3-D clustering to identify multiple oligomerization states by FRET

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Nicotinic Acetylcholine Receptors (nAChRs)

- Pentameric ion channels found throughout the brain
- Composed of a variety of possible subunits in varying stoichiometries
- Open in response to acetylcholine (naturally), nicotine (much stronger!)
- Presumed to underlie the mechanisms of nicotine addiction, tolerance, & withdrawal... plus its protective effect against Parkinson’s

\[(\alpha_4)_3(\beta_2)_2:\]
EC50 ~100μM

100 times as sensitive!
FRET microscopy

(Vogel et al., 2006)
Extending FRET to study stoichiometry

Goal: to estimate the prevalence of distinct nAChR stoichiometries from the distributions of donor, acceptor, and net FRET pixel values.

Challenges:

• Multiple stoichiometries of assembled receptors, partially assembled receptors of unknown geometry, and unpaired donors and acceptors all present.

• Heterogeneous population even within single pixels

• Unknown subcellular localization of FRETing oligomers

(Son et al., 2009)
Current NFRET histogram analysis

Raw data

unmixing

FRET

donor (D)

acceptor (A)

PixFRET bleedthrough compensation

net FRET (nF)

normalization:

\[
\frac{nF}{\sqrt{A \cdot D}}
\]

NFRET

Fig 8H, Moss et al., submitted to JGP
Dangers of fitting NFRET histograms

Single oligomer NFRET values with
\( nF \sim N(1, 0.2) \)
\( A \sim N(10, 4) \)
\( D \sim N(10, 4) \)

\[
NFRET = \frac{nF}{\sqrt{A \cdot D}}
\]

(Xia and Liu, 2001)

1) NFRET distribution from a single oligomer with varying nF, A, and D measurements is skew.
Dangers of fitting NFRET histograms

\[ \text{NFRET} = \frac{nF}{\sqrt{A \cdot D}} \]

(Xia and Liu, 2001)

Given that a fraction \( f \) of the total FRETing constructs are of type A, the NFRET value

\[ T(f) = \frac{f nF_a + (1 - f) nF_b}{\sqrt{(f A_a + (1 - f) A_b)(f D_a + (1 - f) D_b)}} \]

2) NFRET from multiple species combines nonlinearly (sometimes non-monotonically)
Dangers of fitting NFRET histograms

3) Even “ideal” situations (with no variation in nF, A, and D) give skew distributions of NFRET values.
The case for direct clustering instead

• Why collapse 3D information to 1D unnecessarily?
• Clustering automatically assigns pixels to populations.
• Deals more readily with unpaired fluorescence.
Two pure population model

Species A, mean NFRET 0.1:
- \( nF \sim N(1, 0.2) \)
- \( A \sim N(7, 0.7) \)
- \( D \sim N(14, 1.4) \)

Species B, mean NFRET 0.125:
- \( nF \sim N(1.25, .25) \)
- \( A \sim N(14, 1.4) \)
- \( D \sim N(7, 0.7) \)
Segments with unpaired donor/acceptor

- Same species A and B, concentrations [100 50 50] and [50 100 50]
- Total unpaired concentration [25 25 25]
- Unpaired fluorophores have same properties as lesser of donor & acceptor in FRETing species
Choice of clustering algorithm

• Projective k-means
  – Clusters points along lines (representing varying concentrations of a single ratio of species, plus unpaired fluorescence)
  – Doesn’t split high- and low-concentration regions

• Gaussian mixture (GM) model
  – Fits points to a set of Gaussian clusters
  – Doesn’t ignore concentration
  – May be more robust to impure “segmentation”

Both easily extended to probabilistic clustering.
25 images each, 2000 pixels per image. 20% uncertainty in nF, 10% in A and D
Average concentration 10 oligomers (small) in both populations.
Performance of GM clustering

• Accurately and reproducibly clusters pixels from pure-population and segmented models, even with unpaired fluorescence

• Consistently identifies the number of clusters using Bayesian information criterion (introduces a parameter penalty to avoid overfitting)
Next steps

• Next focus is on clustering real data from two experiments: with three and one putative populations of nAChRs

• Use of membrane-specific and non-FRETing distributions to calibrate expected clusters

• Modeling varied transfection ratios and matching clusters across cells
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