

Dear members of the Council,

Thank you for awarding me the “ESC First Contact Initiative Grant”, which offered me the opportunity to travel to the University of Arizona to closely collaborate with Professor Jil Tardiff and her group.

My visit was extremely enriching both professionally and personally, providing me to take my project one step further in the understanding of hypertrophic cardiomyopathy (HCM)-disease onset. During my time there, I had the unique opportunity to learn and work with protein sample preparation, expression and purification of thin and thick filament sarcomeric proteins, and Förster Resonance Energy Transfer (FRET). In addition, I was able to learn about regulated *in vitro* motility assay (R-IVM) and Stop-Flow experiments. I was actively involved in scientific meetings and discussions, which allowed me to thoroughly develop my understanding and importantly, design and develop future projects with Professor Jil Tardiff’s group.

Please find enclosed a report on the experimental work I developed in collaboration with Professor Jil Tardiff during my visit at the University of Arizona.

I sincerely appreciate your trust and support, which granted me the opportunity to interact with a top institution world-wide with exciting young members. In conclusion, my visit was a great success.

Yours sincerely,

Vasco Sequeira

## Scientific Report

Recently, using a homozygous human troponin T (cTnT) mutant (K280N) sample (*TNNT2<sub>mut</sub>*) known to cause hypertrophic cardiomyopathy (HCM) we observed elevated  $\text{Ca}^{2+}$ -sensitivity and reduced length-dependent activation compared to non-failing donor cardiomyocytes.[1] Treatment with protein kinase A (PKA) did not eliminate the difference in  $\text{Ca}^{2+}$ -sensitivity and length-dependent activation between the *TNNT2<sub>mut</sub>* and donor samples.

The homozygous *TNNT2<sub>mut</sub>* necessarily results in 100% mutant cTnT and as such represents a unique tool to assess the level at which mutant protein perturbs sarcomere function. Exchange with 1 mg/mL wild-type (wt) human cTn complex resulted in  $86\pm 1\%$  cTn exchange and was sufficient to restore length-dependent activation back to donor levels when coupled to PKA-treatment. Surprisingly,  $\text{Ca}^{2+}$ -sensitivity remained high when compared to donors. These data supports the great dominance of the *TNNT2<sub>mut</sub>* on  $\text{Ca}^{2+}$ -sensitivity, such that 14% cTnT mutant protein (86% wild-type troponin exchange) presents the full effects on  $\text{Ca}^{2+}$ -sensitivity as observed with 100% endogenous mutant protein.

The purpose of my visit to Professor Jil Tardiff's group was to complement our initial data from human cardiomyocytes with molecular dynamic analysis, including FRET, which would allow for the detection of structural changes between the poorly understood C-terminus of the troponin-tropomyosin (Tn-Tm) complex that includes the cardiac troponin T (cTnT) K280N mutant.

In recent years, Professor Jil Tardiff's group have successfully captured mutant-specific molecular mechanisms of HCM-related alterations and carefully linked their structural changes with the associated pathophysiological effects. Using a complete atomistic model of the Tm-cTn complex developed by their group they have identified the biophysical mechanisms whereby cTnT mutations propagate their effects over distance, and additionally discovered that such effects are inversely correlated to the thin filament  $\text{Ca}^{2+}$ -cooperative activation.[2-4] In addition, the use of informative FRET pairs of thin filament proteins allows the validation of the *in silico* findings with structural observations, which permits to strengthen the generalization that HCM is a "biophysical disease".[5] This is of extreme importance since recent data strongly indicates that the amount of mutant sarcomeric proteins of human HCM patients can deviate from the expected heterozygous 50:50 ratio (<50), demonstrating that significant dosage-dependent effects can harmfully impact the structure and dynamics of sarcomeric proteins.

## Methods

### Förster Resonance Energy Transfer (FRET)

Förster Resonance Energy Transfer (FRET) is a sensitive method that describes the energy transfer between two fluorophores and thus permits monitoring of small conformational changes of proteins. A donor fluorophore will be energetically excited and responsible for the transfer of its energy to an acceptor fluorophore via dipole-dipole coupling. In proteins, because cysteine residues (Cys) are scarce, they are used for specific-binding of

fluorophores. Donor and acceptor proteins are genetically modified, so as to have each an engineered Cys residue to allow the accessibility of the fluorophore. Measurements of FRET efficiency can be used to determine if two fluorophores are within a certain distance of each other, thus allowing the monitoring of alterations associated with a disease mutation.

The different stages for FRET measurements are protein overexpression and purification, protein Cys-labeling with fluorophores, determination of labeling ratio, and thin filament reconstitution and FRET measurements.

### **Protein mutagenesis, expression, purification and reconstitution**

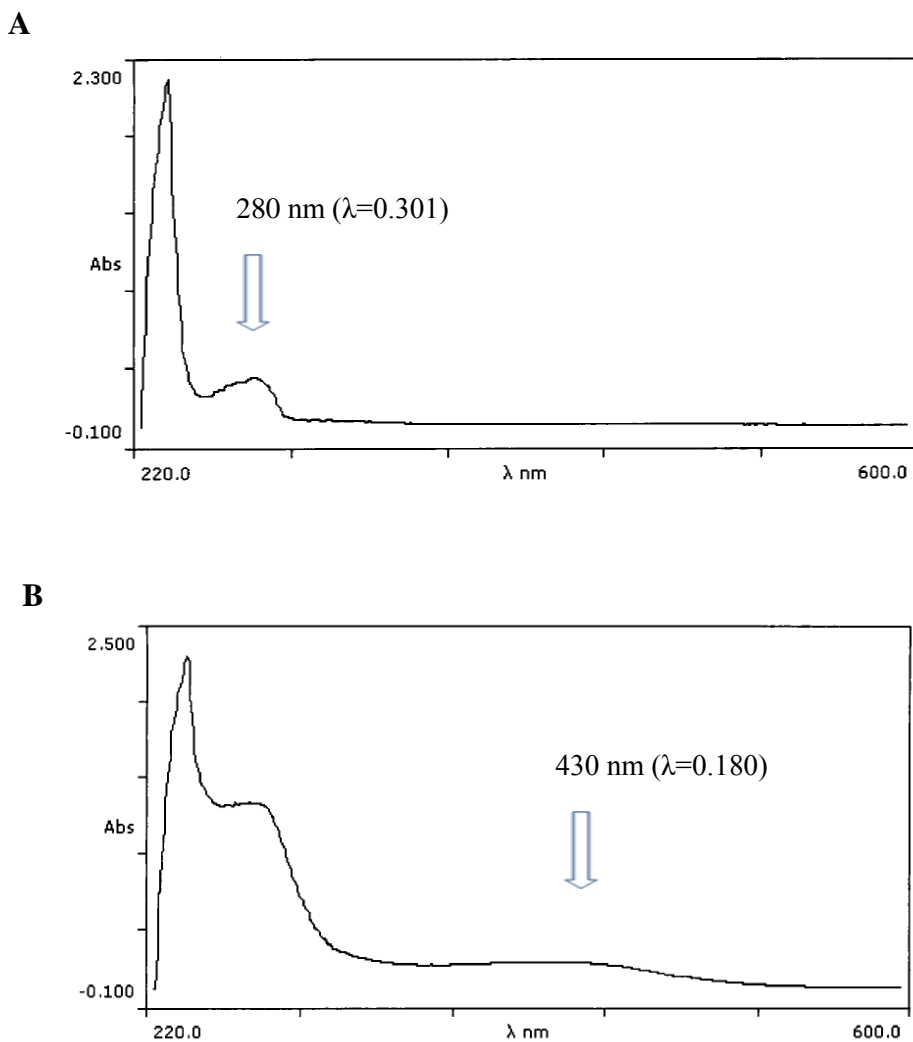
To perform FRET, a series of recombinant wild-type and mutant constructs were generated from cDNA containing the coding sequence for human cTnT, cTnC and cTnI. All HcTn proteins (wild-type or mutants) were cloned into pET3D vector (Novagen cat no: 69421-3) and selectively mutated using the QuikChange II XL site-directed mutagenesis Kit (Agilent cat no: 200521) according to the manufacturer's instructions. Each mutant constructs were transformed into XL10-gold ultracompetent cells and verified by direct DNA sequencing the incorporation of the mutation. Thereafter, the plasmid DNA of the HcTn constructs was transformed into BL21 bacteria cells for overexpression, while solely HcTnT constructs were expressed into Rosetta cells, because these cells are able to express rare codons, which HcTnT contains in great number.

After downstream process, the four HcTn FRET pairs required were purified for FRET. Each mutant fraction generated contained wild-type HcTn subunits and one/two of the following: HcTnT (XxC), HcTnT (XxC/K280N) + HcTnC (XxC), and HcTnC (XxC) [nucleotide type and location are omitted (defined here as "X" or "x", respectively) as work is currently in progress]. The K280N substitution was used to mimic the HCM human cTnT mutant (K280N) sample and endogenous cTnC cysteine residues have been substituted for Serines, as to avoid unwanted Cys-labeling. HcTnC (XxC) was modified with N-(4-dimethylamino-3,5-dinitrophenyl-maleimide) (DDPM) as FRET acceptor and the labeling ratio was assessed spectroscopically at 430 nm for DDPM ( $3500\text{M}^{-1}\text{cm}^{-1}$ ). The label ratio was determined to be 76.3%. The labeling is shown in Figure 1.

During my visit, actin, tropomyosin and all the four HcTn FRET pairs were overexpressed and purified. Tropomyosin was similarly prepared by following downstream protocol. Actin was purified from rabbit skeletal muscle acetone powder. Unfortunately, during my 4-week time stay only one (acceptor) of the four HcTn proteins required for FRET was labelled. In this regard, the group of Professor Jil Tardiff will complete the work, and will accurately determine mutant-specific alterations in the C-terminal region of cTnC.

HcTnT (XxC) and HcTnT (XxC/K280N) donor proteins will be labeled with 5-(2-iodoacetyl-amino-ethyl-amino-naphthalene-1-sulfonic acid) (AEDANS) as FRET donor and will be assessed spectroscopically at 325 nm for DDPM ( $5900\text{M}^{-1}\text{cm}^{-1}$ ). Thereafter, the troponin complex of the four HcTn FRET pairs will be reconstituted by mixing equimolar amounts of cTnT, cTnC and cTnI (1:1:1) and further reconstituted into thin

filament samples using a actin:tropomyosin:troponin molar ratio of 7:1:1. Fluorescence intensity decays will be measured using a FluoroCube lifetime system, which will allow for the detection of FRET.



**Figure 1. Labeling ratio determination.** The labeling ratio was assessed spectroscopically by carefully quantifying first at 280 nm the total purified protein ( $4470\text{M}^{-1}\text{cm}^{-1}$ ) yielding a value  $67.3\ \mu\text{M}$  ( $=0.301/4470$ ) and thereafter at 430 nm for DDPM ( $3500\text{M}^{-1}\text{cm}^{-1}$ ) yielding a labeling of  $51.4\ \mu\text{M}$  ( $=0.180/3500$ ). A labeling ratio efficiency of 0.763 was obtained ( $=51.4/67.3$ ).

## References

- [1] Sequeira V, Wijnker PJM, Nijenkamp LLAM, Kuster DWD, Najafi A, Witjas-Paalberends ER, Regan JA, Boontje N, ten Cate FJ, Germans T, Carrier L, Sadayappan S, van Slegtenhorst MA, Zaremba R, Foster DB, Murphy AM, Poggesi C, dos Remedios C, Stienen GJM, Ho CY, Michels M, van der Velden J. Perturbed length-sepdependent activation in human hypertrophic cardiomyopathy with missense sarcomeric gene mutations. *Circ Res* 2013;112:1491-505.
- [2] Manning EP, Tardiff JC, Schwartz SD. A model of calcium activation of the cardiac thin filament. *Biochem* 2011;50:7405-13.
- [3] Manning EP, Tardiff JC, Schwartz SD. Molecular effects of familial hypertrophic cardiomyopathy-related mutations in the TNT1 domain of cTnT. *J Mol Biol.* 2012;421:54-66.
- [4] Manning EP, Guinto PJ, Tardiff JC. Correlation of molecular and functional effects of mutations in cardiac troponin T linked to familial hypertrophic cardiomyopathy. *J Biol Chem* 2012;287:14515-23.
- [5] Tardiff JC. Thin filament mutations: developing an integrative approach to a complex disorder. *Circ Res.* 2011;108:765-82.