Chiral Single Molecule Localization Microscopy (chiralSMLM)

Aravinth S[1], Neeraj Pant[1] and Partha P. Mondal[1,2]
[1] *Department of Instrumentation and Applied Physics, Indian Institute of Science, Bangalore 560012, INDIA and*
[2] *Centre for Cryogenic Technology, Indian Institute of Science, Bangalore 560012, INDIA*
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Chiral fingerprints are unique to a molecule and convey information related to binding sites, conformation, and its immediate chemical environment. Deciphering these information is expected to aid a better understanding of the biological processes at the very molecular scale. Here, we present a new technique called *chiralSMLM*, to selectively detect chiral enantiomers (left-handed and right-handed) of single molecules and generate a super-resolved map of their distribution in cell. Calibration, characterization, and optimization of the system is carried out using a polarized light source (left and right circularly) and by recording known structures (Actin filaments) in a cell. Subsequently, the distribution of Dendra2-Actin enantiomers on the actin filaments is mapped. Finally, *chiralSMLM* is employed to study the characteristic distribution of viral proteins (Hemaglutinin and NS3) in transfection studies of two diseases (Influenza type-A and Dengue type2) after 48 hrs of transfection. Single-molecule and cluster analysis reveals that left-handed HA and left-handed NS3 molecules have a larger footprint, suggesting the role of chiral molecules in promoting cluster formation during viral infection. This is amazing, considering the fact that Left-handed enantiomers are known to play a crucial role in a range of biological functions. The new classification of single molecules purely based on their chiral nature is expected to advance single-molecule imaging.

Summary & **Significance:** Deciphering the chirality of single molecules and studying its effect on the biological function of a cell is fabulous. This gives a new perspective for understanding functions purely based on the chiral nature of single biomolecules in a cell. We developed a *chiralSMLM* microscopy system to demonstrate the effect of chirality on the clustering behavior of viral proteins in a cell-transfection study. Hence, the findings provide a new way of quantifying molecule-molecule and molecule-organelle interactions.

Corresponding author (Partha Pratim Mondal): partha@iisc.ac.in

I. INTRODUCTION

For decades, molecular chirality has been known to play crucial roles in natural sciences [1] [2] [3]. In biology, the chirality of a molecule enables a better understanding of the biological processes associated with binding, conformation, and recognition of intermediates and catalytic products. This enhances our understanding of the functioning of stereoselective biomolecules in a cellular system. However, these molecules often occur in mixed form (racemic mixture), and hence, detection of the chirality of a single biomolecule is of great interest in a range of fields across all the sub-disciplines of biological sciences. The chirality at the single molecule level is hard to detect as compared to the bulk, calling for specialized optical detection methods. Therefore, a technique capable of deciphering chirality at a single molecule is of great interest to cell biology, drug discovery, and pharmacology.

The availability of chiral information, in addition to its position and localization information, adds a new dimension to single-molecule imaging. In general, super-resolution map gives the location of single molecules as evident from prominent SMLM techniques (PALM, fPALM, STORM, DNA-PAINT), and its variants (BP-fPALM, IML-SPIM, ROSE, SMILE, POSSIBLE, *scanSMLM*, *corrSMLM*) [15] [16] [17] [19] [22] [20] [21] [23] [4] [5]. These techniques, along with other super-resolution techniques, hold the key to unraveling the nanoscopic world of single biomolecules [24] [25] [47] [48] [49] [50] [24] [25]. Although these techniques produce ultra-high resolution map of the distribution of single molecules in a cell, there are no known techniques that can determine their chirality based on fluorescence. Noting that the chirality of a molecule gives key information related to folding, binding, and immediate chemical information, this becomes essential to understand underlying biological mechanism [26] [27] [28]. For example, the three-dimensional structures of proteins depend on the homochirality of amino acids. One of the enantiomers may fit an enzyme (through a binding site or chiral groove), but the other may not [14]. Hence, the chirality of a biomolecule is significant in carrying out the basic functioning of proteins / amino acids. In fact, chirality exists in carbohydrates, DNA, organelles, tissues, and organs and most of the amino acids are left-handed, and most sugars are right-handed [6] [7] [8]. The fact that the structure of a protein, its folding, and its biological functions are intrinsically linked directly exposes the role played by chirality. Moreover, stability and conformational flexibility, which are linked to chirality, are necessary for cargo transport, enzymatic processing, regulatory functions, and communication [29] [30] [31] [32] [33]. These processes have deeper repercussions in a number of diseases (Alzheimer's disease (AD), Parkinson's disease, and Type II diabetes) [34] [35] [36].

Circular polarisation luminescence (CPL) based spectroscopy and microscopy techniques are key for quantifying chiral molecules and its function in cellular systems [37] [38] [39]. The chiral molecules selectively absorb right- or left- circularly polarized light, and this is reflected in their emission pattern. Since CPL heavily depends on the absorbance of the sample, the techniques employing this property makes it insensitive for cell imaging. To date, chirality is explored in many fields ranging from cell biology to material science. For example, the technique is quite useful for the investigation of inorganic substrates generally used in material characterization [40] [41] [42].

Recently, CPL spectroscopy is found to be very useful for studying optically active Europium(III) which can be used as a contrast agent for cell imaging [37] [43] [44]. Moreover, wavelength-dependent left/right circular polarized emission from Europium(III) is observed and the same is used for chiral contrast imaging [45]. Despite these developments, circularly polarised emission microscopy studies are limited to bulk [46]. With the advancement of modern microscopy, it is necessary to develop microscopy techniques that can combine CPL with single molecule imaging.

In this article, we report the detection of chiral single biomolecules in a cell and use it to understand the clustering of single molecules, which is a critical step toward virion maturation. This is enabled by a dedicated chiral detection system where fluorescence from a single molecule is separated based on its chirality. To separate chiral fluorescence, a combination of a quarter-wave plate and a polarizing beam-splitter is used. The whole detection sub-system is realized on a double-channel $4f$ configuration, and the detection of fluorescence (from the single-molecule enentiomers) is observed on a single detection chip. In addition, a series of filters and steering mirrors are used to filter and direct both the beam (left-circularly and right-circularly polarized) onto the same camera chip, thereby enabling simultaneous detection of both the enantiomers. Subsequently, the technique is calibrated, standardized, and validated using fluorescent beads and a known circularly polarized light source. Finally, the method is employed to understand the effect of chirality on the clustering of conjugated viral proteins (Dendra2-HA and Dendra2-NS3) in transfected cells.

II. RESULTS

A. Chiral SMLM System

To investigate the chiral characteristics (left- and right- handedness) of a single biomolecule and its role in clustering during viral pathogenesis, a new kind of super-resolution microscopy system (*chiralSMLM*) is developed. Fig. 1 shows the schematic diagram of the developed *chiralSMLM* system. The system has two major optical sub-systems: illumination and detection. The illumination

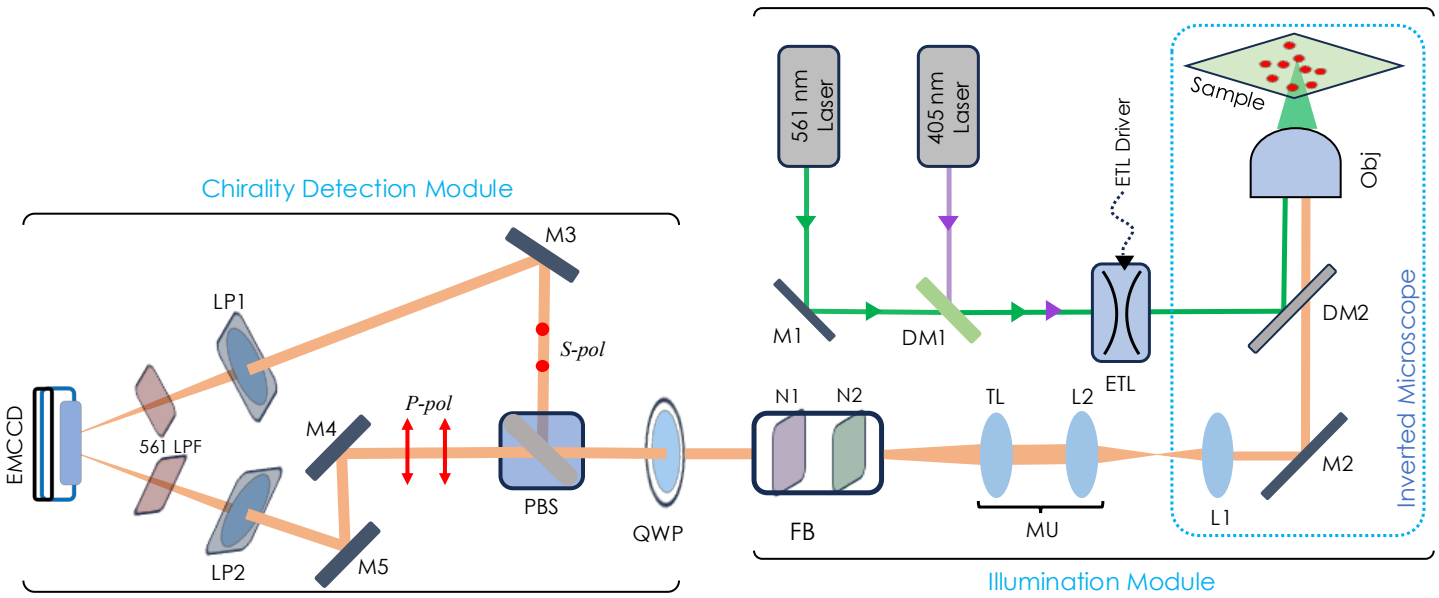

FIG. 1: **Schematic diagram of chiral SMLM:** The figure illustrates the optical setup of chiralSMLM that consists of laser sources, inverted microscope and chiral detection module.

consists of two lasers: an activation laser of wavelength 405 nm for activating the molecule and a second laser of wavelength 561 nm for excitation, causing fluorescence emission. The lasers are combined using a dichroic mirror (DMLP425R, Thorlabs) and directed to the electrically tunable lens (ETL) (Optotune, -1.5 to +3.5 diopters, EL-10-30-CI-VIS-LD-MV), which is placed in front of epifluorescence port of Olympus IX 81 inverted microscope. The ETL focuses the combined beam at the back aperture of the objective lens (Olympus, 100X, 1.3NA) using a dichroic mirror (Di03-R561-t1-25*36, Semrock) placed inside the filter cube of the microscope.

The chiral detection module consists of a Quater Wave Plate (QWP, 10RP54-1B, Newport) mounted on a precision cage rotation mount (CRM1PT, Thorlabs). The fast axis of the QWP is aligned 45° to the s-polarized light (s-polarization vector perpendicular to the optical table). This converts Right Circularly Polarized light (RCP) into s-polarized light and Left Circulary Polarized light (LCP) into p-polarized light. The s-polarized light is reflected by the polarizing beam splitter (PBS)(#49002, Edmund Optics), and p-polarized light is transmitted. This combination of QWP and PBS split the RCP and LCP fluorescence signal emitted by the single molecules into two paths. The mirror M3 directs the RCP light to the right side of the camera sensor (RCP channel), and the mirrors M4 and M5 direct the LCP to the left side of the camera sensor (LCP channel). The mirror $M5$ is placed on a linear translator stage, and it is moved precisely to ensure that the fluorescence signal on both channels is at a focal length of the tube lens (TL). The perfectly aligned linear polarizers (LP1 and LP2) ensure only s and p polarized light enters the camera. Also, the detection subsystem is equipped with two notch filters, N1, and N2 (NF03-405E-25,NF03-561E-25), placed in the filter box (FB) and a long pass filter LPF (561nm Long pass filter - LP02-561RE-25). These filters help eliminate the illumination lights (405 nm and 561 nm) and minimize the background, allowing fluorescence to reach the detector. Overall, the optical setup facilitates simultaneous recording of L and R circularly polarized fluorescence light emitted by left and right-handed single-molecules.

B. Calibration and Standardization

The fluorescence signal from both the channels is collected simultaneously on a single camera chip placed at the focal length of the tube lens. Simultaneous detection requires precise calibration which is performed by imaging fluorescent nanobeads (175nm, Invitrogen $ex : em|505 : 515nm$) embedded in Agarose gel-matrix. First, the bead image is focused on the RCP channel (top portion of the sensor chip), which is followed by precisely changing the distance between M3 and M4 such that the focused image of the bead appears on the LCP channel (see, Fig. 2A). The center of the bead on each channel is calculated, and based on that, lateral shift along the X and Y direction of the RCP channel with respect to LCP is determined. However, for axial shift, the bead was slightly defocused using the microscope nob. This resulted in the defocused image of the beads on both channels, the image of which is captured by the camera (see, Fig. 2B1). This defocused image of a bead consists of airy disk pattern, and accurate matching of this pattern (along with the rings) ensures same optical path for both channels, since slight off-set will result in change in size and position of the rings. Subsequently, the intensity is plotted along a line that passes through the center of each airy disk, and the intensity curve is correlated. The correlation is found to be 95.2%, confirming that sifnal from both channels are at the same focal distance. The fast axis of linear polarizers is aligned using a linearly polarized laser source and a power meter. The LP1 mounted on the precision cage mount is kept on a laser source which emits s-polarized light, and the fast axis of LP1 is rotated in such a way that it transmits maximum laser light. However, for LP2 the fast axis is aligned for the same laser where the transmitted laser power is minimum, and this ensures the proper alignment of linear polarizers to pass s- and p- polarized light. To ensure that the top portion of the camera sensor captures RCP light and the bottom portion captures LCP light, the quarter-wave plate (QWP) is calibrated using circularly polarized light generated by placing a circular polarizer in front of the linearly-polarized laser light source (see, Fig. 2A). The fast axis of QWP is rotated to get the maximum intensity in the top window and the image is recorded by the camera (see, Fig. 2.B2). Later, the right circular polarizer is replaced with the left circular polarizer and this time, the bottom window shows higher intensity compared with the top window. This ensures that the top window records the RCP light whereas the bottom window records LCP light, and this is also confirmed by the contrast between two windows in both cases, i.e., 23.8% and 35.8%, respectively.

C. Actin Filaments in NIH3T3 Cells

The technique (chiralSMLM) is tested on known biological samples with well-known structures such as Actin filaments. Fig. 2B shows the result obtained for Dendra2-Actin transfected NIH3T3 cells. The transfection protocol is discussed in the Methods section III.A. The filaments are imaged in a transfected cell to determine the fraction of chiral Actin molecules / proteins. Initially, the transfected cell image is captured using confocal microscope, as shown in Fig. 2, and a small region of the transfected cell (marked by yellow circle) is imaged using $chiralSMLM$. A sample frame of the recorded data is shown in Fig. 2(C3), which clearly indicates the intensity-difference between the channel for a sample Dendra2-Actin molecule. The reconstructed super-resolved images of the left- and right- handed Dendra2-Actin molecules, along with their merged image (with color code) is shown in Fig. 2(C4 & C5). Note the percentage of molecules detected in both channels (16% for right-handed and 10% for left-handed molecules). The chiral dissymmetry factor (g) of the rest 74% of molecules are

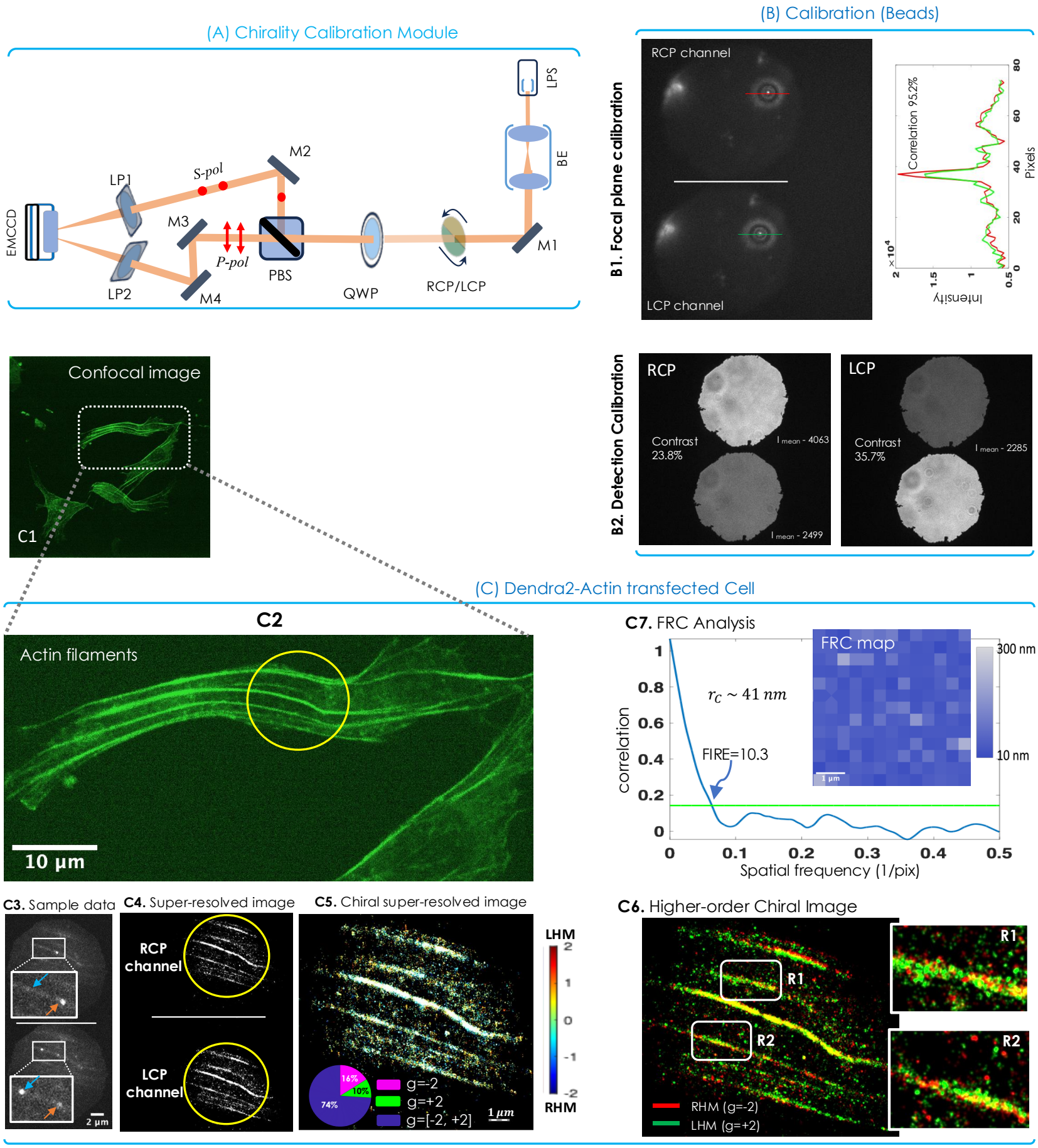

FIG. 2: **System calibration, quantification and image resolution :** (A) Optical setup for calibration system. (B1) The focal plane calibration of *chiralSMLM* system is performed by imaging the fluorescence beads on both the channels. The figure shows the PSF of fluorescent beads in both the channels, and intensity is plotted for the PSF along a line passing through the centre(red and green line) in each channel. The higher correlation(95.2%) of intensity plots confirms that the signal is recorded from a same focal plane. (A2) The detection module is calibrated using circularly polarized. The top channel is brighter when RCP is used, and the bottom channel is brighter when LCP is used. The contrast between the top and bottom channels are mentioned. (B1)Fluorescence image of a transfected cell is recorded using confocal microscopy and super-resolution study is carried out on a marked region(yellow circle). (C1) The fluorescence image of the transfected NIH3T3 cell. (C2) Enlarged view of the cell is shown and a small region (marked by yellow circle) is studied. (C3) Raw data of single molecule blinks shows that single molecule PSF intensity is different with respect to the molecule in two channels as shown by the blue and orange arrows in the enlarged images. (C4) The reconstructed super-resolution image of RCP and LCP channels. From these images, the chiral dissymmetry factor(g) is calculated, and the molecules are color-coded based on their dissymmetry factor value and presented as chiral super-resolved image (C5). The pie chart in fig C5 shows the distribution of the molecule based on the g value (C6). In order to visualize the distribution of molecules that exhibit higher order chirality, the super-resolution images are reconstructed using molecules with $g = -2$ (Right Handed Molecules(RHM)) and $g = +2$ (Left Handed Molecules(LHM)). (C7) The Fourier Ring Correlation analysis. This gives resolution of the chiral super-resolved image (~ 41 nm). Also, the FRC map for the chiral super-resolution image is generated using SQUIRREL analysis, and it is shown with the calibration bar. The corresponding raw data for Dendra2-Actin is shown in **Supplementary Videos 1**.

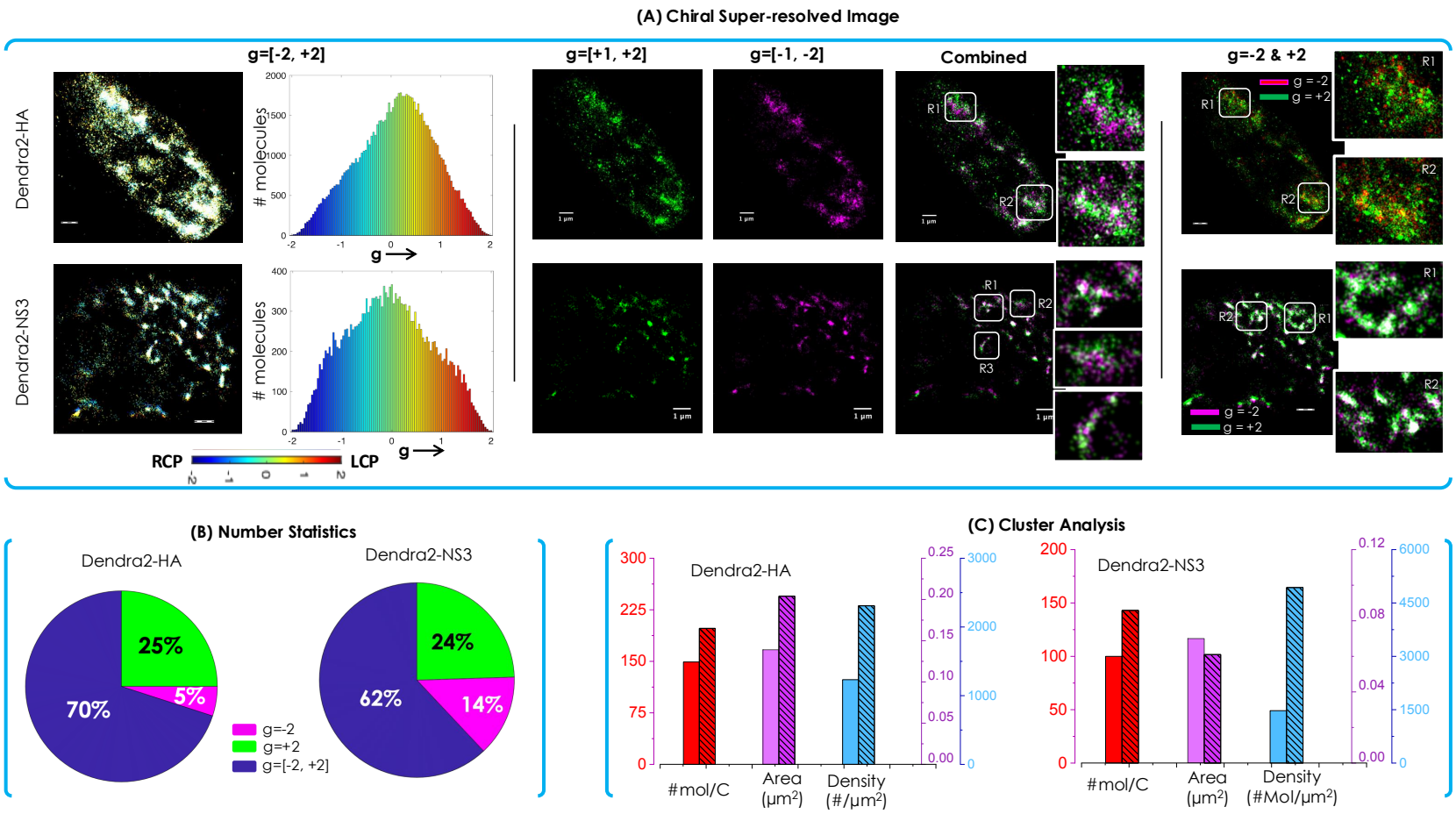

FIG. 3: **Dendra2HA and NS3 images:** (A) The chiral super-resolution images are shown with color code to represent the degree of chirality, and the g-factor histogram shows the distribution of molecules based on g value. Along with that the chiral super-resolution image for the molecules having a higher chiral range($g > 1$ and $g < -1$) is selected and shown. Enlarged sections of the combined images show the distribution of chiral molecules in the single molecule clusters. Images are reconstructed with the $g > 1$ and $g < -1$. Alongside, the combined single super-resolution image reconstructed for the molecules with $g = -2$ & $g = +2$. The percentage of molecules detected in the RCP and LCP channels are shown in the pie chart(B), and the molecules that appear on both channels are shown in the blue region, also these molecules are having the g values between -2 and +2. The other two parts represent the molecules that appear on only one channel, and their g value is -2 or +2. (C)The average cluster-related parameters calculated from chiral super-resolution images of Dendra2-HA and Dendra2-NS3 are shown in the bar graph. The corresponding raw data for Dendra2-HA is shown in **Supplementary Video 2**.

calculated using, $g = \frac{2(I_R - I_L)}{I_R + I_L}$, where I_R and I_L are the intensity of the molecules in the RCP and LCP channels respectively. The fraction of chiral molecules with $g - factor$ of, $g = -2$, $g = +2$, and $g \in [-2, +2] = -2 > g < +2$ are found to be 16%, 10%, and 74%, respectively (see, population statistics in Fig. 2.C5). It may be noted that a significant number of molecules exhibit absolute chirality (g-value, -2 or $+2$). Here, the right-handed molecules are shown in red color, while the left-handed molecules are shown in green color (Fig. 2C6). Fig. 2C7 shows the FRC analysis along with the FRC map [54] [55]. The reconstructed images have a FIRE of 10.3, which corresponds to a resolution (r_{cs}) of 41 nm. The analysis clearly indicates high resolution and the chiral distinguishability of single molecules.

D. Cell Transfection Studies: Disease Biology

Finally, two different disease models are chosen and the chiral distribution of single viral proteins are investigated. NIH3T3 cells were transfected with photoactivable viral proteins (Dendra2-HA, cloned viral protein for Influenza type-A and Dendra2-NS3, a cloned viral protein for Dengue type-2) using the developed protocol. The cells were fixed for imaging after 48 hrs of transfection. The single molecule data is collected using the developed $chiralSMLM$ microscope, and the images are reconstructed along with chiral information (green for RCP with $g \in [+1, +2]$ and magenta for RCP with $g \in [-2, -1]$) tagged on each molecule. Alongside, reconstruction super-resolved map of ideal chiral molecules (with absolute g-values of, $g = -2$ or $g = +2$) is also shown in Fig. 3A. Alongside the population statistics of both types is also shown. A few enlarged regions (R1 and R2) containing HA and NS3 clusters are also shown. This clearly indicates the presence of both left- and right- handed chiral molecules with a dominant presence of left-handed molecules. The corresponding number statistics for idealistic / absolute chiral molecules ($g = -2$ and $g = +2$) and high-degree chiral molecules ($g = [+1, +2]$ and $g = [-1, -2]$) is displayed in Fig. 3B. The combined image shows the distinct chirality of the recorded molecule, an intrinsic property never reported before in single molecule imaging. Also, the images of Dendra2-HA and Dendra2-NS3 shows aggregation of protein molecules as clusters in the cell. It is apparent that left-handed chiral molecules are responsible for clustering in both Influenza type-A and Dengue type-2. This suggests that left-handed chiral molecules promote clustering and may have a crucial role in determining the mechanism of action during viral infection. The observation may have implications to other viral disease falling in the broad family, flavi-virade.

Based on recorded single molecule data, statistical analysis is carried out to determine cluster parameters. To do this, the parameters are calculated for both cases (Influenza and Dengue) using DBSCAN (Density Based Spatial Clustering of Applications with Noise) algorithm. Further, the number of molecules per cluster, the size/area of the cluster and its density are calculated, and their averages are shown in the graph bar where the bars with diagonal strips represent the parameters of left-handed molecules (see, Fig. 3C). This shows that an average cluster has 149 molecules with a density of 1233 $molecules/\mu m^2$ for right-handed molecules whereas, left-handed molecules are relatively large ($\sim$ 198) with a 2-fold more density of 2308 molecules/area. This indicates that the action of left-handed chiral molecules in promoting cluster formation as compared to right-handed ones. This is significant since it depicts the fact that chirality has a significant role in cluster formation.

III. DISCUSION & CONCLUSION

In conclusion, chirality is an intrinsic property of a molecule and an important factor that regulates clustering in viral infection as evident from cell transfection studies. We presented a new technique where the emitted fluorescence from single molecules is split into s and p polarized components to separate out enantiomers. This requires the introduction of a combination of quarter-wave plate and polarizing beam-splitter in the the detection sub-system. The technique is compatible with the existing SMLM systems and does not require the development of new image analysis algorithms. First, we calibrate the detection module using a known source light polarization before integrating it with the SMLM system. Subsequently, the technique is used to investigate known sub-cellular biological structures. Accordingly, NIH3T3 cells were transfected with a conjugate DNA plasmid Dendra2-Actin using the developed protocol. This showed the presence of three major kinds of Actin molecules, categorized into three major subsets: low-order ($g \in [-1, +1]$), high-order ($g \in [-2, -1]$ and $g \in [+1, +2]$) and absolute ($g = -2$ and $g = +2$) chiral molecules. Subsequently, the super-resolved image is reconstructed by combining molecules with absolute chirality.

Next, we demonstrate our technique on disease biology models: Influenza type-A, and Dengue Type-2. The results shows a relatively dominant presence of left-handed molecules than right-handed ones. Specifically, during the clustering phase, left-handed molecules have a larger footprint as compared to right-handed ones. This demonstrates the role played by chirality in the single-molecule clustering process with cluster formation largely promoted by left-handed chiral molecules. This is a new observation and needs more investigation, relating molecular chirality with clustering. It is hoped that the significance will be immediately revealed in the biological process associated with other viral disease models.

IV. METHODS

A. Cell culture and Transfection

NIH3T3 are seeded on No.1(22mm x 22mm) coverslip with a cell count of 90,000. 16 hours post seeding, once the cells are attached properly, the transfection is performed using Lipofectamine 3000 kit(ThermoFisher Scientific, Catalog number:L3000008). The DNA complex is prepared in Opti-MEM media(ThermoFisher Scientific, Catalog number: 11058021). $1\mu g$ of plasmid DNA $2.25\mu L$ of p3000 and $3\mu L$ of Lipofectamine was used. 48 hours post transfection cells were washed with 1x PBS two times and fixed with 3.7% of paraformaldehyde. Then, the coverslip is mounted on the glass slide with Fluorosave mount media.

B. Bead sample preparation

$20mg$ of Agarose is mixed with $5mL$ of milli-Q water and heated using a hotplate at $\sim 100°C$. Once Agarose dissolved in water, the hotplate is turned off, and the temperature of the solution is allowed to decrease. When the temperature is close to 40°C $1\mu L$ of bead solution is added to the Agarose solution and uniformly mixed. Then, the mixed solution is casetted on the live imaging dish.

C. Data Collection and Analysis

EMCCD camera is used to record the fluorescence signal from the single molecules. Before data collection, the temperature of the camera is cooled to $-88°C$ to avoid thermal noise. The frame transfer mode is enabled, and EM gain is kept at 270. The data is recorded in '.tif' file format with an exposure time of $40ms$. The recorded data were analyzed using lab-made MATLAB program, which involves background subtraction using a rolling ball algorithm followed by single-molecule spot detection using the intensity threshold method. The detected spots are fitted with the 2D Gaussian function based on the non-linear least square method. The standard deviation and centroid are measured from the fitted Gaussian, and also the number of photons is calculated. The localization precision of each molecule is calculated using the Thompson formula[52].

For chiral molecule analysis, the molecules detected in the bottom window is separated, and their coordinate is shifted based on the beads data then compared with the molecules detected in the top window of the same frame. Initially, the distance between the centroid of the molecules in both the windows after shift correction is calculated, when the centroid of the molecules is within $120nm$, they are considered as same molecule and chiral dissymmetry factor is calculated based on their number of photons. The super-resolution image is reconstructed by collating all the molecules into a single frame, and each molecule is represented as 2D Gaussian function with the standard deviation of 20nm. For chiral super-resolution image the dissymmetry factor information is incorporated in the molecule color.

D. Cluster Analysis

To identify clusters, DBSCAN clustering algorithm is used. DBSCAN is a density-based technique that identifies groups points that are closely packed together in data space. The inbuilt Matlab function '*dbscan*', along with machine learning toolbox is used to analyze single-molecule clusters. The X and Y coordinates of the single detected single molecules are given as input, and cluster parameters such as the minimum cluster point (~ 50 points) and the minimum distance (2 pixels = 120nm) are chosen for the study. Once the molecules are clustered, the number of molecules per cluster, area of the cluster, and cluster density (number of molecules per unit area) are calculated.

E. Optical Setup

The illumination subsystem of $chiralSMLM$ is similar to $aSMLM$[53] where the ETL is operated at 270 mA current to get a required field of view. The specimen is illuminated with an intensity of $24.4mW/\mu m^2$ for activation and $93.3W/\mu m^2$ for excitation. In the detection subsystem, additional magnification is obtained using magnification unit (MU) which consists of two convex lens of focal length $150mm$ and $400mm$, giving a magnification of $2.66X$. Along with the $100X$ objective lens, the total magnification is about $266X$. This ensures the oversampling of single molecule PSF. Given the pixel-size of 16 μm of the EMCCD camera, and a magnification of 266, the effective pixel size is $60nm$.

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Author Contributions: PPM and AS conceived the idea. AS, NP and PPM carried out the experiments. AS and NP prepared the samples. PPM wrote the paper by taking inputs from all the authors.

Data Availability: The data that support the findings of this study are available from the corresponding author upon request.

Disclosures: The authors declare no conflicts of interest.

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