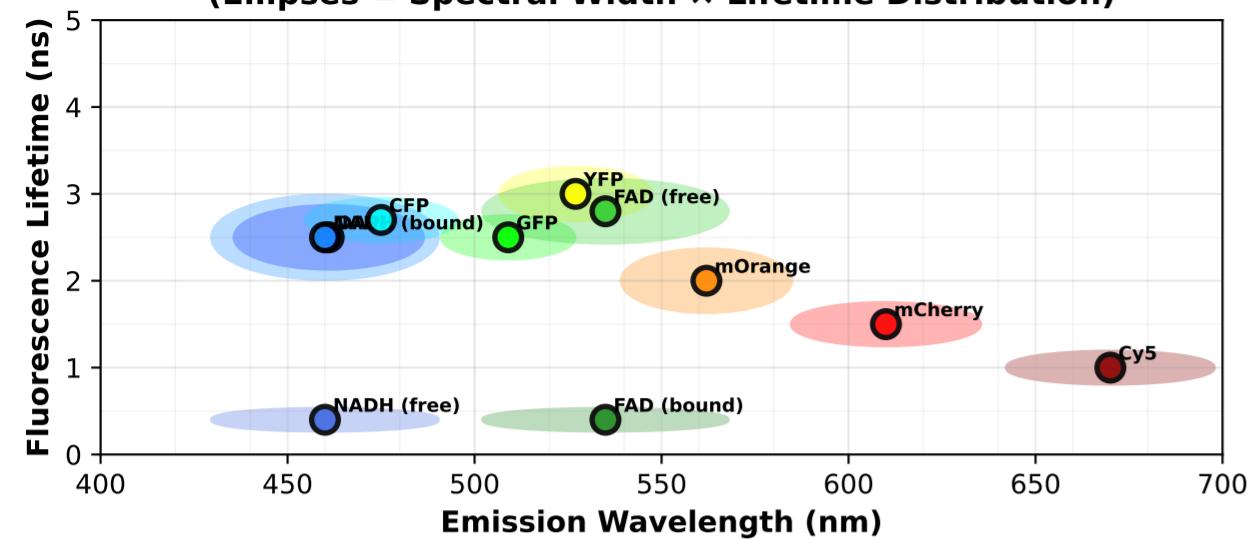


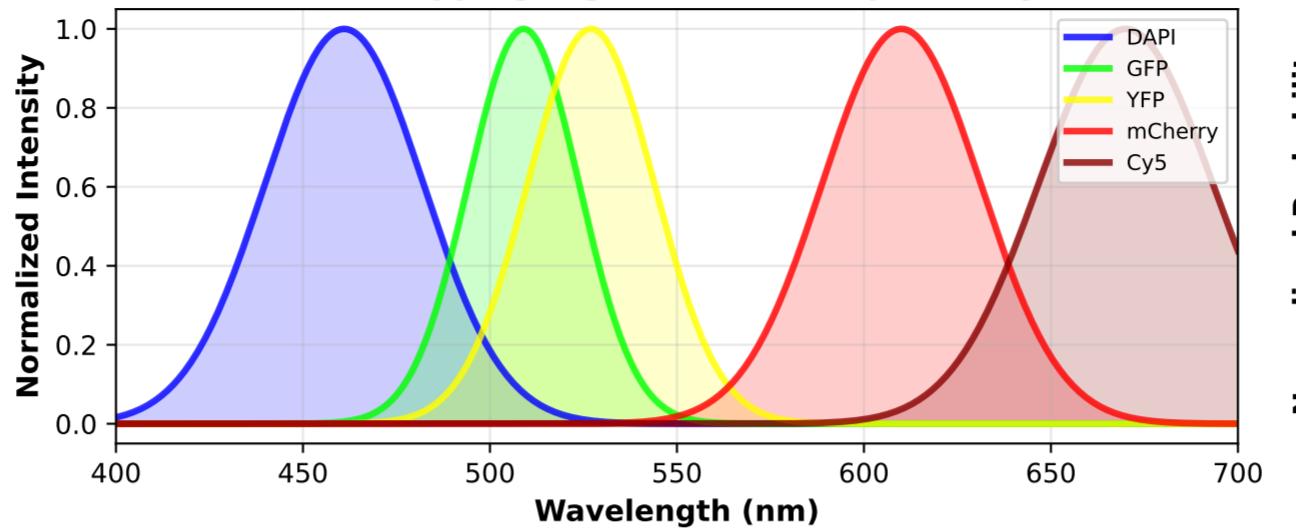
Realistic Fluorophore Separation Capacity Analysis

Accounting for Spectral Overlap and Lifetime Distributions

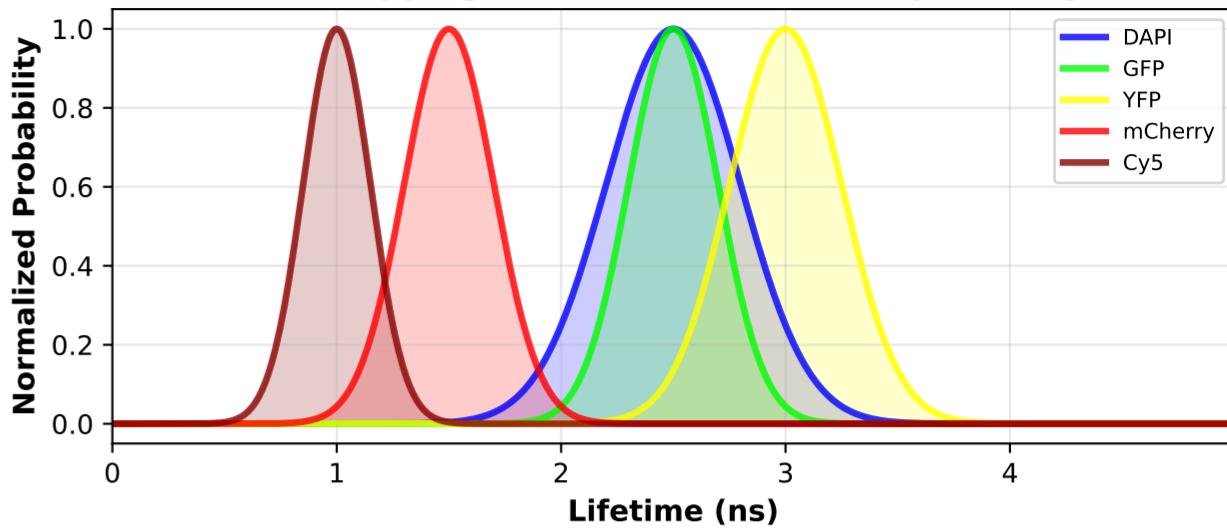
Realistic Fluorophore Separation
(Ellipses = Spectral Width x Lifetime Distribution)



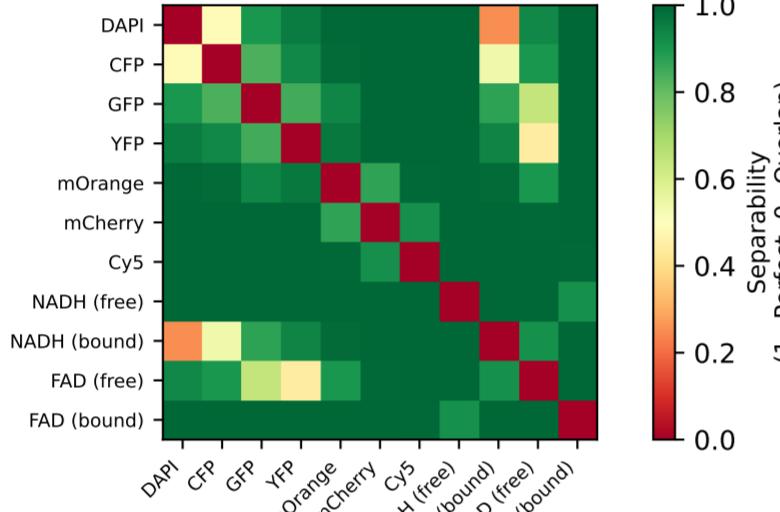
Emission Spectra Overlap
(Overlapping regions reduce separability)



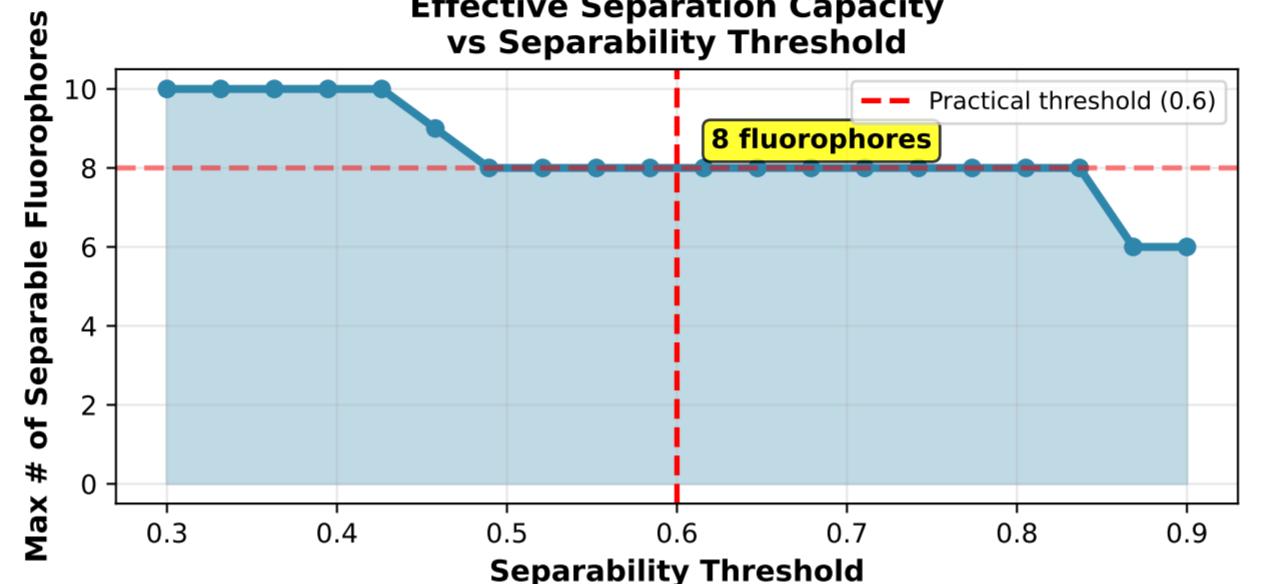
Lifetime Distribution Overlap
(Overlapping distributions reduce separability)



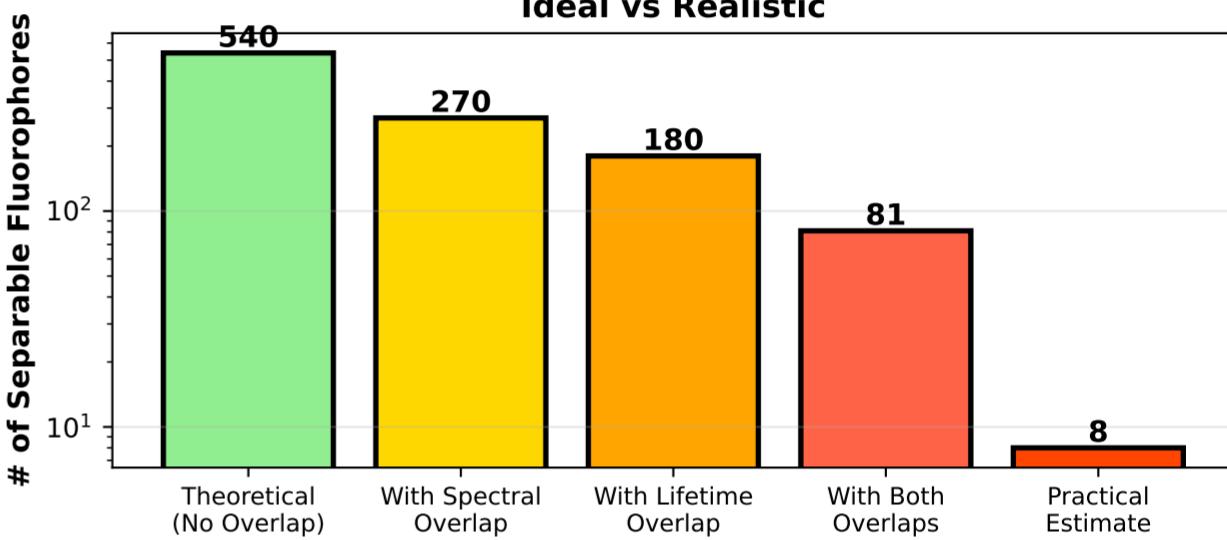
Pairwise Separability Matrix
(Accounting for Spectral & Lifetime Overlap)



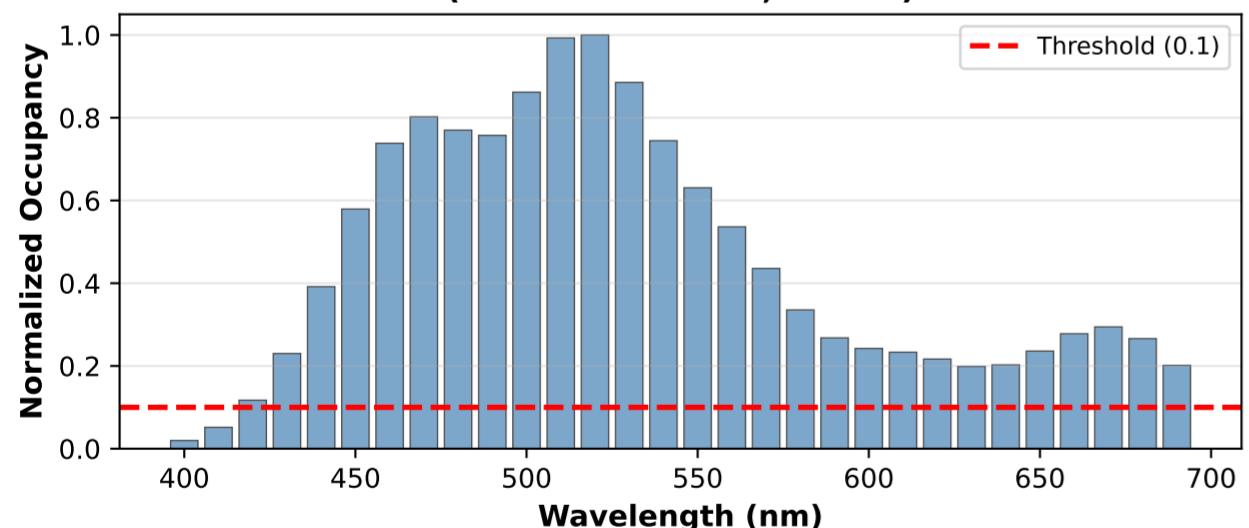
Effective Separation Capacity
vs Separability Threshold



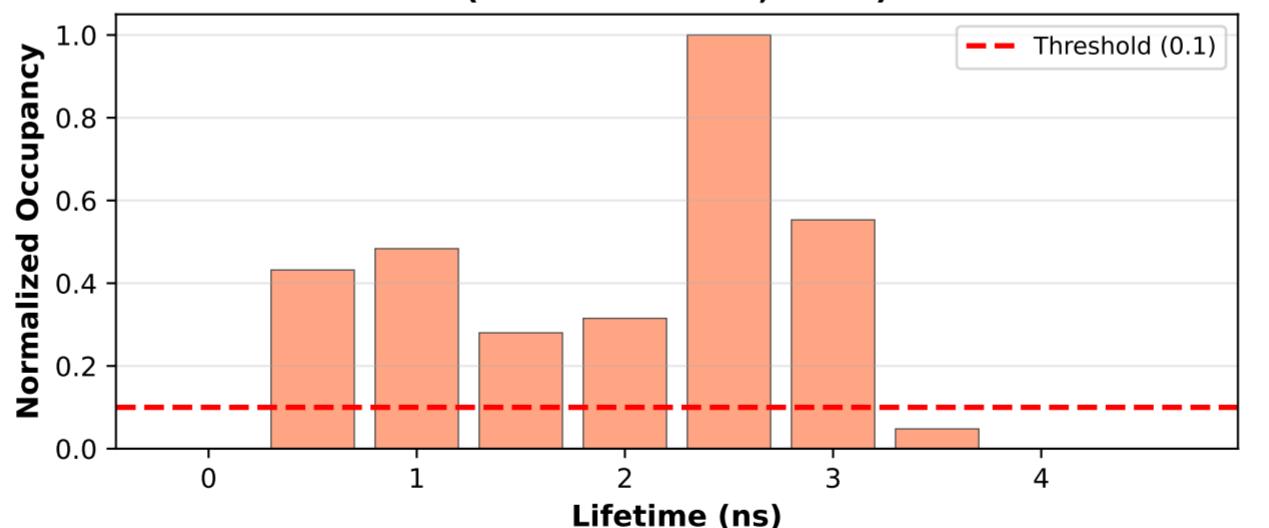
Separation Capacity:
Ideal vs Realistic



Realistic Spectral Bins
(28 effective bins, not 30)



Realistic Lifetime Bins
(6 effective bins, not 9)



REALISTIC SEPARATION CAPACITY ANALYSIS

Instrument Specs:

- Wavelength resolution: 10 nm
- Lifetime resolution: 0.5 ns
- Polarization states: 2

Theoretical vs Realistic:

- Theoretical bins: $30(\lambda) \times 9(\tau) \times 2(\text{pol}) = 540$
- Effective spectral bins: 28 (not 30!)
- Effective lifetime bins: 6 (not 9!)
- Realistic capacity: 8 fluorophores

Key Limitations:

1. Spectral overlap: Emission spectra are broad (FWHM = 35-65 nm, not single wavelength)
2. Lifetime distributions: Not single values ($\sigma = 0.1\text{-}0.4$ ns variation)
3. Crosstalk: Overlapping signals require unmixing

Practical Recommendations:

- Conservative panel: 3-5 fluorophores
- Moderate panel: 5-8 fluorophores
- Aggressive panel: 8-8 fluorophores (requires advanced unmixing)

Best Strategy:

- Maximize wavelength separation (most powerful)
- Use lifetime for same-wavelength species (e.g., NADH free/bound)
- Polarization adds modest 2x benefit

Example Well-Separated Panel (5 fluorophores):

1. DAPI (461 nm, 2.5 ns)
2. GFP (509 nm, 2.5 ns) - 48 nm separation
3. YFP (527 nm, 3.0 ns) - 18 nm + lifetime
4. mCherry (610 nm, 1.5 ns) - 83 nm separation
5. Cy5 (670 nm, 1.0 ns) - 60 nm separation