

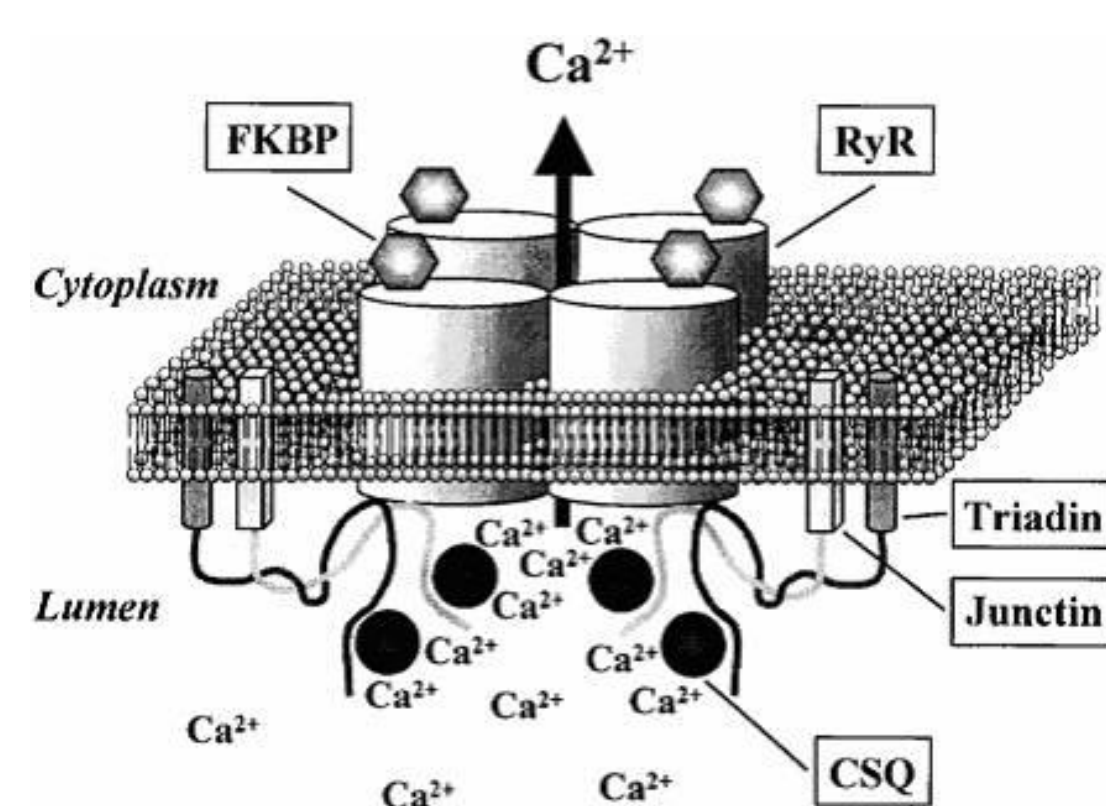


Kinetic Studies on the Polymerization of Cardiac Calsequestrin

Cayla M. Jewett, Emil J. Sanchez, Adam O. Barden, James A. Brozik and ChulHee Kang
Washington State University, Department of Chemistry, Pullman, WA 99164

1

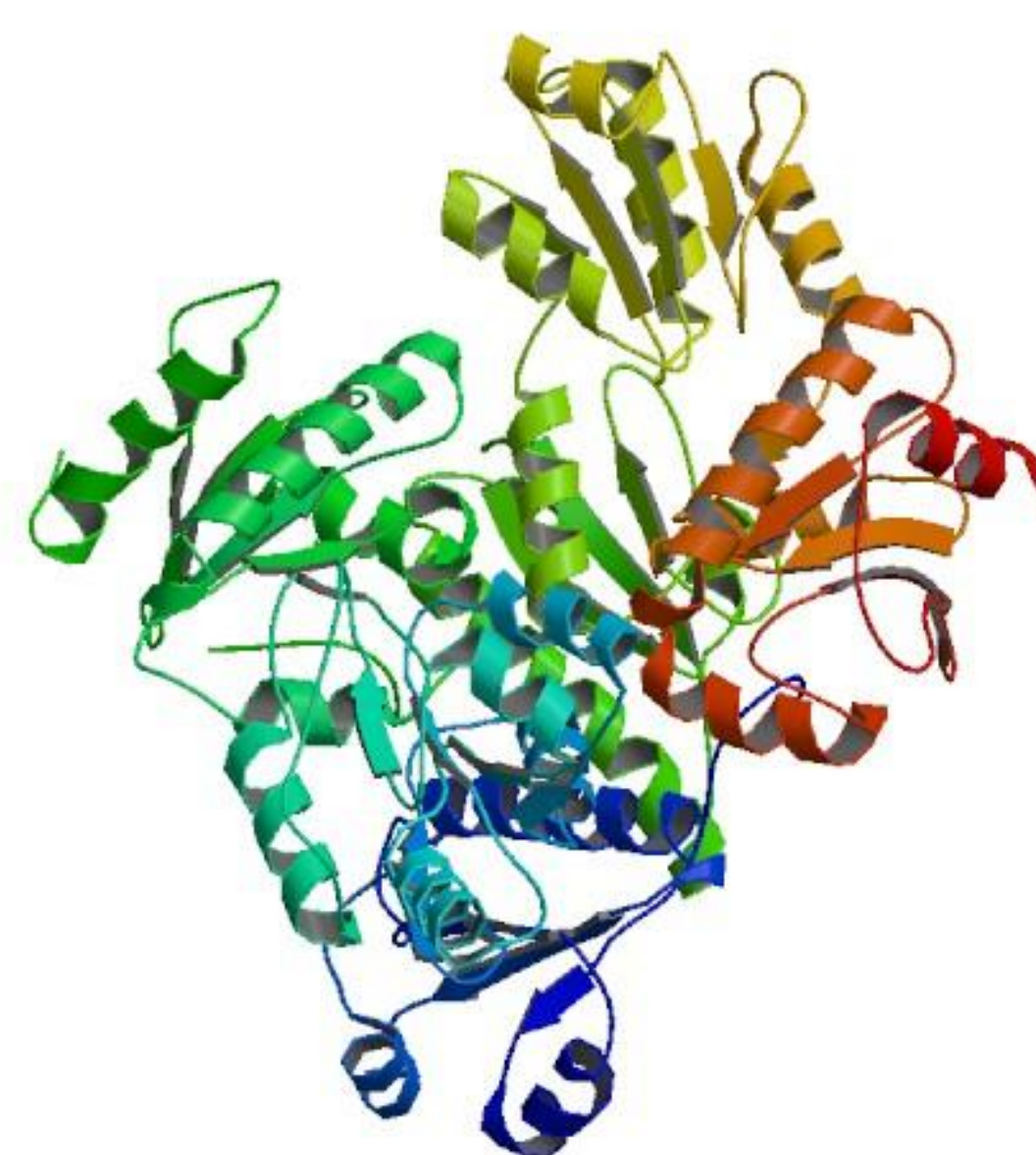
The Biological Role of Calsequestrin



- Calsequestrin is located in the lumen of the sarcoplasmic reticulum, and is part of the calcium protein complex (shown to the left)
- Binds calcium to enable concentrations above inhibitory level of ~1 mM
- Polymerization of calsequestrin binds calcium with higher affinity because entropically favored
- Charged surface created by polymerization enables surface diffusion of calcium out of lumen

2

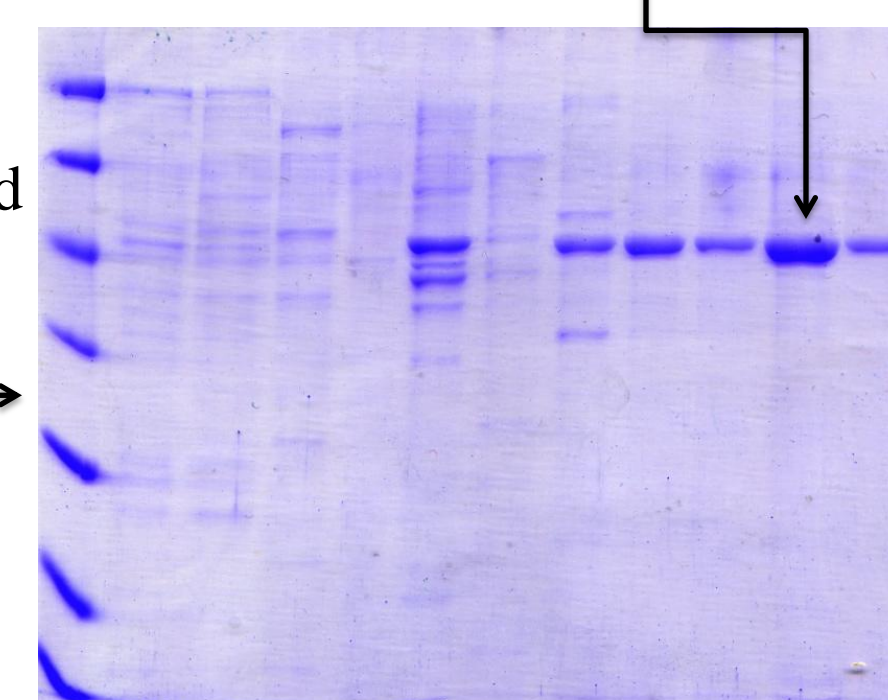
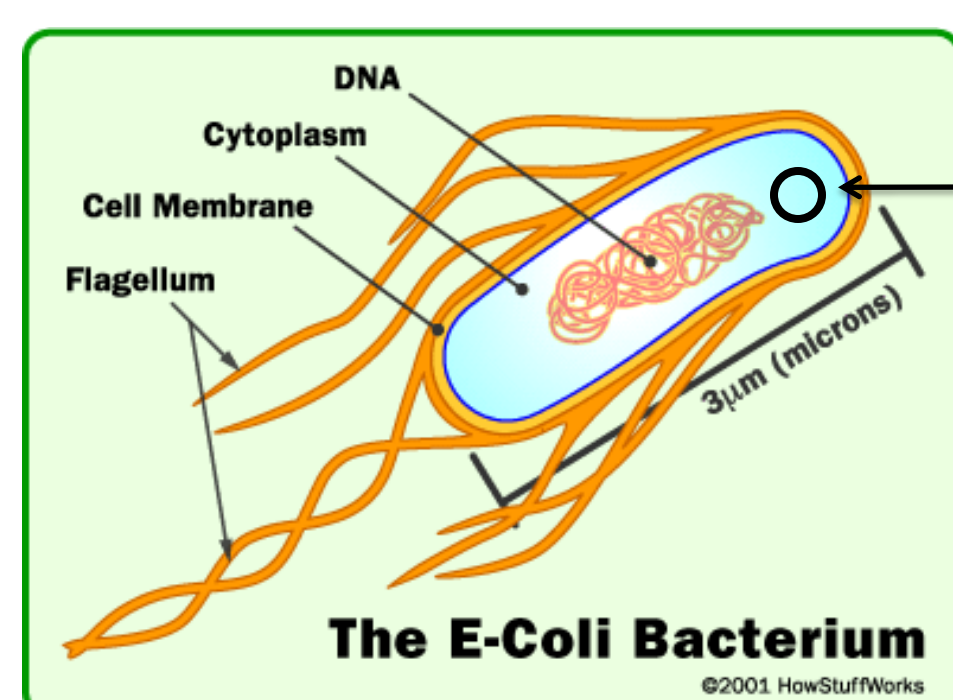
Calsequestrin Specifics



- Two types of calsequestrin present in body, cardiac and skeletal
- One of most acidic proteins in the body
- Cardiac has net charge of -69, while skeletal has net charge of -80
- Made of ~360 residues, 1/3 of which are aspartic or glutamic acid
- Split into 3 domains, each with a thioredoxin fold

3

Production / Purification



- HcCSQ transfected into E. Coli and grown until desired quantity of protein produced
- HcCSQ separated from E. Coli by breaking cells while in buffer solution, then centrifuging to remove cell remnants

- His₆-tag HcCSQ Purification
 - Ni-NTA Column
 - MonoQ Column
- Wild type HcCSQ Purification
 - DEAE Column
 - Hydroxylapatite Column
 - Phenyl Sepharose Column

Introduction

In the body, muscles contract when calcium diffuses from the lumen of the sarcoplasmic reticulum of each muscle cell into the cytoplasm via the calcium protein complex. This process, however, is inhibited when the calcium concentration approaches 1 mM. To circumvent this problem, calsequestrin, part of the calcium protein complex, is also present. As the calcium concentration surpasses 1 mM, the calsequestrin starts to polymerize and the calcium binds to the resulting charged surface. This binding enables surface diffusion of the calcium out of the lumen when muscle contraction is needed, allowing for a faster response time than would liquid diffusion.

Since the polymerization of calsequestrin is such an integral part of proper muscle function, even small changes or problems can have serious consequences. A number of medical drugs have the unfortunate side effect of altering this polymerization process, resulting in fast heartbeat, racing muscle spasms, jerky movements, trembling and shaking of limbs, and tachycardia. In severe cases, these symptoms can lead to a heart attack and even death. Additionally, a mutation in the calsequestrin can lead to catecholamine-induced polymorphic ventricular tachycardia (CPVT), which causes syncope, seizures, and sudden death. To help minimize these drug side effects and counteract CPVT, a better understanding of the calsequestrin polymerization process is needed. Thus, the focus of this research project is to study the kinetics of the polymerization of cardiac calsequestrin (HcCSQ) using single-molecule fluorescence microscopy.

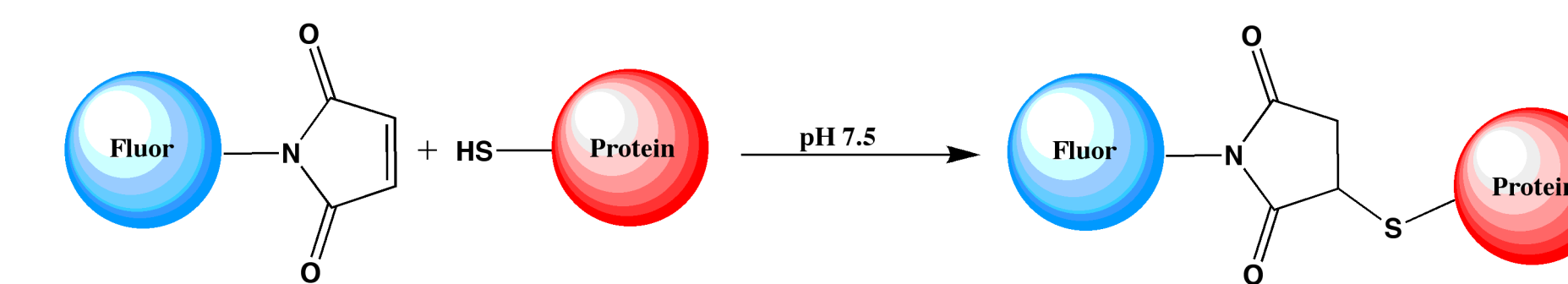
To study the kinetics of the polymerization, two types of HcCSQ, his₆-tag and wild type, were grown, purified and labeled with a fluorescent dye. Translation of the desired protein entailed transfecting E. Coli cells with plasmids containing the HcCSQ DNA. HcCSQ was separated from the E. Coli by centrifuging the cells into a pellet, dissolving into a buffer solution, sonicating, then centrifuging again, resulting in a supernatant containing the HcCSQ. Different purification methods were used for each type of HcCSQ. The his₆-tag HcCSQ was purified using Nickel-NTA and MonoQ columns, while the wild type HcCSQ was purified using DEAE, hydroxylapatite, and phenyl sepharose columns. SDS-PAGE and BCA assays were used to determine the purity and concentration of HcCSQ in the resulting solutions. Finally, the cysteine residues of the his₆-tag and wild type HcCSQ were labeled with Alexa Fluor C₂ 660 Maleimide and Alexa Fluor C₂ 555 Maleimide, respectively, via a maleimide-thiol reaction.

Ongoing / Future Work

In the second stage of this project, single-molecule fluorescence microscopy will be used to study the kinetics of the polymerization of HcCSQ. Currently, different schemes are being analyzed to determine the most effective method for immobilizing the Alexa Fluor C₂ 660 Maleimide labeled his₆-tag HcCSQ on a borosilicate surface. Possible options are immobilizing the his₆-tag of the HcCSQ using Ni-NTA that is attached to a borosilicate surface via 3-glycidyloxypropyl-trimethoxysilane, or immobilizing the his₆-tag using an anti-his antibody that is attached via a crosslinker (N-γ-maleimidobutyryloxy succinimide ester) and a silane (3-Mercaptopropyltrimethoxysilane). Once an immobilization scheme is proven effective, single-molecule experiments will be done to determine proper concentrations for studying both forms of labeled HcCSQ. Next, single-molecule studies of the polymerization of Alexa Fluor C₂ 555 Maleimide labeled wild type HcCSQ to the immobilized labeled his₆-tag HcCSQ in different calcium concentrations will be performed to determine rate constants. Finally, these same studies will be performed under different temperatures to determine the remaining thermodynamic values for the polymerization of HcCSQ.

4

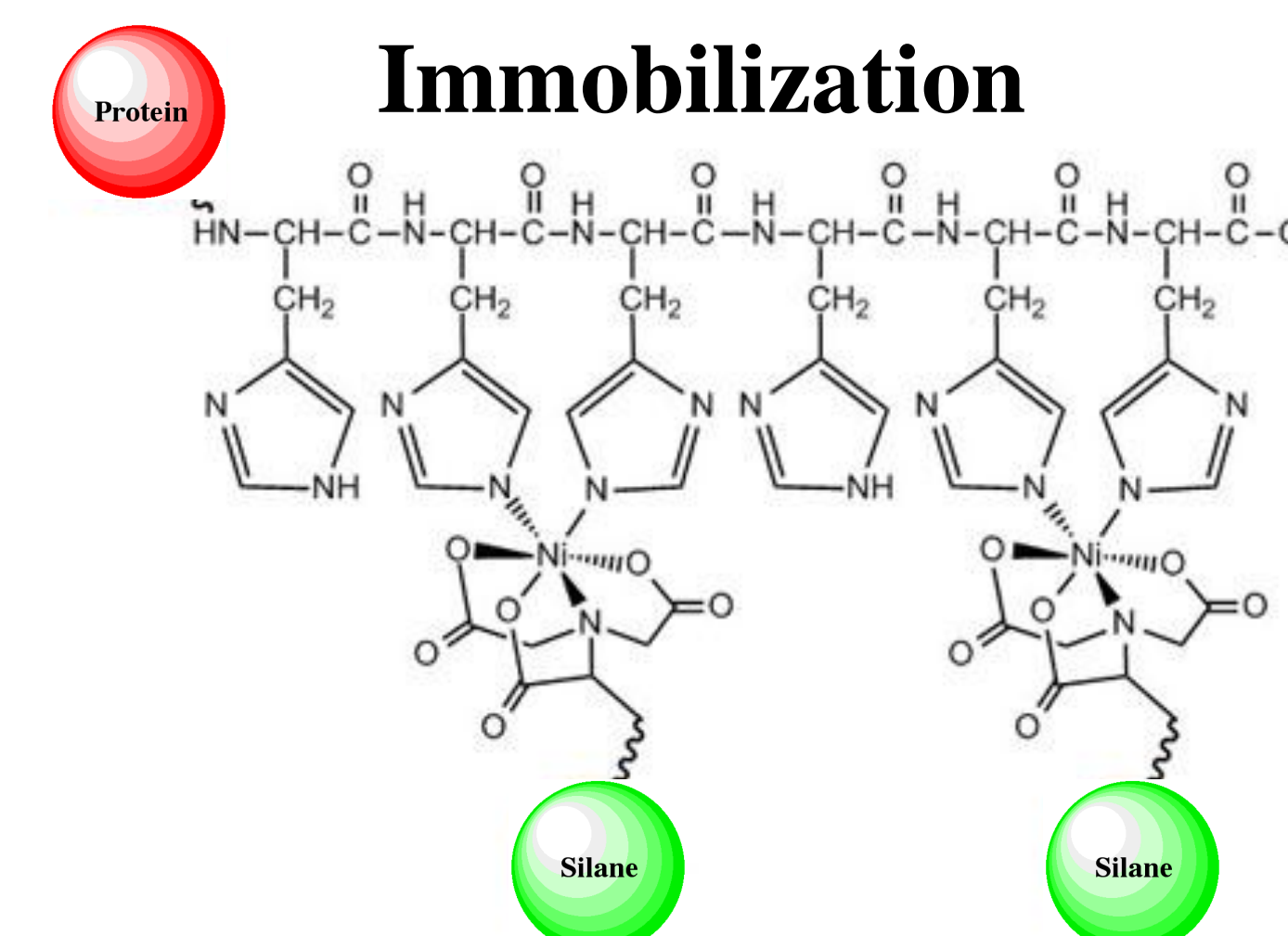
Labeling



- A fluorophore was attached to the protein via a maleimide-thiol reaction between the maleimide of the fluorophore and the thiol on the cysteine of the HcCSQ
- His₆-tag HcCSQ was labeled with Alexa Fluor C₂ 660 Maleimide
- Wild type HcCSQ was labeled with Alexa Fluor C₂ 555 Maleimide
- A Native Gel was run to ensure that labeling did not alter the folding / function of the HcCSQ

5

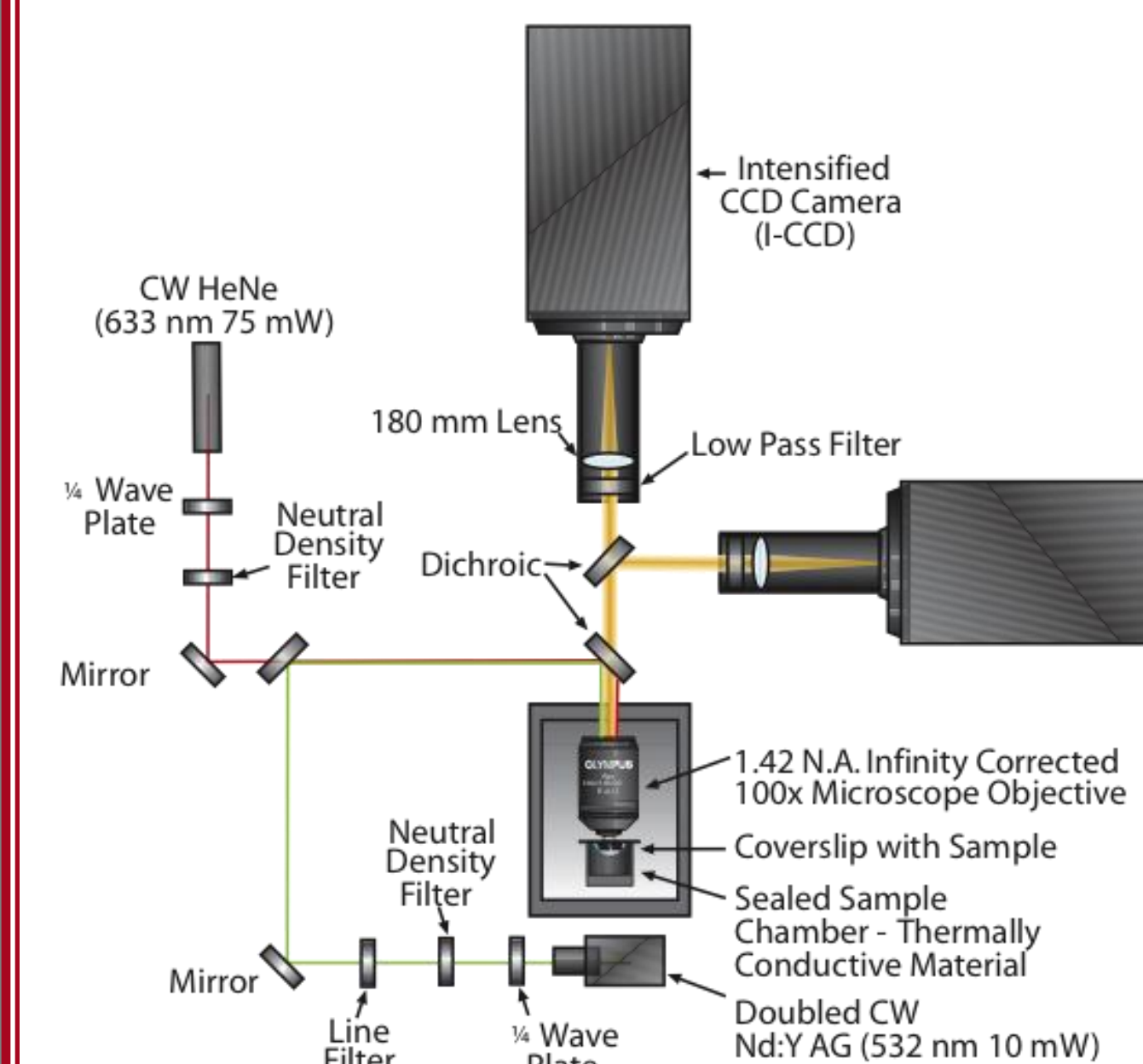
Immobilization



- The his₆-tag on the HcCSQ is immobilized by Ni-NTA, which is attached to a borosilicate surface via 3-glycidyloxypropyl-trimethoxysilane, or
- The his₆-tag on the HcCSQ is immobilized by an anti-his antibody, which is attached to a borosilicate surface via a crosslinker (N-γ-maleimidobutyryloxy succinimide ester) and a silane (3-Mercaptopropyltrimethoxysilane)

6

Single-Molecule Fluorescence Microscopy



- Dual color experiments will be used to study kinetics of HcCSQ polymerization
- HeNe laser excites immobilized Alexa Fluor C₂ 660 Maleimide labeled his₆-tag HcCSQ
- Nd:YAG excites Alexa Fluor C₂ 555 Maleimide labeled wild type HcCSQ