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Probing Differential Binding Mechanisms of Phenylalanine-Glycine-Rich Nucleoporins by Single-Molecule FRET

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Abstract

Phenylalanine-glycine-rich nucleoporins (FG-Nups) are intrinsically disordered proteins, constituting the selective barrier of the nuclear pore complex. They are highly dynamic under physiological conditions and studying their interaction with nuclear transport receptors (NTRs) is key to understanding the molecular mechanism of nucleocytoplasmic transport. Distinct conformational features of FG-Nups interacting with diverse NTRs can be detected by multiparameter single-molecule fluorescence energy transfer (smFRET), which is a powerful technique for studying the dynamics and interactions of biomolecules in solution. Here we provide a detailed protocol utilizing smFRET to reveal differential binding mechanisms of FG-Nups to NTRs, with a focus on practical considerations on sample preparation of unglycosylated and glycosylated FG-Nups, site-specific dual-labeling, smFRET measurements, and data analysis.

Abbreviations

AcF	<i>p</i> -acetylphenylalanine
Amp	ampicillin
CBD	chitin binding domain
Cm	chloramphenicol
Cys	cysteine
ddH₂O	double-distilled water
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid

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FCS	fluorescence correlation spectroscopy
FG-Nup	phenylalanine-glycine nucleoporin
FRET	fluorescence resonance energy transfer
GdHCl	guanidine hydrochloride
IDP	intrinsically disordered protein
MgAc	magnesium acetate
MgCl₂	magnesium chloride
NaAc	sodium acetate (anhydrous)
NaCl	sodium chloride
ncAAs	noncanonical amino acids
NTR	nuclear transport receptor
OD₆₀₀	optical density measured at a wavelength of 600nm
O-GlcNAc	O-linked-N-acetylglycosamine
OGT	O-linked N-acetylglucosaminyl transferase
PBS	phosphate-buffered saline
PMSF	phenylmethylsulfonyl fluoride
POI	protein of interest
rpm	revolutions per minute
S	stoichiometry
sm	single molecule
smFRET	single-molecule fluorescence resonance energy transfer
TCEP	tris(2-carboxyethyl)phosphine
UDP-GlcNAc	uridine 5'-diphospho-N-acetylglucosamine sodium salt

1 Introduction

1.1 Nuclear Pore Complex

The nuclear pore complex (NPC), which resides in the nuclear envelope, mediates the molecular exchange between the nucleus and the cytoplasm. The metazoan NPC is around 125MDa large and formed by 30 different nucleoporins (Nups) where roughly one-third of them are largely intrinsically disordered proteins (IDPs) rich in phenylalanine-glycine motifs, termed FG-Nups (Denning, Patel, Uversky, Fink, & Rexach, 2003; Hurt, 1988; Ori

et al., 2013; Wentz, Rout, & Blobel, 1992). FG-Nups have high conformational flexibility and multiple binding sites (FG motifs) which allows them to interact with transport receptors (Aramburu & Lemke, 2017; Gorlich & Kutay, 1999; Lim et al., 2006; Tetenbaum-Novatt & Rout, 2010; Tran, King, & Corbett, 2014). In metazoan they have been shown to be targets of O-linked β -*N*-acetylglucosamine (O-GlcNAc) modification, an omnipresent posttranslational modification in metazoan NPCs, which may play a role in regulating nucleocytoplasmic transport (Favreau, Worman, Wozniak, Frappier, & Courvalin, 1996; Labokha et al., 2013; Zhu et al., 2016). FG-Nups form the permeability barrier of the NPC and selectively control molecular transport, e.g., the barrier is permeable for small molecules below ~ 40kDa that can cross via passive diffusion and enables the translocation of large macromolecules when the transport is mediated by nuclear transport receptors (NTRs) (Cook, Bono, Jinek, & Conti, 2007; Gorlich & Kutay, 1999; Timney et al., 2016). While structured Nups contributing to the eightfold symmetric ring of the NPC have been extensively studied by X-ray crystallography, cryo-electron tomography, and biochemical methods (Brohawn & Schwartz, 2009; Chug, Trakhanov, Hulsman, Pleiner, & Gorlich, 2015; Eibauer et al., 2015; Kim et al., 2018; Kosinski et al., 2016; Lin et al., 2016; von Appen et al., 2015), conformational features of FG-Nups and changes imposed by their interactions with NTRs during nucleocytoplasmic transport remain unclear.

1.2 Coupled Reconfiguration-Binding vs Archetypal-Fuzzy Complex Formation in the NPC

Recently, we showed that at least two differential binding mechanisms between FG-Nups and NTRs coexist (Fig. 1A) (Milles et al., 2015; Tan et al., 2018). First, in vitro, NTRs like Importin β were found to interact with FG-Nups (e.g., FG-Nup153) via the formation of an archetypal-fuzzy complex, in which the conformational ensemble of the unbound FG-Nup153 is binding prone and ready to bind Importin β without substantial conformational change on the length scale probed by single-molecule fluorescent measurements. Such a binding mechanism can explain how fast, yet specific transport of molecules across the NPC is possible, in agreement with complementary results and other techniques from us and other labs (Hayama et al., 2018; Hough et al., 2015; Milles et al., 2015; Raveh et al., 2016; Sparks, Temel, Rout, & Cowburn, 2018; Tan et al., 2018). Second, we found that FG-Nup214, which localizes to the cytoplasmic side of the NPC, undergoes a clear and distinct conformational change when binding the CRM1•RanGTP export complex. This indicated that the complex formation of FG-Nup214•CRM1•RanGTP follows rather a coupled reconfiguration-binding mechanism which can regulate spatial localization of export complexes that help cargo and NTR undocking processes (Tan et al., 2018).

Much of the conclusions drawn in these studies were driven by multiple experimental tools such as molecular dynamics simulations, stopped-flow kinetics, nuclear magnetic resonance, thermodynamic measurements, scattering, and (single-molecule) fluorescence measurements. Nevertheless, a key indication of these differential binding mechanisms can be easily derived from single-molecule fluorescence resonance energy transfer (smFRET) experiments. Here we compare side by side the FG-Nup153•Importin β complex (archetypal-fuzzy complex formation) with the FG-Nup214•CRM1•RanGTP complex (coupled reconfiguration-binding mechanism) to showcase how smFRET enables to distinguish

characteristic fingerprints of these binding mechanisms. We provide a detailed step-by-step protocol of sample preparation, including FG-Nup expression, purification, and labeling steps. Then we discuss smFRET, including experimental setup, measurement, and data analysis, and how evidence for coupled reconfiguration-binding mechanism can be distinguished from archetypal-fuzzy complexes formation. We also report on the preparation of glycosylated FG-Nups and their study using smFRET.

2 Site-Specific Dual-Labeling of Fg-Nups/ Glycosylated Fg-Nups

FRET is the nonradiative transfer from a fluorophore-labeled donor molecule in the singlet excited state to another fluorophore-labeled acceptor molecule via a dipole–dipole coupling mechanism. The prerequisite to perform quantitative smFRET experiments as a structural biology tool is to site-specifically introduce a FRET dye pair into the protein. This permits to probe conformational features in the region sandwiched between the two dyes. The FRET efficiency (E_{FRET}) reports the distance between dye pair (r), described by the equation

$$E_{\text{FRET}} = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6}$$

where R_0 being the Förster radius of this dye pair at the distance at which $E_{\text{FRET}} = 0.5$. Due to the power 6 dependence, probing intra-dye distance changes is most sensitive around R_0 (Roy, Hohng, & Ha, 2008).

Here we designed an FG-Nup214 mutant for smFRET measurements to probe the part of Nup214 involved in CRM1 binding as seen from the crystal structure of FG-Nup214•CRM1•RanGTP•SNUP1 (Port et al., 2015). We site-specifically labeled the FG-Nup214 mutant at cysteine (Cys, aa 1905) with Alexa 594-maleimide (acceptor dye) and at the incorporated noncanonical amino acids (ncAAs) *p*-acetylphenylalanine (AcF, aa 2043) with Alexa 488-hydroxylamine (donor dye) via maleimide–cysteine coupling and oxime ligation reaction, respectively (Fig. 1B). In this way, specific labeling of the reactive group can be achieved (Brustad, Lemke, Schultz, & Deniz, 2008; Lemke, 2011) (see Note 1 for FG-Nup153).

2.1 Materials

- Codon-optimized FG-Nup gene (Mr. Gene, Regensburg, Germany)
- pEVOL-*p*-AcF plasmid (Addgene)
- *Escherichia coli* (*E. coli*) BL21(DE3) AI cells, arabinose-inducible promoter araBAD upstream of T7 RNA pol (Invitrogen)

Note 1 The codon-optimized FG-Nup153 disordered region (amino acids 875–1475 of the full-length Nup153; numbering with respect to the full-length protein as in UniProt: P49790) is cloned into a pTXB3-6His-intein-CBD vector, generating pTXB3-6His-Nup153-intein-CBD. The His-tag is cleaved via TEV cleavage and the intein-CBD is cleaved via intein cleavage. We site-specifically labeled FG-Nup153 mutant at the cysteine (Cys, aa 1312) with Alexa 594-maleimide (acceptor dye) and at the incorporated noncanonical amino acids (ncAAs) *p*-acetylphenylalanine (AcF, aa 1391) with Alexa 488-hydroxylamine (donor dye) via maleimide–cysteine coupling and oxime ligation reaction, respectively.

- LB agar plates with 50µg/L ampicillin (Amp) and 33µg/L chloramphenicol (Cm)
- Luria–Bertani (LB) growth medium
- Terrific Broth (TB) growth medium
- *p*-acetylphenylalanine (p-AcF)
- 20% L-arabinose, filter-sterilized through a 0.22µm Millipore® Stericup
- Lysis buffer (2*M* Urea, 4 × phosphate-buffered saline (PBS), 5mMimidazole, 1mM phenylmethylsulfonyl fluoride (PMSF), and 0.2mM tris (2-carboxyethyl)phosphine (TCEP))
- Storage buffer (4*M* GdHCl, 1 × PBS, pH 7.4)
- Ni-NTA superflow resin (Thermo Fisher Scientific: 88223)
- Chitin resin (NEB)
- TEV cleavage buffer (50m*M* Tris, 150m*M* sodium chloride (NaCl), 1m*M* PMSF, 0.2m*M* TCEP, pH 7.5)
- Glycosylation buffer (50m*M* Tris, 200m*M* NaCl, 20m*M* magnesium chloride (MgCl₂), 0.2mM TCEP, and 1% Tween20, pH 7.5)
- TEV protease
- O-linked N-acetylglucosaminyl transferase (OGT)
- Uridine 5'-diphospho-N-acetylglucosamine sodium salt (UDP-GlcNAc) (Sigma: U4375)
- β-mercaptoethanol (Sigma-Aldrich: M6250)
- Polypropylene chromatography column with filter (Qiagen)
- Oxime labeling buffer (4*M* GdHCl, 0.05*M* anhydrous sodium acetate (NaAc), 0.15*M* NaCl, pH 4)
- Maleimide labeling buffer (4*M* GdHCl, 1 × PBS, 0.1m*M* ethylenediaminetetraacetic acid (EDTA), pH 7)
- EDTA-free protease inhibitor cocktail (Roche: 11873580001)
- Alexa Fluor® 488-hydroxylamine dye (Thermo Fisher Scientific)
- Alexa Fluor® 594-maleimide dye (Thermo Fisher Scientific)
- Dithiothreitol (DTT) (Biomol: 04010)
- Anhydrous acetonitrile (Thermo Fisher Scientific)
- S200 chromatography column (GE Healthcare)
- NuPAGE denaturing SDS-PAGE gel (Thermo Fisher Scientific)
- SDS-PAGE MOPS running buffer
- SDS-PAGE loading dye

- SDS-PAGE molecular weight markers
- 3kDa MWCO Amicon® ultra centrifugal filter units (Merck)
- Sonicator or microfluidizer
- UV–visible spectrophotometer

2.2 Expression and Purification of FG-Nup214 Containing ncAAs

1. Perform site-directed mutagenesis of codon-optimized FG-Nup214 disordered region (see ^{Note 2}) to introduce Amber stop codon at the site of the interest. Mutate the codon for amino acid 2043 in the FG-Nup214 sequence to TAG and 1905 FG-Nup214 to Cys. The gene of interest containing a single Cys and a TAG codon at the desired positions is then cloned into a pBAD-intein-chitin binding domain (CBD)-12His plasmid, generating pBAD-Nup214^{1905C,2043TAG}-intein-CBD-12His.
2. Cotransform *E. coli* BL21 (DE3) AI cells with pEVOL-*p*-AcF plasmid, which encodes for the *p*-AcF Amber suppressor tRNA/tRNA synthetase pair and Cm resistance with plasmids pBAD-Nup214^{1905C,2043-TAG}-intein-CBD-12His, which encodes for protein of interest (POI) and Amp resistance (see ^{Note 3}). Spread transformed colonies on LB agar plates with 50µg/L Amp and 33µg/L Cm. Let the cells grow overnight in a 37°C incubator.
3. Pick an individual colony and grow overnight in LB medium (containing Amp and Cm) at 37°C.
4. Add 20mL (1:100) overnight culture and 2mL (1:1000) Amp/Cm in 2L TB medium. Let the cell grow at 37°C, 180 revolutions per minute (rpm) in the incubator.
5. Add 1mM (final) *p*-AcF when the optical density measured at a wavelength of 600nm (OD₆₀₀) = 0.4.
6. Induce the recombinant expression of the POI with 0.02% arabinose at 0.7 < OD₆₀₀ < 1.2. Let cells grow at 37°C for 6–8h.
7. Harvest cells after expression time at 4°C, 4000rpm for 20min.
8. Dispose of supernatant in S1 waste, resuspend cell pellet with one pellet volume of lysis buffer (see ^{Note 4}).
9. Sonicate the sample 3 × 30s on ice. Let the cells rest on ice for at least 30s between each cycle, or microfluidize sample 3 times for smaller volumes (4–8L

^{Note 2}The codon-optimized FG-Nup214 disordered region: aa 1392–2090 is numbered with respect to the full-length protein (Uniprot: P35658).

^{Note 3}We use two plasmids for ncAAs incorporation in *E. coli*, one for the expression of the gene of interest, containing the Amber (TAG) stop codon mutation, and a second that encodes the orthogonal tRNA and aminoacyl-tRNA synthetase pair. When the plasmid containing the Amber codon (UAG) is being translated in the ribosome, the Amber codon is recognized by the orthogonal tRNA charged with the ncAA and subsequently the ncAA is added to the growing polypeptide chain. In this chapter, FG-Nup214^{1905C, 2043TAG} is used as an example and the protocol can be applied to other FG-Nup variants unless otherwise stated.

^{Note 4}Usually 10mL of lysis buffer/L expression at pH 8.5.

expression/less than 200mL volume of lysate) or 4–7 times for larger volumes (12–24L expression/more than 200mL volume of lysate). Spin down the lysate at 4°C, 15,000rpm for 1h.

10. Wash Ni-beads with double-distilled water (ddH₂O) (1mL beads per 1L expression) and equilibrate in lysis buffer. Take the clear lysate (supernatant) and load it on the Ni-beads. Incubate 2h at 4°C on the shaker or roller.
11. Collect flow-through with polypropylene column in the column rack in the following step (see ^{Note 5}). For in vitro glycosylation of FG-Nup, jump to step 12.
 - a. Wash each column with ddH₂O and once with lysis buffer.
 - b. Wash with 20mL/mL Ni-bead with lysis buffer.
 - c. Wash with 20mL/mL Ni-bead with lysis buffer of 15mM imidazole.
 - d. Wash with 20mL/mL Ni-bead with lysis buffer of 20mM imidazole.
 - e. Wash with 10mL/mL Ni-bead with lysis buffer of 25mM imidazole.
 - f. Wash with 10mL/mL Ni-bead with lysis buffer of 35mM imidazole.
 - g. Elute protein with 10mL elution buffer (lysis buffer with 400mM imidazole) per 2mL Ni-beads.
12. In vitro glycosylation of FG-Nup, adapted from previously published protocol for in vitro glycosylation of Nup98 (Labokha et al., 2013).
 - a. Wash the Ni-bead with 1.5 times volume of Ni-beads of glycosylation buffer.
 - b. Add 20 time excess Ni-bead volume of glycosylation buffer containing 5μM OGT (see ^{Note 6}) and 1mM UDP-GlcNAc.
 - c. Stir the Ni-beads at room temperature for at least 16h.

^{Note 5}For FG-Nup153, collect flow-through with polypropylene column in the column rack in the following steps. Additional chitin beads purification steps are required for higher protein purity.

- a. Wash each column with ddH₂O and once with lysis buffer.
- b. Wash with 10mL/mL Ni-bead with lysis buffer of 10mM imidazole.
- c. Wash with 10mL/mL Ni-bead with lysis buffer of 15mM imidazole.
- d. Elute protein with 10mL elution buffer (lysis buffer with 400mM imidazole) per 2mL Ni-beads.
- e. Pour Ni-bead elution on chitin beads (2–5mL per liter expression).
- f. Close column(s) and incubate elution on chitin beads for at least 8h to overnight at 4°C on shaker.
- g. Discard the flow-through and wash the chitin beads with lysis buffer and equilibrate on TEV cleavage buffer.
- h. Add 0.5mg TEV protease per liter expression and supplement 100mM BME in the TEV cleavage buffer.
- i. Perform the cleavage for at least 6h at room temperature under mild rotation.
- j. Collect the flow-through and continue with the step 14.

^{Note 6}OGT, CRM1, Importin β , and RanGTP are expressed in *E. coli* BL21 (DE3) AI cell and purified using standard nickel affinity and size exclusion chromatography purification procedures as described in Tan et al. (2018).

- d. Wash Ni-beads with glycosylation buffer and follow step 11.
13. Add 100mM β -mercaptoethanol and 1 $\times$ EDTA-free protease inhibitor cocktail to the eluted protein. Incubate overnight at room temperature on a shaker (see Note 7).
14. Dialyze the sample in a suitable dialysis bag and put it in 2L of lysis buffer (without imidazole) for 4h (on a magnetic stirrer in the cold room). Repeat dialysis in fresh 2L lysis buffer (without imidazole) for another 2h (see Note 8).
15. Wash 0.5mL Ni-beads/L of expression with ddH₂O and then with lysis buffer.
16. Load buffer exchanged protein on the Ni-beads and incubate 2h at 4°C on a shaker.
17. Collect flow-through with polypropylene column in the column rack. Concentrate the protein and run size exclusion chromatography.
18. Check purification success by SDS-PAGE. Concentrate fractions of interest and buffer exchange to storage buffer.

2.3 Fluorescent Labeling of FG-Nup214

2.3.1 Oxime Ligation of FG-Nup214 With Alexa 488-Hydroxylamine Dye

1. Exchange the protein buffer with oxime labeling buffer, filtering the buffer before use.
2. Dissolve 1mg of Alexa 488-hydroxylamine dye in pH 4 oxime labeling buffer for 1mM final concentration. Store frozen in the dark at –80°C and thaw prior to use.
3. Label protein with fivefold molar excess of Alexa 488-hydroxylamine dye.
4. Allow the reaction to proceed for 12h at room temperature in the dark (without stirring).

2.3.2 Coupling of FG-Nup214 With Alexa 594-Maleimide Dye

1. Exchange buffer to maleimide labeling buffer with 10mM DTT at pH 7, filter the buffer before use.
2. Wash sample (5 times) with maleimide labeling buffer with 0.2mM TCEP in 3k Centricon® device (see Note 9).
3. Solubilize 1mg of Alexa 594-maleimide dye in anhydrous acetonitrile. Aliquot the solution in 25nmol fractions into dry microcentrifuge tubes. Lyophilize the aliquots and store at –80°C.
4. Label protein with twofold molar excess of Alexa 594-maleimide dye.

Note 7 This step is performed to achieve intein cleavage in order to remove the intein-CBD-12His tag of the protein.

Note 8 This step is to eliminate β -mercaptoethanol and imidazole in the sample.

Note 9 This step is to remove the excess of DTT which would quench the maleimide dye.

5. Allow the reaction to proceed for 2h at room temperature in the dark.
6. Quench the labeling reaction with maleimide labeling buffer containing 10mM DTT.
7. Run size exclusion chromatography (Superdex 200). Check labeling success by SDS-PAGE and UV-visible spectrophotometry. Concentrate fractions of interest and buffer exchange in storage buffer.

3 Single-Molecule Fret Studies of Fg-Nup and NTR Interactions

3.1 Single-Molecule Fluorescence Resonance Energy Transfer

In contrast to ensemble FRET measurement, which provides the average E_{FRET} value of a high number of molecules, smFRET is able to resolve the FRET signal of individual molecules. Hence, it can provide direct measurements of conformational distributions and stochastic dynamics even in the context of complex structural landscapes and mixtures of subpopulations (Ferreon, Gambin, Lemke, & Deniz, 2009; Mukhopadhyay, Krishnan, Lemke, Lindquist, & Deniz, 2007; Muller-Spath et al., 2010). Our smFRET measurement is performed on a custom-built confocal detection-based microscope as shown in the schematic diagram (Fig. 2A). A pulsed laser diode (LDH 485, Picoquant, Germany), pulsed at 26.67MHz, is used for donor excitation; a supercontinuum white light laser source (SuperK Extreme, NKT photonics, Denmark) is used for acceptor excitation. We use pulsed interleaved excitation (PIE) scheme (Muller, Zaychikov, Brauchle, & Lamb, 2005) to alternately excite donor and acceptor fluorophores to retrieve the stoichiometry (S) information. Both lasers are cleaned up with excitation filters (Brightline FF01-482/18 and Brightline FF01-572/15, Semrock) and passed through the polarizer (GL-10A, Thorlabs) before entering the objective lens (60 $\times$, water immersion, Nikon) through the central dichroic mirror (ZT488/561 Chroma). The diffusing doubly labeled proteins are excited within a confocal volume (Fig. 2B). The excited fluorescent light is reflected into the emission pathway, through the tube lens ($f = 200\text{mm}$, Nikon) via the central dichroic mirror. The emission light is then focused to a 100 μm pinhole to block the out-of-focus signal and directed onto a lens $f = 160\text{ mm}$, Thorlabs) for beam collimation. After that, the fluorescence emission of the donor and the acceptor dyes is split into parallel and perpendicular polarization directions via a polarized beam splitter (BS), followed by being separated into donor and acceptor color channels via the dichroic mirror (zt561rdc-UF2, Chroma), and detected by avalanche photodiodes (APDs) (Picoquant, Berlin, Germany). Photon signals are counted using a Hydrharp400 (Picoquant, Berlin, Germany). Acquired data are subject to multiparameter fluorescence analysis and processed burstwise for fluorescence intensities, donor lifetime, and donor anisotropies (Eggeling et al., 2001; Sisamakias, Valeri, Kalinin, Rothwell, & Seidel, 2010). In this chapter, we will focus on the analysis of the fluorescence intensity to calculate E_{FRET} and S values.

3.2 Experimental Measurement and Analysis

All sm experiments are performed at protein concentration of 50 μM (to ensure the detection of single molecule) (see ^{Note 10}). CRM1, Importin β , and RanGTP concentrations are 1 μM (see ^{Note 6}). The sample is placed in 8-well chamber slides with a final volume of 200 μL .

Traces are recorded for 30min and each fluorescent molecule is identified by the start and the end of a fluorescent burst (Fig. 2C) (see Note 11). A burst search algorithm (Eggeling et al., 2001) is used and detected photons are collected into 1ms bins. After burst recognition, a threshold of 80 photons is set for every detected burst (see Note 12). All analyses are performed using a custom-written code in IgorPro (Wavemetrics, Lake Oswego, OR).

E_{FRET} and S values are then calculated burstwise using the following equations

$$E_{\text{FRET}} = \frac{I_A}{\gamma I_D + I_A}; S = \frac{I_A + I_A^{\text{dir}}}{I_D + I_A + I_A^{\text{dir}}}$$

where I_D and I_A are the donor and acceptor intensity, respectively. I_A^{dir} is the intensity from directly excited acceptor molecules by acceptor laser, and γ is a correction factor dependent on quantum yields and detection efficiencies of donor and acceptor. The γ value can be estimated from leakage and direct excitation-corrected E_{FRET} and stoichiometries following a basic procedure developed by Lee et al. (2005) and described in further detail by Fuertes et al. (2017) with the preassumption that the quantum yields of both donor and acceptor do not vary across samples.

For every single-molecule burst E_{FRET} and S are calculated and plotted into 2D histogram. The histogram is color-coded for number of events. The laser power of donor and acceptor laser is adjusted such that a FRET-labeled molecule (1:1 donor to acceptor dye-labeled sample) lies at $S \sim 0.5$. In such 2D frequency histogram of E_{FRET} vs S (Fig. 2C) at least two peaks emerge. A $S = 1$ and $E_{\text{FRET}} = 0$ molecules can be found that are labeled with donor only (or where the acceptor dye bleached or was photophysically inactive for other reasons (Kong, Nir, Hamadani, & Weiss, 2007; Sisamakos et al., 2010)), while a population shifting toward zero along the x axis (E_{FRET} value) and staying constant at $S \sim 0.5$ indicates an increase of the end-to-end distance between the dyes.

In the absence of CRM1•RanGTP complex doubly labeled FG-Nup214 molecules showed a single FRET population with an E_{FRET} value of 0.6 (Fig. 3A, top panel). If the CRM1•RanGTP complex is added ($1\mu\text{M}$), we observed a single population with significant shift in the E_{FRET} values of FG-Nup214 corresponding to a bound ($E_{\text{FRET}} = 0.1$ and $S \sim 0.5$) state. This suggests a much more expanded and docked conformation than in the unbound state. FG-Nup214 appears to undergo a drastic conformational change upon binding the CRM1•RanGTP complex, which is distinct from the core signature of archetypal-fuzzy complex formation of FG-Nup153•Importin β which did not show any substantial change in the E_{FRET} peak positions upon binding (Fig. 3B, top panel).

When there is a conformational change detected in smFRET, it usually indicates that binding occurred. However, when there is no substantial conformational change detected, it can be

Note 10 The doubly labeled protein is diluted to $1\mu\text{M}$ from stock solution to prevent protein from sticking to the wall of Eppendorf tube. The protein is further diluted to a final concentration of 50pM in solution in $1 \times \text{PBS}$ pH 7.4 containing 2mM DTT and 2mM MgAc.

Note 11 In the cases where very few bursts were detected per measurement, signal from different replicates can be accumulated.

Note 12 In order to remove aggregated clusters from analysis, all bursts longer than 8ms and brighter than 600 photons were discarded.

that no binding occurred, which is very common due to the inherently low concentrations used in smFRET experiments. Hence, one needs additional experimental validation to confirm that under single-molecule concentrations the complex is indeed formed, before the absence of detecting a conformational change can be taken as an indication for formation of an archetypal-fuzzy complex. Among other tools, fluorescence correlation spectroscopy (FCS), stopped-flow kinetics, or fluorescence anisotropy measurement as used by us previously can be recruited for such purposes, and we refer the reader to the literature to learn more about those (Milles & Lemke, 2014; Milles et al., 2015; Tan et al., 2018). In particular FCS analysis can also be performed on the same single-molecule data set as obtained for smFRET and can be directly used to confirm binding interactions. The acceptor burst signal excited by the donor laser (corresponding to FRET signal) is extracted from smFRET measurement and autocorrelated to obtain information about the translational motion of the FG-Nups. When the translational diffusion of FG-Nup is slower in the presence of NTR, it indicates that FG-Nup•NTR complexes formed (Milles et al., 2015; Sisamakidis et al., 2010).

We repeated the smFRET experiments using glycosylated variants of FG-Nups (FG-Nup214^{Glc} and FG-Nup153^{Glc}). As shown in Fig. 3A (bottom panel), a mildly lower E_{FRET} was measured in comparison to unglycosylated FG-Nup214 in the unbound form, indicating that the glycosylated Nup is more extended. In the presence of CRM1•RanGTP, we detected a single population with an E_{FRET} nearly identical to the unglycosylated bound state. Likewise, the FG-Nup153^{Glc} behaves similar in binding to Importin β (Fig. 3B, bottom panel). The smFRET data strongly suggest that the glycosylated and unglycosylated Nups behave remarkably similar in their respective binding mechanisms, highlighting that glycosylation only mildly tunes the binding mechanism between FG-Nup and NTR. Taken together, we summarize that in the NPC at least two fundamentally different binding mechanisms coexist between FG-Nups and NTRs (Tan et al., 2018). The conformational features of these binding mechanisms can be distinguished well by smFRET.

4 Summary

In this chapter, we report a workflow combining smFRET and sitespecific dual-labeling method to study the binding mechanism of intrinsically disordered FG-Nups. SmFRET allows to differentiate the conformational features of FG-Nup upon interacting with NTRs at the singlemolecule level, while the labeling technique enables us to site-specifically probe specific regions of interest. The ability of the presented smFRET technique to resolve structural and dynamic heterogeneity is particularly well suited for the investigation of IDP structure and function. It provides not only detailed information about the folding and ligand/protein binding of IDPs, but could also be used to detect folding intermediates and pathways which are very challenging to extract from ensemble-average data. The presented technique should enable the design and construction of novel tools for biological studies.

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Fig. 1. Differential binding modes of FG-Nup•NTR interactions and site-specific labeling of FG-Nups.

(A) Disordered FG-Nup214 undergoes coupled reconfiguration-binding with the NTR CRM1•RanGTP complex, in contrast to archetypal-fuzzy complex formation of FG-Nup153•Importinβ (Tan et al., 2018). (B) Top: scheme of FG-Nup214 constructs and labeling sites. The different FG motifs are color-coded as distributed across the FG-Nup214 sequence. F residues are only shown in the zoom-in region. Bottom: scheme of labeling FG-Nup214 with p-AcF via oxime ligation with Alexa 488-hydroxylamine dye and coupling of Alexa 594-maleimide dye to the Cys residue of FG-Nup214. Panels (A) and (B) are adapted/modified from Tan, P. S., Aramburu, I. V., Mercadante, D., Tyagi, S., Chowdhury, A., Spitz, D., et al. (2018). Two differential binding mechanisms of FG-nucleoporins and nuclear transport receptors. *Cell Reports*, 22(13), 3660–3671. <https://doi.org/10.1016/j.celrep.2018.03.022>.

Fig. 2. Scheme of sm multiparameter setup.

(A) The PIE pathway is highlighted as *green* (donor excitation) and *orange* (acceptor excitation) *dotted lines*. A confocal volume is used to excite singly freely diffusing FRET-labeled proteins as shown in (B). The emitted photons (highlighted in *thick, light blue*) are collected by the objective lens, passed through a pinhole, split based on their emission wavelengths and polarization states (parallel: $\parallel$, perpendicular: $\perp$), and eventually detected by APDs (see details in the text). (C) Separate bursts of photons, each corresponding to a single-molecule event, are collected by avalanche photon-counting modules, and analyzed using custom-written software. For each burst, E_{FRET} and S are calculated and the results are placed in the E_{FRET} vs S 2D histogram. DM1: dichroic mirror (R488-Di01, Semrock); DM2: dichroic mirror (FF605-Di02, Semrock); DM3: dichroic mirror (ZT488/561, Chroma); DM4: dichroic mirror (ZT561rdc-UF2, Chroma); L1: lenses ($f = 200$ mm, Nikon); L2: lenses ($f = 160$ mm, Thorlabs); L3: lenses ($f = 50$ mm, Thorlabs); BS: beam splitter (Thorlabs); APD: avalanche photodiode; F1: laser clean-up filter (FF01-572/15, Semrock); F2: laser clean-up filter (FF01-482/18, Semrock); F3: emission filter (FF01-525/50, Semrock); F4: emission filter (FF01-612/69, Semrock).

Fig. 3. Two differential binding mechanisms of FG-Nups•NTRs detected by smFRET.

(A) E_{FRET} vs S histograms of 50 pM FG-Nup214 (bottom panel: FG-Nup214^{Glc}) in the absence and presence of CRM1•RanGTP (from left to right, at 1 μM for both CRM1 and RanGTP concentration). Donor-only labeled population ($E_{\text{FRET}}=0$) is shown in black dotted line, indicating the imperfect labeling efficiency or photobleaching of acceptor dye. In the presence of CRM1•RanGTP, one distinct conformation with very low E_{FRET} state (blue solid line) is seen suggesting a much more expanded conformation than in the unbound state (blue dotted line) for both FG-Nup214 and FG-Nup214^{Glc}. (B) E_{FRET} vs S histograms of 50 pM FG-Nup153 (bottom panel: FG-Nup153^{Glc}) in the absence (blue dotted line) and presence of Importinβ (blue solid line), indicating no conformational change in FG-Nup153 (and FG-Nup153^{Glc}) as detectable by smFRET, in the presence of Importinβ. In both cases, FG-Nup214^{Glc} and FG-Nup153^{Glc} show a mildly lower E_{FRET} compared to unglycosylated case in the unbound form, indicating that the glycosylated FG-Nup is more extended. However, the two differential binding mechanisms are conserved. Panels (A) and (B) are adapted from Tan, P. S., Aramburu, I. V., Mercadante, D., Tyagi, S., Chowdhury, A., Spitz, D., et al. (2018). Two differential binding mechanisms of FG-nucleoporins and nuclear transport receptors. Cell Reports, 22(13), 3660–3671. <https://doi.org/10.1016/j.celrep.2018.03.022>.