Digital Assays

ELEC6205
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Digital assays – analysing large numbers of **single** events

- DNA
- Proteins
- Cells
What is a digital assay?

Molecules are diluted into a set of discrete containers (pL) so that ON AVERAGE each container has 1 molecule or 0 molecules – hence digital
Analog Assay

Done in a test tube

Readout (A) proportional to concentration of bulk sample e.g. fluorescence intensity from a labelled DNA probe tells you the amount of DNA in a sample
Digital Assay

Sample diluted and partitioned into containers. Each small volume contains a discrete number of molecules, e.g. 0, 1, 2, 3. Each container is separately assayed giving a 1 or 0 for molecule present or not.

Data evaluated using Poisson statistics and the sample concentration counted by counting the number of EMPTY containers.
Advantages:

No need for complex detection systems that measure large dynamic changes in signal.

Only need to detect 1 or 0 (signal or no signal).

Ratio of 1 to 0 is then related to number of molecules using statistics.
Two notable effect of partitioning:

1. **Concentration:** Limit of Detection (LoD) improved – small reaction volume increase the effective concentration.

   Single molecules of DNA can be amplified accurately OR single cells can be processed with compounds at a high local concentrations.
2. **Enrichment**: Target molecules in complex mixtures are isolated from myriad interfering compounds. Droplet enrichment increase the ratio of target molecules vs background.
How do we make small chambers and how small is small?

1. Small test-tubes pL or fM wells
e.g. plastic or PDMS

2. Droplets of water suspended in oil
e.g. emulsions
Miniature wells in Si or Polymer

Expensive

Static (can’t manipulate chambers)

Lithography resolution

Volume variability
Droplet microfluidics

Oil and water don’t mix
Water stays as small droplets in oil

- Can make >10 million droplets at kHz
- Polydispersity low <5%
- Avoid absorption of molecules to interfaces: use surfactants
Partitioning statistics

- Number of target molecules = $m$
- Number of containers = $n$ with $m<n$
- Average number of molecules $\lambda$

Number of molecules depends on the sample concentration $C$ AND number of containers $n$

- $\lambda = m/n = C \, V_d$ (where $V_d$ is container volume)

$$\lambda = \frac{\text{Number of molecules}}{\text{Number of containers}}$$
The probability that a droplet will contain \( x \) copies is governed by the \textbf{Binomial distribution}.

This is a discrete probability distribution that gives the likelihood of \( x \) successes in \( m \) trials, each with a probability \( p \).

For example, when rolling a dice, the binomial distribution will give the probability of rolling a 4 in exactly 7 out of 10 trials.

\textbf{Poisson distribution:}

A digital assays typically employ a large number of containers. When \( n \) is large, and the probability of a successful trial \((1/n)\) is small, the binomial distribution is approximated by the \textbf{Poisson distribution}. 
Poisson distribution

Let $X =$ the number of events in a given interval

Probability of $x$ events is: $p(X = x) = e^{-\lambda} \frac{\lambda^x}{x!}$ \quad x = 0, 1, 2, 3, ...

We say $X \approx P o (\lambda)$

The probability of an empty container $E$ is for $X = 0$:

$E = p(0) = e^{-\lambda}$

Therefore, the number of molecules $(m)$ and the sample concentration $C$ can be calculated from the percentage of empty drops $(E)$:

$\lambda = -\ln(E)$ \quad \text{And the number of empty drops:}$

$C = \frac{\lambda}{V_d} = -\frac{\ln(E)}{V_d}$ \quad $m = n\lambda = -n \ln(E)$

Mean and variance of the Poisson distribution are both equal to $\mu = \sigma^2 = \lambda$
Poisson distributions for various values of sample concentration $\lambda$.

Recall $\lambda = \frac{\text{Number of molecules}}{\text{Number of containers}}$

$\lambda = 0.1$ is 1 molecule for every 10 containers (10%)

As number of molecules ($\lambda$) increases, the proportion of empty vessels falls to zero, making quantitation impossible.

To statistically have at least one empty container ($nE > 1$), the number of targets must be less than $n \ln(n)$.
EXAMPLE Calculation

Births in a hospital occur randomly at an average of 1.8 births per hour.
What is the probability of observing 4 births in a given hour?

Let $X =$ No of births in a given hour

(i) Events are random
(ii) Mean rate $\lambda = 1.8 \implies X \approx Po(1.8)$

So probability of exactly 4 births is

$$P(X = 4) = e^{-1.8} \frac{1.8^4}{4!} = 0.0723 \quad 7\%$$
Example II

Concentration of target DNA is 1pM
A 2μL sample is divided into 1 million equal sized droplets
Calculate the probability of a droplet containing a single DNA molecule

Total number of molecules in a 2μL sample at 1pM concentration is:

\[6 \times 10^{23} \times 2 \times 10^{-6} \times 10^{-12} = 1.2 \times 10^6\]

1 Million droplets means that each droplet contains on average 1.2 molecules

\[P(X = 0) = e^{-1.2} = 0.3\]

\[P(X = 1) = e^{-1.2} \frac{\lambda}{x!} = 0.3 \frac{1.2}{1} = 0.36\]

\[P(X = 2) = e^{-1.2} \frac{\lambda}{x!} = 0.3 \frac{1.2^2}{2!} = 0.26\]
EXAMPLES

1. DNA
2. Proteins
3. Cells
1. DNA: Digital PCR

(a) Droplet generation using flow-focusing microfluidic chip; Droplets contain sample DNA and all the reagents. PCR amplification produces fluorescence 
(b) Serial droplet fluorescence reading; 

Alternatively 
(c) Minute volumes of PCR sample are partitioned in micro-well microfluidic chip; 
(d) Planar imaging for fluorescence positive channels
Example application
2. Digital protein analysis

Digital enzyme-linked immunoassays (ELISAs): Quantify proteins with 2 to 3 log lower detection limit than conventional ELISA, making them suited for extremely-abundance biomarkers.

Workflow:
1) isolate single cells or proteins in droplets,
2) detect protein activity via enzymatic amplification (ELISA),
3) Tag droplets individually by co-encapsulating molecular barcodes.
Measures single-enzyme kinetics.

The fluorescence in each vessel increases over time at a rate dependent on the number of encapsulated enzymes molecules.

Green shaded region illustrates variation which may occur due to differences in volume or enzymatic efficiency.
Digital ELISA

First demonstrated in 2010

Quantifies single proteins by a digital enzyme-linked immunosorbent assay.

Histogram showing rate of fluorescence increase in each partition (for $\lambda<1$). Line shows Poisson distributions - characteristic of discretized encapsulation.
Digital bead based ELISA

Beads with enzyme-labeled target are partitioned on a single molecule array. After sealing and incubation, fluorescence accumulates in chambers containing the labeled target.
(B) Brightfield images of beads in wells.

C) Fluorescent images, showing positive wells.

(D) Limit of detection with PSA, showing the % of fluorescent wells vs. protein concentration (fM).
Clinical applications of digital ELISA

1. Detect extremely low-concentration protein biomarkers in blood

Examples

- Biomarkers for cancer (PSA)
- Neurological disorders (tau protein),
- Inflammation cytokines (TNF-α and IL-6)
- Early stage human immunodeficiency virus (HIV)

2. Quantifying protein expression in single cells
SUMMARY

In digital PCR, partitioning helps detect rare nucleic acid targets in the presence of a wild-type population.

In digital ELISA, partitioning enables the detection of low abundance proteins in the presence of those at much higher concentrations.
Digital Cell Assay – single cell biology

Encapsulation of discrete numbers of cells in small-volume partitions.
Compared to digital assays with nucleic acids and proteins, digital cell assays have broad applications:

Measure many aspects of cell phenotype and genotype:
• Intracellular and extracellular gene expression,
• Surface biomarkers,
• Kinetic activity of cellular proteins,
• Metabolic activity,
• Cell secretions.
• Genome and transcriptome sequencing
Why?

In conventional systems a cell is like a fish in the sea — any secretions are quickly diluted in an infinite volume and may even be masked by the secretions of other cells within the culture.

If a single cell is encapsulated in a tiny volume comparable to the cell, any secretions and uptake will remain at a larger concentration and cause detectable changes in the composition.

Examples: metabolic exchange of nutrients and waste products; enzymatic activity of secreted or surface proteins.

With digital encapsulation, these changes can be detected with high sensitivity.
Two methods

(1) encapsulating single cells in droplets with fluorescent substrates for enzymatically amplified detection of proteins or metabolites

(2) co-encapsulation of single cells with barcoded beads for sequencing and transcriptomics – not discussed further
Advantages and examples

High-throughput digital cell assays can statistically quantify genomic or phenotypic differences between cells and classify cells into subpopulations.

In antibiotic resistance studies, adaptive resistance of bacteria is known to be correlated with phenotype heterogeneity.

In infection pathology, the spatial organization of a cell is linked to phenotypic traits that enhance resistance to viral infection.

In immunology, genetically identical cells with diverse phenotypes together mount a host immune response.

In oncology, tumors are known to have a diversity of phenotype due to genetic mutations, environmental cues, and other factors. Understanding the mechanisms of tumor heterogeneity can lead to better monitoring and the development of targeted therapies.
Some examples of single cell assays

(A) Time-resolved measurement of **intracellular** gene expression.
(B) **Extracellular** gene expression.
(C) Quantification of cell **surface** biomarkers.
(D) Enzymatic efficiency of surface-display proteins
Summary

• Digital Assays for DNA (PCR) and Protein (Enzyme) quantification
• Only requires (Signal) or (No Signal) measurement
• Extremely sensitive
• Requires very large number of containers (droplets)
• Output governed by Poisson statistics

• Single cell “omics” – emerging new technology
• Problem is that most of the containers are empty!