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## Analysis of VHH dynamics

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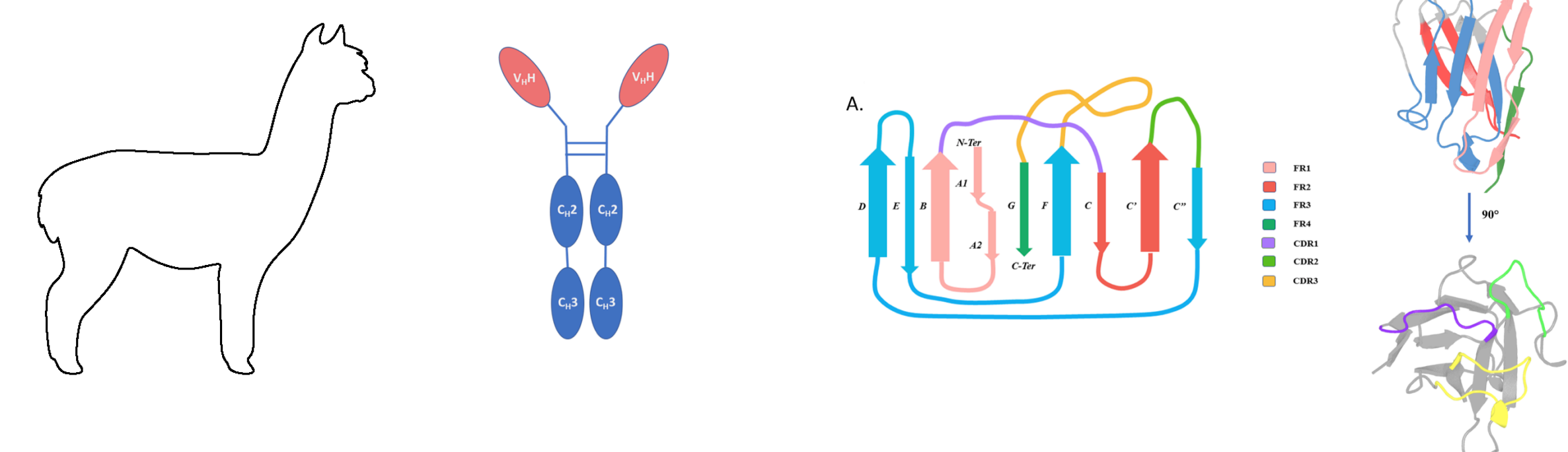
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**Abstract:** Protein conformational flexibility is crucial for its structural stability and function. The concerted displacements of residues in an antigen-antibody complex facilitate and determine their interactions' strength, making their study indispensable to modulating their function. Members of the family *Camelidae* express a unique subset of Immunoglobulin Gamma called the Heavy Chain only Antibody (HCAbs), consisting of one Variable domain (V<sub>H</sub>H) at the N-terminus of each heavy chain. Each V<sub>H</sub>H domain comprises two types of amino acid regions varying in sequence identity arranged alternately called the Framework Regions (FRs) and Complementarity Determining Regions (CDRs). Even when expressed independently *in-vitro*, V<sub>H</sub>H domains exhibit excellent solubility and thermostability compared to the V<sub>H</sub>-V<sub>L</sub> complexes, so they present a valuable opportunity to exploit their biophysical and biochemical properties to generate the next generation of therapeutic and diagnostic molecules. Recent studies have reported sequence and structural features of V<sub>H</sub>H domains contributing to these abilities in comparison to classical V<sub>H</sub>-V<sub>L</sub> complexes. In this study, we performed large-scale classical molecular dynamics simulations for a dataset of unrelated V<sub>H</sub>H structures to understand the local and global differences in their dynamics. We used classical metrics such as the Normalised B-factors of C $\alpha$  atoms, RMSF of C $\alpha$  atoms and an in-house method called the Protein Blocks (PBs) to investigate flexibility in V<sub>H</sub>H domains and trajectories. We have classified the trajectories based on C $\alpha$  Root Mean Squared Fluctuation, which revealed four main clusters of the V<sub>H</sub>H trajectories. We observed various local changes in CDRs but within different ranges in trajectories within the same cluster as well as from other clusters. The FR-CDR boundary regions showed distinct local backbone conformational diversity when assessed using PBs. This study sheds light on region-wise changes in flexibility during dynamics which could aid in improving the design and function of V<sub>H</sub>H domains.

## Introduction

Heavy Chain only antibodies from Camelids and their Variable domains (V<sub>H</sub>H).

2D and 3D organisation of a V<sub>H</sub>H domain



- Are the FRs as conformationally similar as they have been previously observed?
- Are the CDRs as conformationally diverse as they have been previously observed?
- Can we have other perspectives on flexibility in the V<sub>H</sub>H domains?

## Results:

- Flexibility in 88 V<sub>H</sub>H PDB structures and corresponding 1 $\mu$ s trajectories.

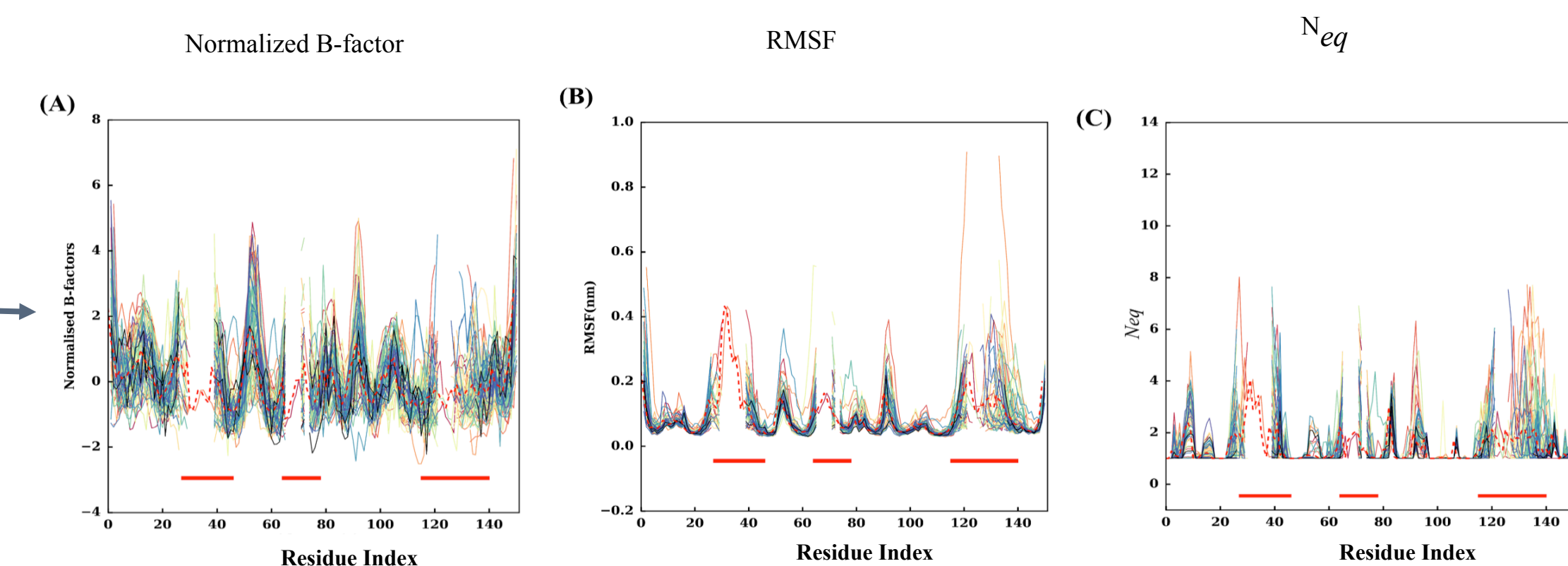


Figure 2: Flexibility metrics at each residue position. (A) Representation of normalized B-factors of C $\alpha$  atoms, the x-axis is the residue position in the MSA and the y-axis the Normalised B-factor values, (B) Representation of all RMSF values C $\alpha$  atoms, (C) Representation of  $N_{eq}$  values. The three CDR regions are highlighted using three red-coloured regions at the bottom of the plots. The average values of each metric are shown in dotted red lines.

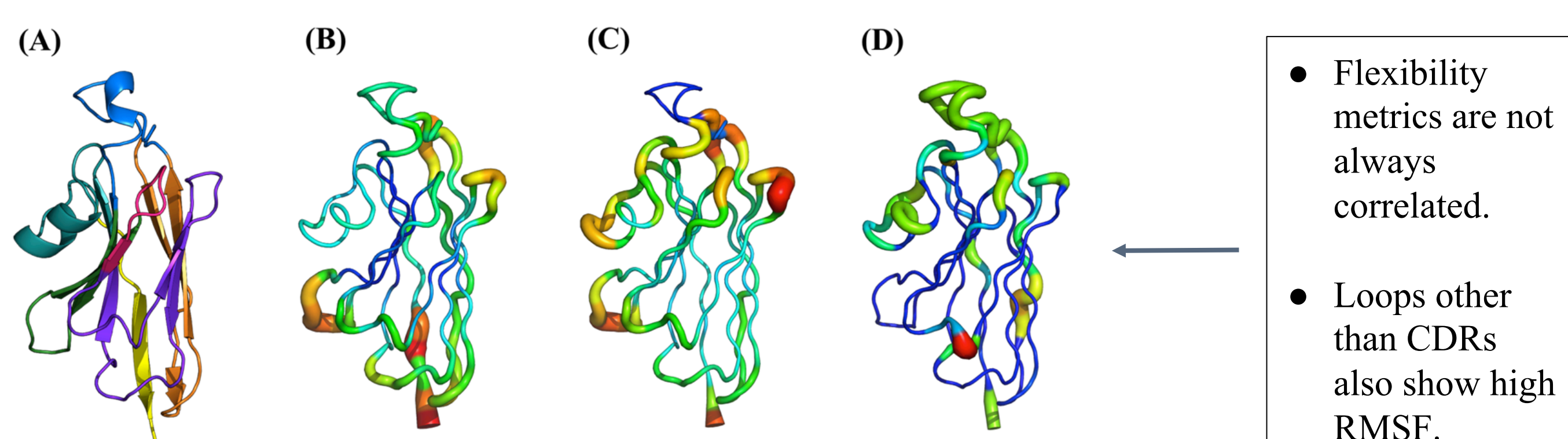


Figure 3: Representation of mean values of flexibility metrics onto a 3D structure of a V<sub>H</sub>H. (A) FRs and CDRs coloured

- Flexibility metrics are not always correlated.
- Loops other than CDRs also show high RMSF.

- Classification of Flexibility in CDRs

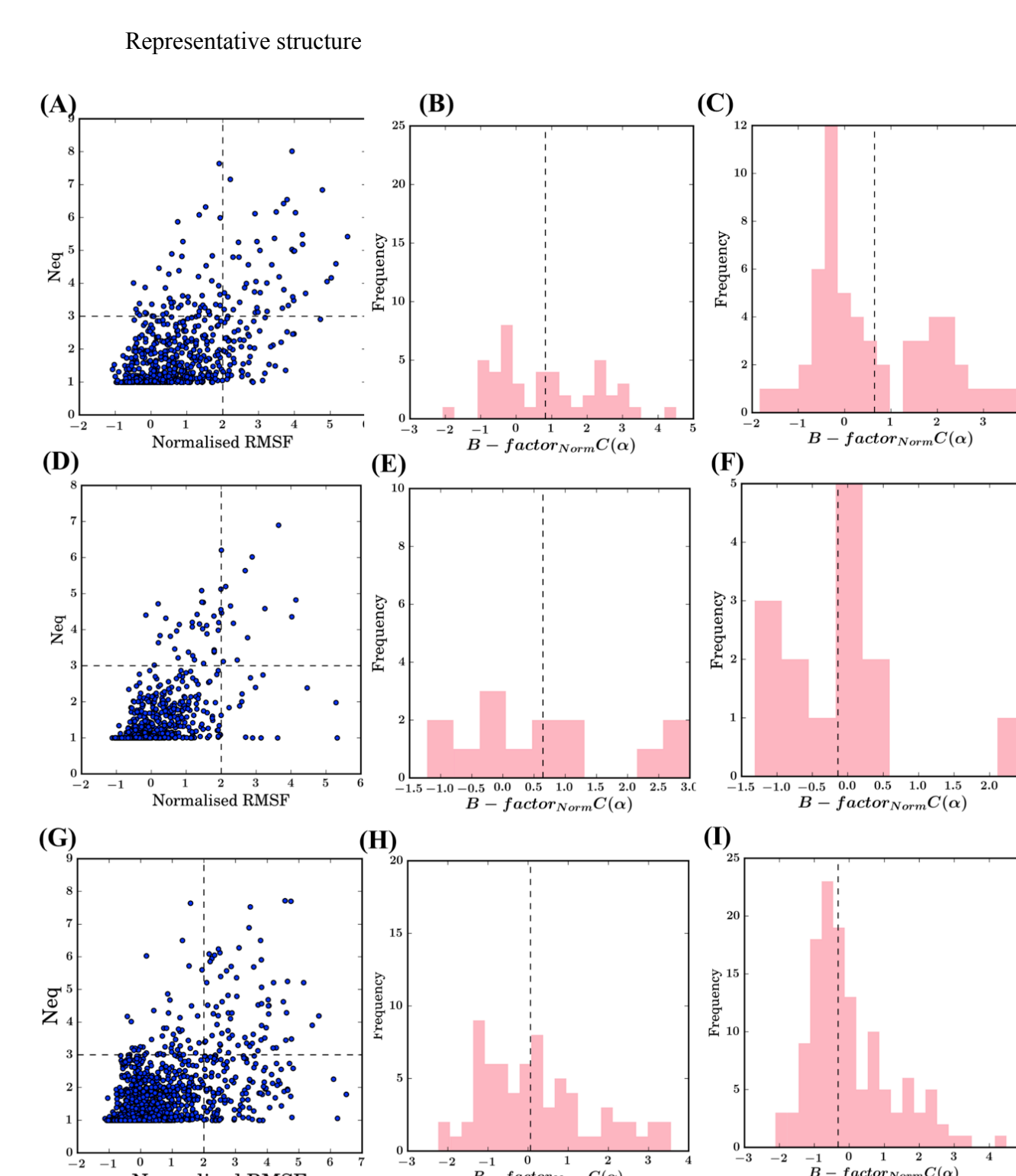


Figure 4: CDR flexibility. (A) to (C) is CDR1, (D) to (F) is CDR2 and (G) to (I) is CDR3. (A), (D) and (G) show the normalized RMSF vs  $N_{eq}$  while (B), (E) and (H) are the normalized B-factors of flexible quadrant (RMSF > 2 and  $N_{eq}$  > 3), and (C), (F) and (I) for mobile quadrant (RMSF > 2 and  $N_{eq}$  < 3). The dotted line in each B-factor distribution represents the mean value.

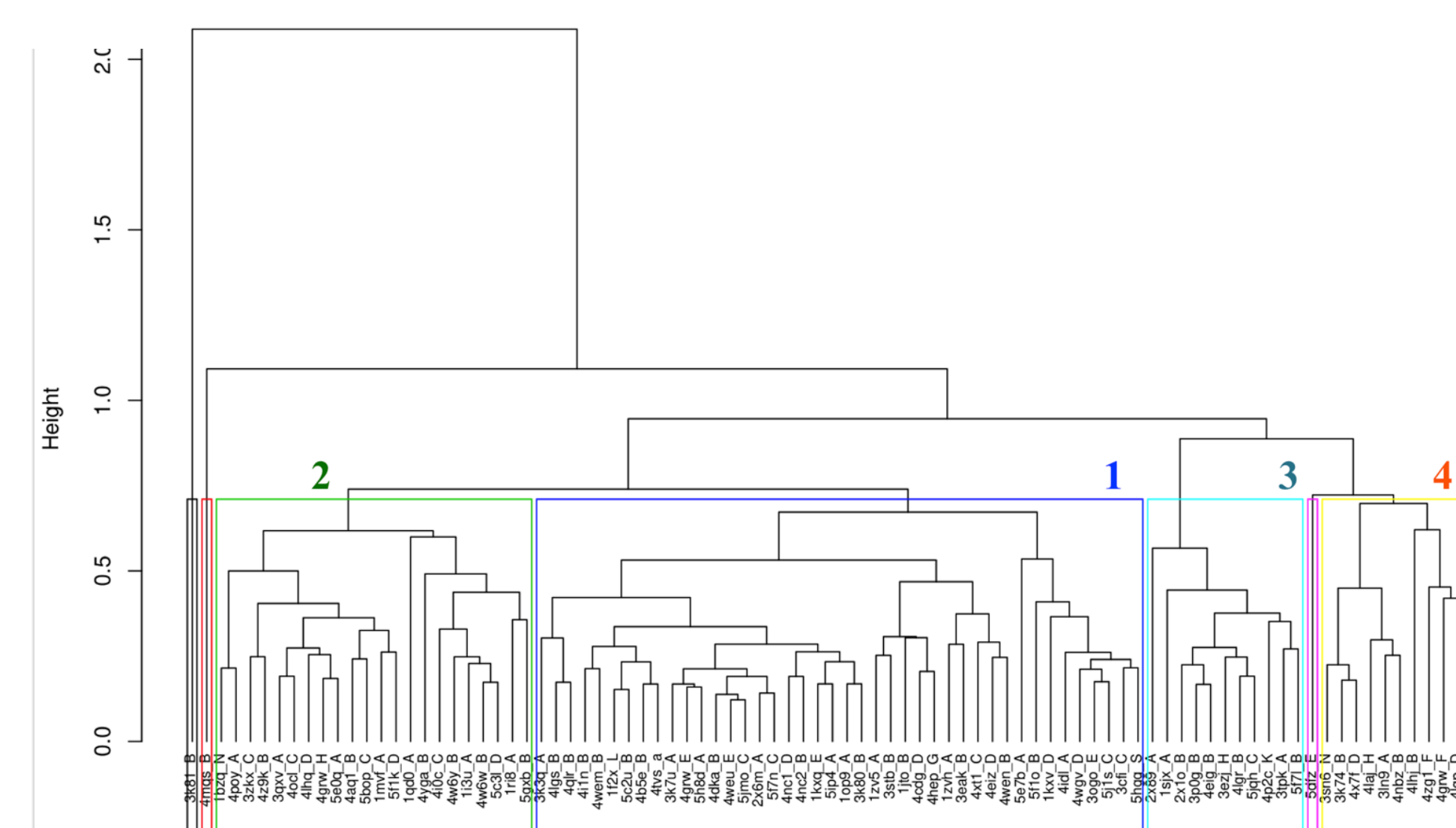


Figure 5: Hierarchical clustering of V<sub>H</sub>H trajectories using RMSF. The different RMSF clusters are demarcated using coloured boxes, and their cluster number is marked accordingly.

- Hierarchical clustering of 88 1 $\mu$ s trajectories Distance between two RMSF vectors were calculated using .

$$d(v, w) = \sqrt{\frac{1}{n-m} \sum_{i=1}^m (v(i) - w(i))^2}$$

Four dense clusters and 3 outliers were obtained from the clustering.

## Methods:

We used three different flexibility metrics to dissect conformational changes in V<sub>H</sub>H

- 1: Normalised B-factors
- 2: C $\alpha$  RMSF
- 3: Protein Structural alphabet called as Protein Blocks (PBs) (de Brevern et al. 2000). PB assignment for a given protein using PBxplorer (<https://github.com/pierrepo/PBxplorer>).

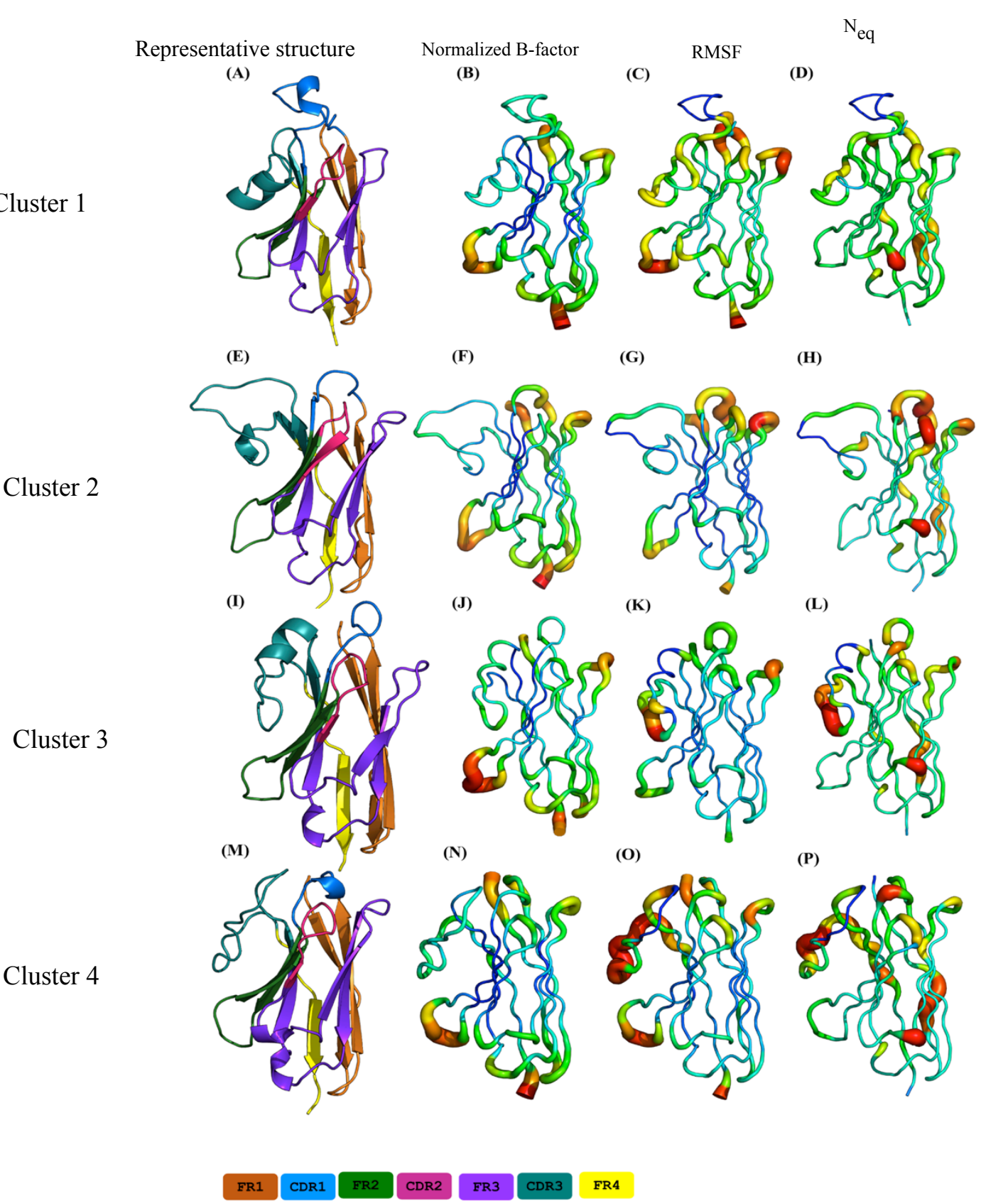
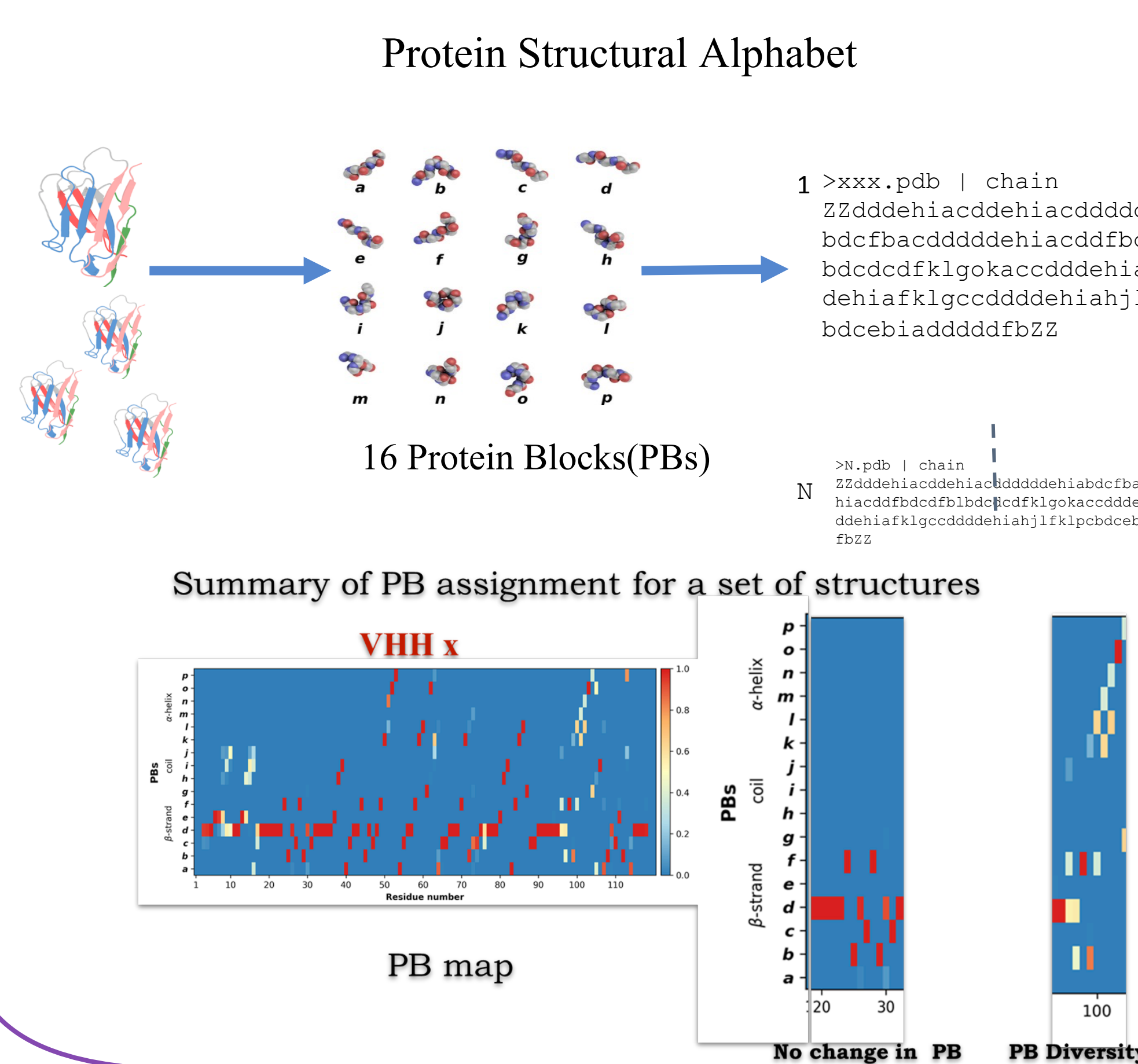
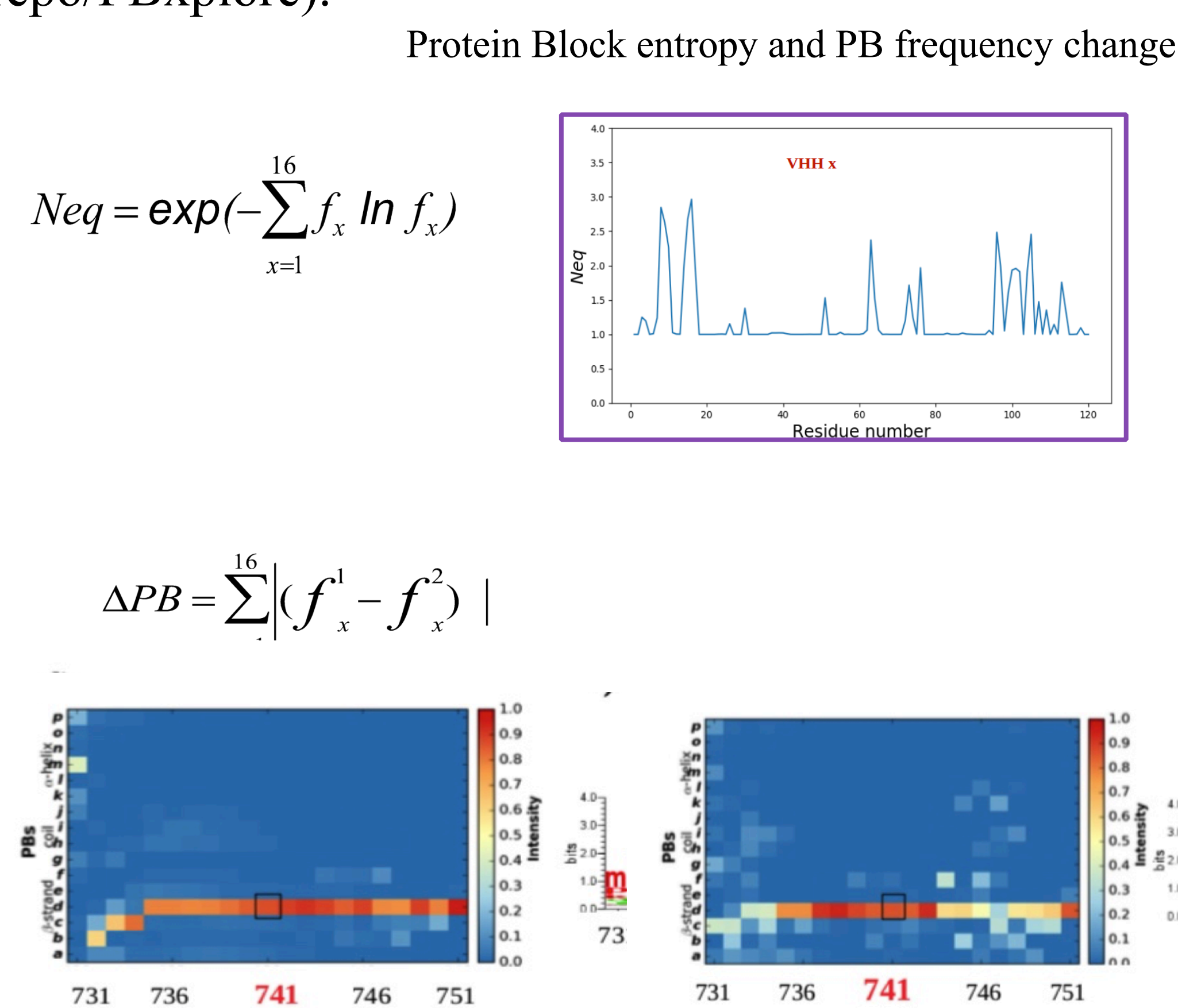


Figure 6: Representation of mean values of flexibility metrics onto a 3D structure of a V<sub>H</sub>H from each cluster.

- Mean flexibility metrics represented for each cluster.
- Not all the residue positions of CDRs are diverse.
- FRs show relatively less diversity when compared to CDRs except in the C-terminal loop regions and the 4th N-terminal loop.



$$N_{eq} = \exp\left(-\sum_{x=1}^{16} f_x \ln f_x\right)$$

$$\Delta PB = \sum_{x=1}^{16} |f_x^1 - f_x^2|$$

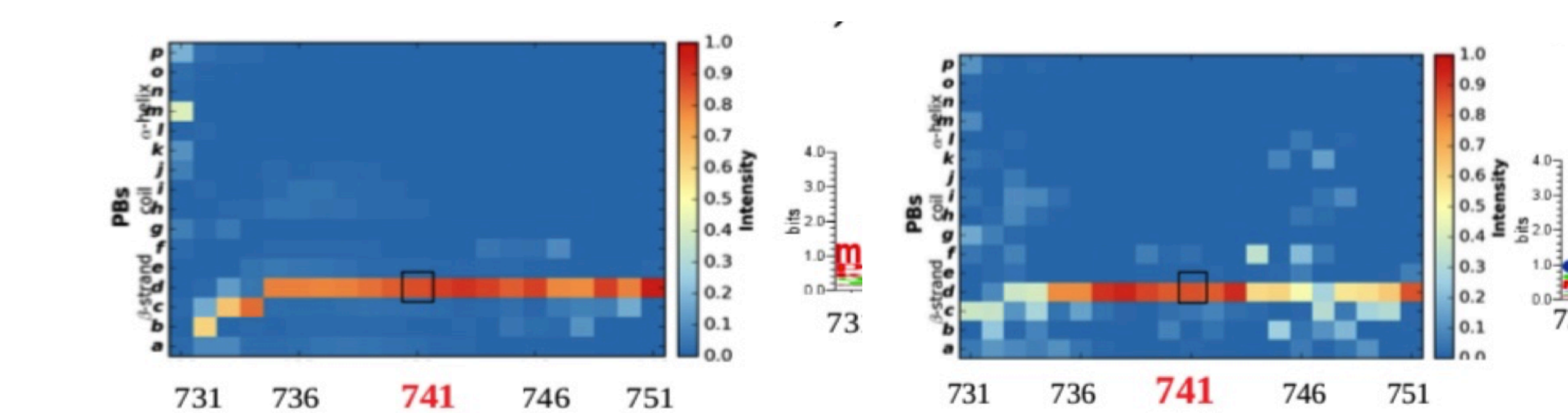


Figure 7: Local backbone diversity at the light of Protein Block maps. (A) PB map of all V<sub>H</sub>H trajectories aligned according to MSA, (B)  $N_{eq}$  values (red line - all V<sub>H</sub>H trajectories, sky blue - V<sub>H</sub>H trajectories belonging to RMSF cluster 1, green - V<sub>H</sub>H trajectories belonging to RMSF cluster 2, purple - V<sub>H</sub>H trajectories belonging to RMSF cluster 3 and pink - V<sub>H</sub>H trajectories belonging to RMSF cluster 4), (C) PB map of V<sub>H</sub>H trajectories belonging to (C) from RMSF cluster 1, (D) from RMSF cluster 2, (E) from RMSF cluster 3 and (F) from RMSF cluster 4. The x-axis represents the residue positions, and the y-axis represent the types of PBs of the  $N_{eq}$ .

- Total conformational diversity observed in various V<sub>H</sub>H trajectory groups quantified using Protein Blocks.
- The  $\beta$ -core is well conserved in the FRs represented using the PB 'd', except in some regions of FR2 and FR3.
- CDR1 also exhibits higher conformational diversity in terms of PBs.

## Dataset

Sequence and structural features of 88 non redundant V<sub>H</sub>H domains.

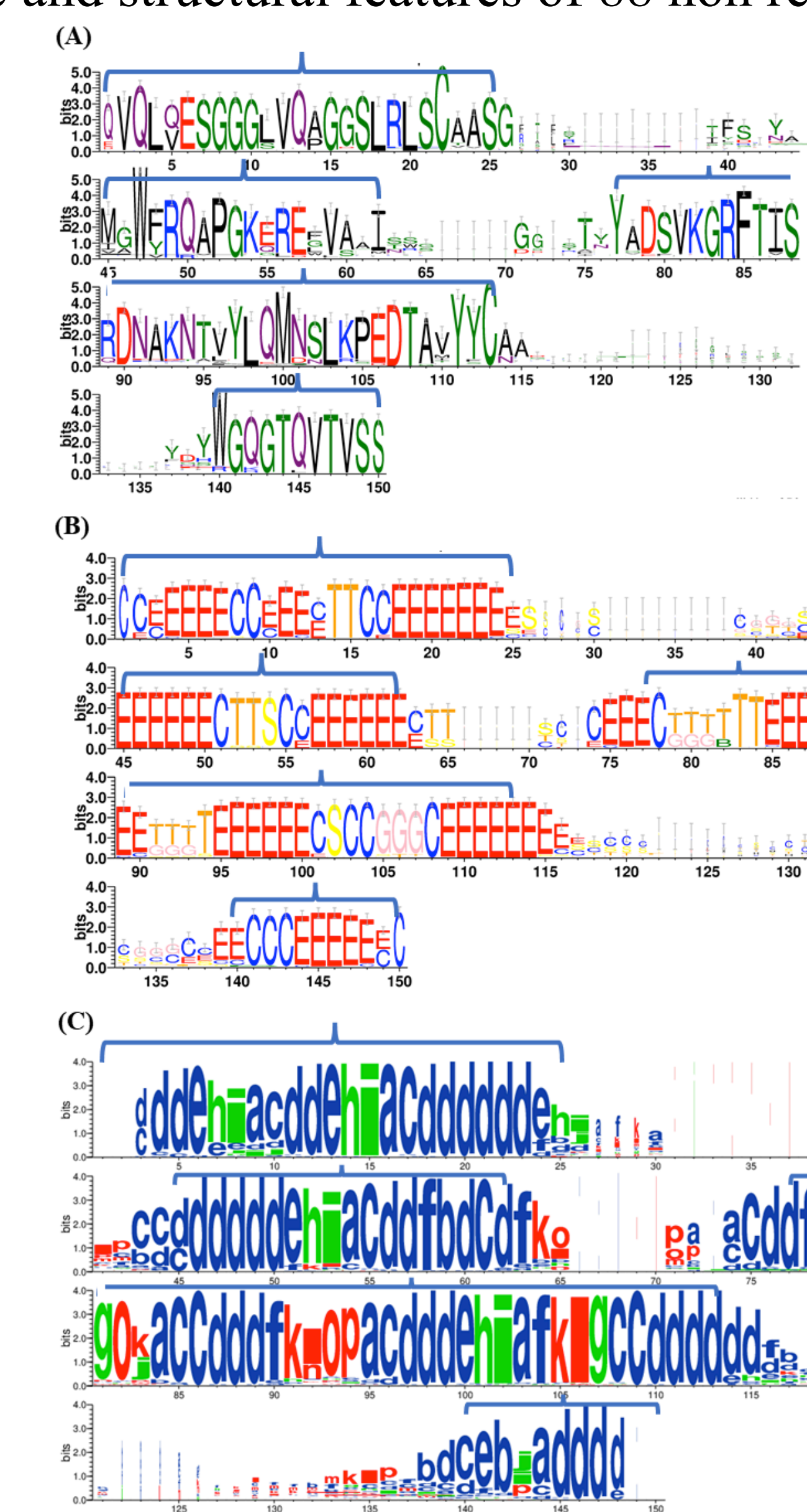


Figure 8: Sequence and structural characteristics in V<sub>H</sub>H dataset. Construction of (A) Amino acid residues, (B) Secondary structures, and (C) Protein Blocks. The four Framework regions are delimited in each figure.

## Conclusions:

- Protein Blocks offer a unique perspective which is both qualitative and quantitative to characterize backbone diversity in structures and trajectories of V<sub>H</sub>H domains.
- Higher RMSF values need not be always considered as flexible residues.
- There are few residue positions in CDR3 that do not show much diversity.
- The 4th N-terminal loop exhibits unexpected backbone diversity.

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