REPORT

Report on participation of the ICMR International Fellow (ICMR-IF) in Training/Research abroad.

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2. Address : Department of Corelab,
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3. Frontline area of research in which training/research was carried out : Genomics and Biomarker Discovery

4. Name & address of Professor and host institute : Prof. Joseph Irudayaraj
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5. Duration of fellowship with exact date : 10th Oct 2019 to 10th Dec 2019
6. Highlights of work conducted :

a. **Technique/expertise acquired:**

Fluorescence Correlation Spectroscopy (FCS)

Micro and Nanotechnology Laboratory in the University of Illinois Urbana-Champaign houses a Scanning Confocal time-resolved microscope system which is used for confocal fluorescence imaging and fluorescence correlation spectroscopy (FCS). FCS provides information on the mobility of fluorescing molecules at single molecule resolution and also helps to assess the role of the mobility and diffusion kinetics of epigenetic enzymes on methylation programming inside a cell. Imaging experiments to evaluate cell function and receptor aggregation were performed to generate data for a collaborative publication with the host institution. Live cell monitoring of diffusion of DNMTs/TETs was performed using the FCS and analysis modules of the above system.

Around 10,000 ARPE-19 cells were seeded on round 18-mm, No 1.5H coverslip. Coverslips were coated with poly-L-lysine in 12 well plates under UV and rinse at least 3 times with sterile MQ water. After around 48 hours of cell culture, the cell culture medium was replaced and transfected with DNMT3A-EGFP. Around 300 ng of DNMT3A-EGFP was transfected with lipofectamine 3000 according to the manufacturer's instruction overnight. The next morning, the cell culture medium was replaced with fresh medium and incubated with DEX for 4 more hours. Cells were then washed with warm PBS before transferring to an in-house 3D printed coverslip holder in a stage top incubator at 37°C for FCS data collection. Data were collected at five 20-second measurements per location to minimize photobleaching effects in each segment of data. Cells were measured within one hour before switching to other samples.

The autocorrelation function G(t) of FCS is defined as:

$$G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t+\tau) \rangle}{\langle \delta F(t) \rangle^2} \quad (1)$$

Where $< F >$ is the average fluorescence intensity and $\delta F(t) = F(t) - < F(t) >$.

For solution measurements, $G(\tau)$ was fitted with:

$$G(\tau) = \frac{1}{<N>} \cdot \frac{1}{1+\frac{\tau}{\tau_D}} \cdot \frac{1}{\sqrt{1+\frac{1}{\kappa^2}\frac{\tau}{\tau_D}}} \quad (2)$$

Where $\kappa = \frac{z_0}{\omega_0}$, represents the structure parameter of the confocal profile. τ_D is the characteristic diffusion time of the fluorescence molecules. K was calibrated using 10 nM Rhodamine110 ($D=430\mu m^2\ s^{-1}$) at the beginning of each experiment. $< N >$ is the average number of fluorescent particles in the effective volume.

For live-cell FCS, the acquired $G(\tau)$ was fitted with the standard equation for free, 3D Gaussian 2-component model:

$$G(\tau) = \frac{\gamma}{<N>} \cdot \left[f_1 \left(\frac{1}{1+\frac{\tau}{\tau_{D1}}} \cdot \frac{1}{\sqrt{1+\frac{1}{\kappa^2}\frac{\tau}{\tau_{D1}}}} \right) + (1-f_1) \left(\frac{1}{1+\frac{\tau}{\tau_{D2}}} \cdot \frac{1}{\sqrt{1+\frac{1}{\kappa^2}\frac{\tau}{\tau_{D2}}}} \right) \right] \quad (3)$$

Here f_1 represents the fraction of its corresponding component. All FCS analysis was performed with VistaVision software (ISS, Champaign, USA).

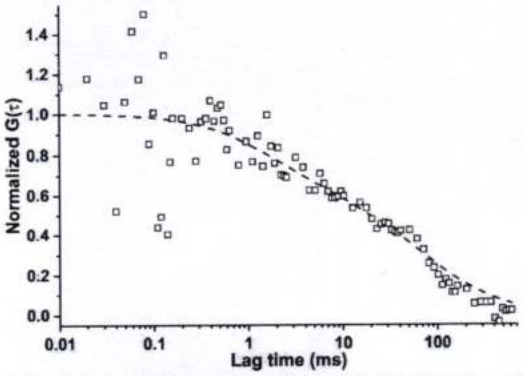

Fig: *Typical FCS curve of DNMT3A-EGFP in ARPE-19 cells fitted with a 3D 2-component free diffusion model (dashed line).*

Briefly instrument setup includes, a 488 nm picosecond pulsed laser at 50 MHz repetition rate used as the excitation source, and the emission passed through an apochromatic water immersion objective. Photons were collected by the same objective, reflected by a 561 nm band pass filter, a 525/50 emission filter, and a 50 µm pinhole before reaching an avalanche photodiode. Samples were then prepared and imaged. Fluorescence information was recorded using the time-correlated single photon counting module in the time-tagged time-resolved mode using TimeHarp200 tool. Raw fluorescence images and autocorrelation data were analyzed using SymPhoTime software package.

This work is part of the collaborative publication with Dr. Joseph Irudayaraj (host Supervisor) and Vision Research Foundation (parent institute) which was written and submitted to Journal of Molecular Vision during the fellowship period.

Pyrosequencing

PyroMark Q24 Advanced system from Qiagen is a robust and latest instrument used for methylation and mutation quantification in long sequence runs. This advanced technology provides real-time, sequence based detection and quantification of DNA methylation at CpG and CpN sites and is also helps in analyzing complex mutations and de novo sequencing applications such as microbial typing.

Complete training on the principle and operation of the instrument was obtained with the help of graduate student in the host lab. As the machine was not in use for a very long time me with the help of the student initiated the machine and successfully brought it to up and running stage. We fist did the performance check of the instrument and vacuum station with the PyroMark Q24 Advanced Validation Oligo using the guided protocol by Qiagen Application Scientist. After three successful performance checks, Linearity, Bias and Repeatability of the measurements was checked using PyroMark and Control DNA kits using manufacturer's protocol. Two test runs were performed to troubleshoot the bias in repeat samples.

We then proceeded to evaluate gene-specific methylation for the gene Acetyl co-enzyme-A synthetase (ASCl 1) in mouse tissue samples. Forward Reverse and Sequencing primers were designed using Pyromark Assay Design tool 2.0 of Qiagen and primers were obtained from the company. Genomic DNA isolated from mouse tissue samples using Qiagen tissue DNA extraction kit. Extracted DNA is first bisulfite converted using the EZ DNA methylation kit and then PCR amplified using the Pyromark PCR kit per manufacturer's specification with 20 ng of bisulfite converted DNA. A biotinylated PCR product is generated from the bisulfite converted DNA template and is bound to streptavidin-coated Sepharose beads. The beads are captured with the vacuum tool on the PyroMark Vacuum Workstation, where they are thoroughly washed and subsequently denatured, generating single-stranded DNA suitable for Pyrosequencing. This template DNA is released into the Pyrosequencing reaction plate containing the sequencing primer provided with PyroMark CpG Assays. After primer annealing, the plate is placed into the PyroMark instrument, and the sequencing reaction is started. Pyrogram is read and analyzed for CpG sites using PyroMark Q24 Advanced software. As the experiments performed are part of PhD thesis of the graduate student in the host institute who trained me I could not incorporate the results in this report.

GWAS data analysis using R program

Training on the R programming based GWAS data analysis to identify statistically significant genetic variations between populations was obtained from the Bioinformatics core at the Roy J Carver Biotechnology Centre. Using the learnt methodology data analysis for identifying the Single Nucleotide Polymorphisms between steroid responders and Non-responders from Indian population was performed. The data is part of the running ICMR project in which Genome Wide Association Study (GWAS) was performed by low-pass whole genome sequencing from patient blood samples in VRF, India. Blood samples from a total of 52 patients receiving steroid treatment for various retinal complications were collected and analysed in this study. Association testing was already performed to identify the statistically significant SNPs between groups using the Chi-Square Test implemented in Plink software. A Q-Q plot of p-values across all SNPs was produced using R program, demonstrating that results were not confounded by population structure or relatedness among individuals. Although power to detect associations was low due to small sample size, elbows and gaps in the Q-Q plot suggested –log10(P) thresholds of 4.75 and 5.5 for identification of putatively associated SNPs for further investigation (Figure). Manhattan plot (Figure) was generated to

describe the distribution of association p-values across variant loci. The points above grey and red lines represent statistically significant SNPs above threshold 4.75 and 5.5 obtained from the QQplot. Primary Analysis revealed a total of 20 SNPs to be significant above the threshold. Further analysis to understand the role of the significant genes is being processed after coming back to India.

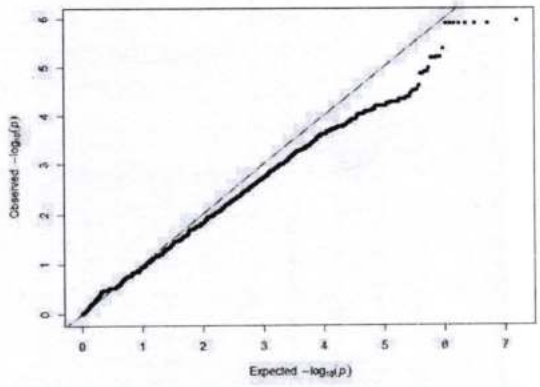

Figure: *Q-Q plot of p-values across all SNPs showing elbows and gaps suggesting –log10(P) thresholds of 4.75 and 5.5 for identification of putatively associated SNPs for further investigation*

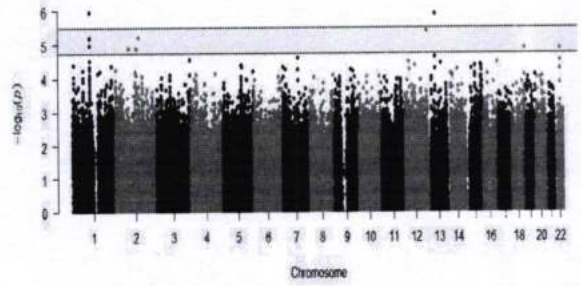

Figure: *Manhattan plot showing the distribution of associated p-values across variant loci. Points above Grey and red line represent statistically significant SNPs above thresholds 4.75 and 5.5 respectively*

Fluorescence Lifetime-Imaging Microscopy Förster resonance energy transfer (FLIM-FRET)

As part of the fellowship training I got an opportunity to observe the instrumentation and workflow of advanced FLIM-FRET technology using Scanning Confocal time-resolved microscope system with a Microtime200 housed in Micro and Nanotechnology Laboratory in the University of Illinois Urbana-Champaign. I was allowed to observe the flow of experiments being conducted for an ongoing project in the host lab. FLIM FRET is a technique used to understand the direct interactions between cellular proteins and various epigenetic modifications occurring inside the cell. These can provide insights into the role of epigenetic modifications in disease development which can be used as potential therapeutic targets. FRET screening has been used in applications such as to identify molecules or drugs that dissociate DNA-protein or protein-protein interactions in search of possible inhibitors and evaluation of apoptosis or protein aggregation for drug screening with known molecules. FLIM-FRET also allows for the observation of interactions at single molecule resolution and other proximity-based evaluation of molecules directly.

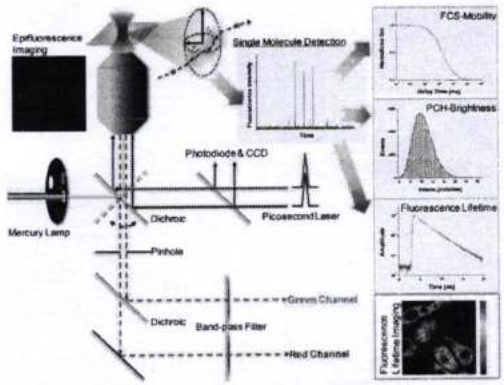

Fig: Functional modules of implemented single-molecule platform. The single-molecule system is integrated into a confocal fluorescence scanning microscope. Other than the conventional fluorescence imaging, the power of this system comes from simultaneously determining multiple parameters of the fluorescent molecules. The optic "femtoliter" detection volume created by μm-level pinhole or multi-photon laser, in combination with user-friendly interface for data analysis, makes it possible to obtain and visualize the diffusion dynamics (FCS/FLCS), molecular stoichiometry (PCH), and inter-molecule/molecule environment interactions (FLIM, FLIM-FRET) within one single measurement. Image Source: *Cui Y, Irudayaraj J. Dissecting the behavior and function of MBD3 in DNA methylation homeostasis by single-molecule spectroscopy and microscopy. Nucleic Acids Res. 2015;43:3046–55*

The instrument workflow observed involves excitation of GFP tag and Alexa488 labeled antibodies using a 465 nm picosecond pulsed laser. Cy3 was excited by a 532 nm laser for in vitro Photon Counting histogram (PCH) calibration. The excitation beam was delivered to the sample stage through an apochromatic water immersion objective and the fluorescence was collected by the same objective, after which the emission was separated by a dual band dichroic. A 50 m pinhole was employed to block the off-focus photons and the final signal was additionally filtered by a band-pass filter (520 ± 20 nm for green emission, 610 ± 30 nm for red emission) before reaching the single photon avalanche photodiode detectors (SPAD). Fluorescence information was recorded using the time-correlated single photon counting module in the time-tagged time-resolved mode using TimeHarp200 tool. Raw fluorescence images and autocorrelation data were analyzed using SymPhoTime software package.

b. Research results, including any papers, prepared/submitted for publication

- NIH-DBT Indo-US proposal on "Epigenome regulation and evaluation of targets in Steroid Induced Glaucoma" has been written and submitted to both NIH and DBT on the 9th of November 2019 during my visit as a Collaborative grant between the host institute and parent institute. Dr. Joseph Irudayaraj is the PI from the USA and Dr. Sharada Ramasubramanyam from Vision Research Foundation is the PI from India. I am part of the grant as Co-Investigator from India.
- A publication titled "Epigenetic alterations associated with Dexamethasone sodium phosphate through DNMT and TET in retinal pigment epithelium cells" has been written and submitted to Journal of Molecular Vision with collaborative work done with the US PI including the FCS experiments performed during my visit.

c. **Proposed utilization of the experience in India**

The experience greatly helps in extending my current research of steroid induced Glaucoma from genomics to epigenetics. From the training obtained in GWAS data analysis all the whole genome sequencing data to be developed by the fellow in the current project can be analyzed for more in-depth understanding of the population genetics and polymorphisms

unique to south Indian population. The same can be extrapolated to the GWAS studies currently being carried out at VRF for various Ocular disease. I intend to share the knowledge with my colleagues for them to get benefitted from the focused data analysis. Pyrosequencing is an advanced technique that helps in evaluating and quantifying the methylation pattern in targeted genes. As in the proposed INDO-US grant we aim to identify the epigenetics markers involved in steroid induced glaucoma, the knowledge and experience gained in understanding the principle and running the machine will help in processing the patient sample DNA for targeted methylation marks in future projects. Single cell microscopy using FCS involves high end state of the equipment which helps in evaluating the protein interactions at single cell resolution. This technique can be extrapolated to various ocular research projects that are focusing on identifying therapeutic targets for diseases. Intend to use the technique in the proposed INDO-US project and also incorporate the same in extension of the current project where in after identifying significant genes that are playing a role in steroid induced glaucoma, plan to evaluate the interaction of the genes with membrane proteins and explore their usage as therapeutic targets at single cell resolution level. Overview, advantages and applications of the technology will be shared with colleagues in parent institute for them to adapt the protocol in future projects.

Signature of ICMR-IF

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