instead of only two. After that is figured out, it would be fascinating to conduct studies in neurons to study the effect of inhibiting subunit exchange during LTP and its potential physiological consequences.

Lastly, by looking into the dynamics of CaMKII subunit exchange, my data suggest that occupying the ATP binding pocket may affect holoenzyme stability and thus exchange. The data shown was only using FRET bulk assay. In order to confirm these results, the corresponding single-molecule TIRF experiments should be performed. Consequently, if the single-molecule TIRF confirms that the ATP binding pocket is somehow regulating subunit
exchange, then the current model of CaMKII subunit exchange will need to be modified to include this new compelling evidence.

6.3 CaMKII holoenzyme stability

In Chapter 3, a comprehensive study of CaMKIIα holoenzyme stability was presented. Here, we showed that the hub is more thermally stable compared to the kinase. It was also shown that that the holoenzyme presents an intermediate thermal stability. Two crystals structures of the kinase with and without regulatory segment were published. Finally, we showed that the hub is very stable over a range of dilutions compared to the holoenzyme. For that publication, we focused on only CaMKIIα, yet it would be intriguing to characterize the other CaMKII variants and compare. The kinase and the hub have 90% and 75% identity, respectively. Thus, it be would be expected to show similar results as with CaMKIIα. However, CaMKII never ceases to amaze us, and it would be enthralling to compare the whole panel of stability of holoenzyme stability: DSC, MP, and crystal structures of the kinases. The linker length for CaMKIIα did show to be essential for neither thermal stability nor dissociation patterns, but it would be curious to see if this holds true for the other CaMKII variants.

6.4 CaMKII oligomerization state

Chapter 4 dives into how the oligomerization state of CaMKII is related to thermal stability and possibly regulating calcium sensitivity. All of these experiments were done using unactivated CaMKII. Hence, comparing unactivated CaMKII with active CaMKII and observing how their oligomerization states vary or not would be delightful! It would be expected that indeed there would be a difference because active CaMKII is going through subunit exchange once it becomes active, and doing this experiment would provide a clue as to what happens to CaMKII oligomerization state. The current model states that it happens
via a dimeric subunit; however, there is no direct evidence of this. As mentioned before, it would also be interesting to see if the linker affects how this happens. For CaMKIIα, it is not necessary for subunit exchange.

### 6.5 CaMKII FRET biosensor

Chapter 5 allowed me to work with Camui, a FRET biosensor for CaMKII activity and FLIM-FRET. Currently, there is another FRET biosensor, FRESCA, which measures endogenous CaMKII activity. FRESCA was tested in eggs in collaboration with Rafael Fissore’s laboratory. It would be fascinating to put this sensor into neurons and be able to measure CaMKII endogenous activity. Camui has been previously used in neurons, but it has its limitations. Of course, it would be excellent if we can get the FLIM-FRET set up to work in conjunction with FRESCA in neurons!

### 6.6 Conclusions

CaMKII is an enzyme that keeps delighting me and surprising me. It helped me become a scientist, and I am looking forward to keeping up with its upcoming discoveries done in the Stratton lab. There is still a lot to know about this engaging protein. Doing all of these biochemical and biophysical characterizations of CaMKII always opens the door for me to think about if everything we see in vitro can also be observed in vivo. Specifically, with subunit exchange, I am looking forward to the day I get the email about how it does have a role during LTP (hopefully!). Most of the focus should be on cells using fluorescence microscopy, CRISPR, and FRET biosensors. All the best CaMKII, it was fun.
A1. Mass spectrometry data showing partial biotinylation of purified CaMKII.

A2. Western blot for CaMKII using pan CaMKII and pThr286 antibody. We used panCaMKII to detect CaMKII monomer as a positive control and ULP1 as a negative control. And for pThr286, we use ULP1 as a negative control, activated CaMKII as a positive control,
dephosphorylated CaMKII and CaMKII from the purification. No background phosphorylation was detected.


spectroscopy and dynamics of intrinsically fluorescent proteins: Coral red (dsRed) and yellow (Citrine). *Proceedings of the National Academy of Sciences of the United States of America, 97*(22), 11996–12001. https://doi.org/10.1073/pnas.97.22.11996


fluorescent proteins. Nature Methods, 5(6), 545–551. https://doi.org/10.1038/nmeth.1209

Improve


human oocyte activation and infertility that is not rescued by the WW-binding protein PAWP. Human Molecular Genetics, 25(5), 878–891. https://doi.org/10.1093/hmg/ddv617


https://doi.org/10.1371/journal.pbio.1000426

https://doi.org/10.1038/nmeth.4431


targeting module that localizes CaMKIIα/β heterooligomers to dendritic spines.
*Neuron*, 21(3), 593–606. https://doi.org/10.1016/S0896-6273(00)80569-3


