OUTLINE

➢ N&B Analysis

➢ Aggregation of protein in live cells: the case of Huntington’s disease

➢ Conclusion
N&B ANALYSIS

Purpose: to provide a map with pixel resolution of aggregation of molecules in cells

Method: uses the first and second moment of the intensity distribution at each pixel

Source: any raster scan image obtained with a laser scanning microscope, TIRF with fast camera, spinning disk confocal microscope

Interface: the N&B page of the SimFCS program

Output: brightness map, brightness vs intensity 2D histogram

Tools: cursor selection of pixels with similar brightness quantitative analysis of center and std dev of the $\varepsilon$ and $n$ distribution
Given two series of equal average, the larger is the variance, the less molecules contribute to the average. The ratio of the square of the average intensity ($<k>^2$) to the variance ($\sigma^2$) is proportional to the average number of particles N.

$$G(0) = \frac{\sigma^2}{<k>^2} = \frac{1}{N}$$

Average (first moment)  \( < k > = \frac{\sum k_i}{K} \)

Variance (second moment)  \( \sigma^2 = \frac{\sum (k_i - <k>)^2}{K} \)

Qian and Elson (1990)
Digman, Dalal, Horwitz, Gratton Biophys J. 2008
N&B analysis provides a map of number N and brightness B for every pixel in the image by calculating average and variance of the intensity distribution in each pixel.

\[ N = \frac{\langle k \rangle^2}{\sigma^2} \]

\[ B = \frac{\langle k \rangle}{N} = \frac{\sigma^2}{\langle k \rangle} \]

\[ \sigma^2 = \text{Variance} \]

\[ \langle k \rangle = \text{Average counts} \]

N = Apparent number of molecules

B = Apparent molecular brightness
counts/time/molecule
The *average intensity* at each pixel is given by

\[
\text{Average intensity} = \frac{\sum k_i}{K} = \varepsilon n
\]

In the N&B analysis two *sources of fluctuations* are considered: particles fluctuations and detector shot noise.

**Variance due to particle fluctuations**

\[
\sigma_n^2 = \varepsilon^2 n
\]

**Variance due to detector shot noise**

\[
\sigma_d^2 = \varepsilon n
\]

The *measured variance* is given by the sum of the two terms

\[
\sigma_{\text{measured}}^2 = \sigma_n^2 + \sigma_d^2 = \varepsilon^2 n + \varepsilon n = \varepsilon n(\varepsilon + 1)
\]
N and B are recovered from average and variance

\[ N = \frac{<k>^2}{\sigma^2} = \frac{<k>^2}{\sigma_n^2 + \sigma_d^2} = \frac{\varepsilon^2 n^2}{\varepsilon^2 n + \varepsilon n} = \frac{\varepsilon}{\varepsilon + 1} n \]

\[ B = \frac{\sigma^2}{<k>} = \frac{\sigma_n^2}{<k>} + \frac{\sigma_d^2}{<k>} = \frac{\varepsilon^2 n}{\varepsilon n} + \frac{\varepsilon n}{\varepsilon n} = \varepsilon + 1 \]

The “true” number n and the molecular brightness \( \varepsilon \) for mobile particles can be obtained from

\[ n = \frac{<k>^2}{\sigma^2 - <k>} \]

\[ \varepsilon = \frac{\sigma^2 - <k>}{<k>} \]

Digman, Dalal, Horwitz, Gratton Biophys J. 2008
Dhal, Digman, Horwitz, Vetri, Gratton. Microsc Res Tech. 2008
Variance due to particle fluctuations \( \sigma^2_{n} = 0 \)

Variance due to detector shot noise \( \sigma^2_{d} = \varepsilon n \)

\[
N = \frac{< k >^2}{\sigma^2} = \frac{< k >^2}{\sigma^2_{n} + \sigma^2_{d}} = \frac{\varepsilon^2 n^2}{0 + \varepsilon n} = \varepsilon n
\]

\[
B = \frac{\sigma^2}{< k >} = \frac{\sigma^2_{n}}{< k >} + \frac{\sigma^2_{d}}{< k >} = 0 + \frac{\varepsilon n}{\varepsilon n} = 1
\]

The apparent brightness of immobile particles is 1.
IDENTIFICATION OF MOBILE AND IMMOBILE MOLECULES

Changing the laser power the plot of the ratio variance/intensity vs intensity can distinguish the mobile from immobile fraction.
IDENTIFICATION OF MOBILE AND IMMOBILE MOLECULES

Fluorescein

Beads

B > 1

B = 1
To increase the apparent brightness we could increase the dwell time, since the brightness is measured in counts/dwell time/molecule.

**BUT** Increasing the dwell time decrease the amplitude of the fluctuation.
ANALOG DETECTOR

\[
\langle k \rangle = \frac{\sum k_i}{K} = S \varepsilon n + \text{offset}
\]

\[
\sigma^2 = \sigma_n^2 + \sigma_d^2 = S^2 \varepsilon^2 n + S^2 \varepsilon n + \sigma_0^2
\]

\[
N = \frac{(\langle k \rangle - \text{offset})^2}{\sigma^2 - \sigma_0^2} = \frac{\varepsilon}{\varepsilon + 1} n
\]

\[
B = \frac{\sigma^2 - \sigma_0^2}{\langle k \rangle - \text{offset}} = S(\varepsilon + 1)
\]

Bleaching and macromovements are other sources of variance that are not considered in the N&B equations.

Solution: high pass filter

![Intensity plots before and after detrend](image)
N&B: HOW DATA ARE REPORTED

- Stack of frames
  - average
  - variance/average

- Intensity map

- Brightness map

- Brightness vs intensity graph

- Brightness 2-D histogram

- pixels with same B

- Map of selected pixels

- selection of B
Stack of images $\xrightarrow{\text{Intensity distribution at each pixel}}$ Calculation of $N$ and $B$

$N = \frac{\langle k \rangle^2}{\sigma^2}$

$B = \frac{\langle k \rangle}{N} = \frac{\sigma^2}{\langle k \rangle}$

$\sigma^2$ = Variance

$\langle k \rangle$= Average counts

$N$ = Apparent number of molecules

$B$ = Apparent molecular brightness

Digman, Dalal, Horwitz, Gratton Biophys J. 2008
N & B

CALCULATING PROTEIN AGGREGATES IN IMAGES

N → average number of molecules in the observation volume

B → aggregates size

pixel resolution map of molecular aggregation in live cells

follow aggregation process and detect transient intermediate states
Study of Huntingtin aggregation

Giulia Ossato, Michelle Digman, Charity Aiken, Lawrence Marsh, Enrico Gratton
PROTEIN MISFOLDING AND AGGREGATION DISEASES

Dobson Nature 2003
PROTEIN MISFOLDING AND AGGREGATION DISEASES
COMMON THEME

Poly Q inclusion in SCA3, HD and other polyQ diseases

Alpha synuclein ‘Lewy Bodies’ in Parkinson’s

β amyloid plaques in Alzheimer’s

Neurofibrillary tangles of Tau protein inside nerve-cells of the Alzheimer’s afflicted brain
PROTEIN MISFOLDING AND AGGREGATION DISEASES
POLY Q DISEASES

Huntington’s disease (Huntingtin)
Dentatorubral pallidoluysian atrophy (Atrophin-1)
Spinocerebellar ataxia-1 (Ataxin-1)
Spinocerebellar ataxia-2 (Ataxin-2)
Spinocerebellar ataxia-3 (Ataxin-3)
Spinal and bulbar muscular atrophy (Androgen Receptor)
Spinocerebellar ataxia-6 (Ca$^{2+}$ channel)*
Spinocerebellar ataxia-7 (Ataxin-7)
Huntington’s disease is a dominant, late onset neurodegenerative disease.

Stages of HD:
1. Subtle changes in coordination, depression, irritable mood
2. Difficulty in speaking and swallowing
3. Inability to walk, speak. Death from complications (infection..)
HUNTINGTON’S DISEASE

AGE OF ONSET

Anyone who inherits the faulty gene will, at some stage, develop the disease. The longer the mutant polyglutamine sequence the earlier the disease onsets.

Age at neurological onset (blue) and the time from onset to death (red) are plotted against CAG repeat length

WHAT ARE THE TOXIC SPECIES?

Are inclusions toxic?
Could oligomeric forms be the principal toxic species?

IF SO, can we observe and quantify these species?

Antibodies
Electron Microscopy
Number & Brightness (N&B)
EXPERIMENTAL METHODS

- Olympus FluoView1000 using an incubator with CO$_2$ control: stacks of 100 frames, pixel time 20 $\mu$s

- COS7 cells transfected with mEGFP for B calibration, measurements before and after the experiments

- COS7 cells transfected with Huntingtin of three different polyglutamine sequence lengths (Httex1-25QP-EGFP, Httex1-46QP-EGFP, Httex1-97QP-EGFP) followed for few hours to observe the aggregation process

- N&B analysis
CALIBRATION

COS7 cell transfected with mEGFP

intensity | brightness | B vs intensity

heterogeneous | homogeneous | selection

B = 1.15
CALIBRATION

COS7 cell transfected with mEGFP

intensity  
brightness  
B vs intensity

B plot  
selection

B=1.15

monomers =1.15
CALIBRATION
COS7 cell transfected with mEGFP

intensity  brightness  B vs intensity

B plot  selection

B=1.15

monomers =1.15

dimers B=1.30
CALIBRATION

COS7 cell transfected with mEGFP

intensity  brightness  B vs intensity

trimers $B=1.45$
dimers $B=1.30$
monomers $B=1.15$

$B=1.15$
DATA:
COS7 cell with Httex1-EGFP

Httex1-25QP-EGFP

Httex1-46QP-EGFP

Httex1-97QP-EGFP
DATA:
COS7 cells transfected with Httex1-97QP-EGFP

0 | 1hr | 2hr | 3hr | 4hr
---|-----|-----|-----|-----

Intensity map

B map

B vs intensity

B plot
DATA:

COS7 cells transfected with Httex1-97QP-EGFP

0 1hr 2hr 3hr 4hr
DATA:

COS7 cells transfected with Httex1-97QP-EGFP
RESULTS

**Httex1-25QP-EGFP**
- no aggregation (essentially only monomers)
- no change in brightness with time

**Httex1-46QP-EGFP**
- limited aggregation (no aggregates larger than 4 Htt)

**Httex1-97QP-EGFP**
- substantial aggregation starting with monomers, and moving to smaller aggregates (8-10 Htt). (could be larger due to immobile fraction)
- mass action after sufficient protein expression plaque formation begins. Protein not recruited by the plaques is monomeric or in small aggregates (dimers, trimers)
- after a lag phase the formation of the plaque is fast (around 30 minutes)
- the aggregation process can be followed with the graph of brightness versus intensity
MODEL OF HTTEX1 AGGREGATION

1. Expression of Httex1 but the protein concentration is low. During this period there are only monomers in the cell.

2. At higher concentration of monomers, small oligomers (8-15 proteins) form. Monomers and oligomers are in a rapid equilibrium until one or more nuclei of aggregation are formed.

3. Formation of nucleation sites, and one inclusion for each nucleation site close to the membrane starts to grow.

4. Inclusions recruited most of the protein present in the cell.
CONCLUSION

N&B analysis can be used to

✔ Detect oligomers
✔ Quantify oligomers
✔ Follow aggregation process

in **LIVE** cells
THANKS!