From single molecules to heart disease
A thought experiment involving time travel

How long would it take for them to understand how a Ferrari works?
How far are we from understanding the heart?
A single amino acid substitution can cause cardiovascular disease.

Sickle-cell anemia

And channelopathies, cardiomyopathies, hypercholesterolemia...
A SINGLE AMINO ACID SUBSTITUTION CAN CAUSE CARDIOVASCULAR DISEASE!!!!

Number of base pairs in the genome: $3 \times 10^9$

Number of AT pairs

WT

Sickle-cell anemia

Number of people in the world: $7 \times 10^9$
Genetics vs. risk factors

Risk factors

Sudden death
Genotype to phenotype: *why* do we want to know?

**Diagnostic reasons**

Your kids are going to be OK!

Polymorphism or pathogenic mutation?

**Therapeutic reasons**

Drugs that *restore* healthy phenotype
Why a particular missense mutation causes disease?

1. **Decreased thermodynamical stability/protein levels (haploinsufficiency)**
   It is considered to be a major driver of pathogenesis JMB 353, 459 (2005).

2. **Defective activity (catalytic activity of an enzyme)**
   Difficult to predict, challenging to determine experimentally

3. **New toxic properties (poison peptide)**
   e.g. Hemoglobin

4. **Pre-protein effects**
   RNA levels, alternative splicing leading to truncations, etc.

5. **Defective mechanical properties**
   Highly relevant for proteins or the contractile machinery of the sarcomere
Why single molecules?

Maritime routes between New York and San Francisco
Average vs single trajectories

By averaging we lose information

Example listened to Steve Block, a pioneer in single-molecule optical tweezers
Advantages of single-molecule approaches

1. Novel information that cannot be obtained in bulk

- Access to individual properties that are not accessible to bulk experiments
- Synchronization
- Access to vectorial properties: movement, force, etc...

Random orientation of molecules in bulk

Molecular Motors
Advantages of single-molecule approaches

2. Less material is needed/parallelization: next-generation DNA sequencing

Single-molecule fluorescence

Nanopore technology (Oxford Nanopores)
Main single-molecule techniques: classification

- **Manipulation**: application of mechanical forces
  - Atomic Force Microscopy (AFM), magnetic tweezers, optical tweezers, nanopores

- **Observation**
  - fluorescence
First single-molecule techniques

Electron Microscope
E. Ruska
Nobel Prize in Physics, 1986

Single-channel patch clamp
E. Neher y B. Sakmann
Nobel Prize in Physiology or Medicine, 1991
Challenges associated with single-molecule experiments

- **Complex** instruments and experiments

- **Signal is small**: how can we tell apart signal from noise?

- **New mindset**

- **New methods** of analysis and interpretation of results
Mechanical forces and proteins: from the cradle to the grave

Muscle activity

Oxidative folding

Proteasomal degradation
The **sarcomere** is the functional unit of striated muscle.

![Diagram of sarcomere and cardiac muscle](image)

- **Skeletal muscle**
  - Sarcomere
  - Z line
  - M band
  - I band
  - A band
  - H zone
  - Myofibril

- **Cardiac muscle**

- **Sarcomere**

- **Titin**
Sarcomere organization as observed by electron microscopy
Sarcomere organization as observed by electron microscopy

9-3 Organization of the myofibril. (A) Diagram of three sarcomeres, showing thick and thin myofilaments forming I, A, and H bands and Z lines. (B) Imaginary sections through the sarcomere at different levels show profiles of thin (left) and thick (right) filaments, and both types (center). (C) Electron micrograph of a cross section in which the sarcomeres of adjacent myofibrils are out of register and can thus be matched with the corresponding profiles shown above. Spider monkey extracocular muscle. Magnification 100,000×. [Courtesy of L. D. Peachey]
The **sliding filament hypothesis** of muscle contraction

Both authors independently proposed the sliding filament hypothesis in 1952
Testing the sliding filament hypothesis

Figure 19.17. Myofilament dimensions in frog muscle. The lower diagram B shows the myofilament arrangements at different lengths; the letters a, b, c and z refer to the dimensions given in the upper diagram A. The sarcomere lengths corresponding to the positions labelled 1 to 6 are indicated by the arrows in fig. 19.16. (From Gordon et al., 1966b.)
It’s not only about contraction...

Muscle is elastic!
We need **passive elasticity**

“Compared with other carnivores, [humans] are slow, weak and lack natural weapons such as fangs and claws.

However, [humans] were eating meat at least 2.6 million years (Myr) ago, and were probably hunting large prey 1.9 Myr ago…”

Titin is **BIG**, a molecular **Titan**

Titin is the **largest protein** in the human proteome (up to 4 MDa)

Wang et al. PNAS 76, 3698 (1979)
The titin gene

(0.28 Mbp, 363 exons)
Titin has **beads-on-a-string** appearance
The length of titin changes during contraction/extension cycles.

Protein elasticity is determined by protein unfolding/refolding.
Mechanical forces and exposure of cryptic binding sites

Mechanosensing and mechanotransduction
The mechanics of the myocardium is defective in cardiomyopathies

- **Defective contraction:** impaired systole
  - Dilated cardiomyopathy (DCM)
  - Hypertrophic cardiomyopathy (HCM)

From “Pathophysiology of Heart Disease”, 5th Edition, Ed. Leonard S. Lilly
Mutations in sarcomeric proteins lead to familial cardiomyopathy

Dilated cardiomyopathy (~25% of cases)

Hypertrophic cardiomyopathy (~70% of cases)

Genotype to phenotype?
Single-molecule techniques: manipulation
AFM

Force spectroscopy by Atomic Force Microscopy

The pioneer technique to measure mechanical properties of proteins

Reversible Unfolding of Individual Titin Immunoglobulin Domains by AFM
Matthias Rief, Mathias Gautel, Filipp Oesterhelt, Julio M. Fernandez, Hermann E. Gaub*

Science (1997) 276, 1109
Constant-velocity experiments: \textit{force-extension}

Photodiode (A-B→Force, picoNewtons)

laser

Cantilever

protein

Lineal actuator (extension, nanometers)
A more realistic view of an AFM pulling experiment

Polyproteins are “minititins”
Polyprotein engineering for force spectroscopy
Atomic Force Microscopes/Spectrometers

Home-made AFM

Commercial AFM

At CNIC
AFM cantilevers for single-molecule experiments

Spring constant: 5-20 pN/nm
Worm-like chain model of polymer elasticity

\[ F(x) = \frac{kT}{p} \left[ \frac{1}{4} \left( 1 - \frac{x}{L_c} \right)^{-2} - \frac{1}{4} \frac{x}{L_c} \right] \]

- \( x \): extension
- \( L_c \): contour length (length at infinite force)
- \( p \): persistence length (~internal flexibility)

Graph showing force vs. extension with \( L_c = 100 \text{ nm} \).
The importance of **fingerprinting** single-molecule data

**Bond rupture**

Rupture?

**Protein unfolding**

Repetitive recording (sawtooth)
Non-specific interactions happen close to the surface
Constant force experiments: *force-clamp*

- Better approach to determine force dependencies
- Feedback systems to keep the force at a predefined set point
A force-clamp experimental trace

Unfolding events (staircase)

Feedback response time: 5 ms
New mindset: Single-molecule events are stochastic

**SINGLE-MOLECULE**  Vs.  **BULK**

**Length (20 nm/ref line)**

**Time (s)**

4 different unfolding trajectories

<table>
<thead>
<tr>
<th>Variant</th>
<th>Melting temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>66.3</td>
</tr>
<tr>
<td>D122Y</td>
<td>50.4</td>
</tr>
<tr>
<td>G130V</td>
<td>43.2</td>
</tr>
<tr>
<td>G137V</td>
<td>46.0</td>
</tr>
<tr>
<td>I154F</td>
<td>50.7</td>
</tr>
<tr>
<td>W155R</td>
<td>61.4</td>
</tr>
</tbody>
</table>
Crossing of energy barriers at the single-molecule level

\[ k_u = k_u^0 e^{F\Delta x / kT} \]
Measuring kinetics of mechanical protein unfolding

\[ F = 140 \text{ pN} \]

\[ \text{Fit to a simple exponential function} \]

\[ P(U) = 1 - e^{-k(F) \cdot t} \]
Force-dependent mechanical unfolding

The higher the force, the faster proteins unfold
Mechanical refolding by AFM

Mechanical stability

Elasticity!!

Unfolding

Refolding pulse

Probe

Mechanical refolding

Refolding rate

3 refolded

4 unfolded

5 refolded

0 unfolded

Protein Length

Δt Refold = 3 s

Δt Refold = 10 s

25 nm

0.5 s
Molecular determinants of the mechanical stability of proteins

Mechanical stability: (β-shearing > β-unzipping > alpha)
Molecular determinants of the mechanical folding of proteins

Folding?
An example of single-molecule experiments informing about biology: **Thiol chemistry controlling titin elasticity**
Titin’s buried *(cryptic)* cysteines
**Redox posttranslational modifications in muscle**

- ROS (superoxide, $H_2O_2$)
- Xanthine oxidase
- NAD(P)H oxidase

**Oxidative modification of cysteines in titin**

What’s the functional relevance?

- NO
- Nitric oxide synthase
S-glutathionylation inhibits protein folding

Inhibition of folding

Softening of the tissue
The elasticity of cardiomyocytes is modulated by S-glutathionylation of titin’s cryptic cysteines

In collaboration with Nazha Hamdani and Wolfgang Linke (Bochum University, Germany)

MT

Magnetic tweezers
Magnetic tweezers
**Magnetic tweezers** to examine the mechanical properties of proteins

Advantages
- No need for feedback to get constant force
- Stability
- Good sensitivity at < 20 pN
- Parallelization

Disadvantages
- Low temporal resolution
- You need to buy your own
Optical tweezers

Folding-Unfolding Transitions in Single Titin Molecules Characterized with Laser Tweezers

Miklós S. Z. Kellermayer,*† Steven B. Smith,*
Henk L. Granzier,‡ Carlos Bustamante*

Science (1997) 276, 1112
Trapping small objects using light
Optical tweezers

Advantages
• Good sensitivity at < 20 pN
• Controlled manipulation: versatility

Disadvantages
• Complex instrumentation for high resolution studies
• Need of molecular handles

Double trap

https://youtu.be/gOA7wvycV-Q
Measuring the activity of molecular motors using OT
From single molecules to heart disease: take home messages

• Single-molecule methods provide new information that may be relevant to understand the pathophysiology of (heart) diseases.

• Many key biomolecules experience or produce mechanical force

• Single molecules behave stochastically

• Main single molecule manipulation techniques: AFM, MT, OT

• A new mindset and novel analysis tools
Recent findings in the field

RESEARCH ARTICLE

BIOCHEMISTRY

Contractility parameters of human β-cardiac myosin with the hypertrophic cardiomyopathy mutation R403Q show loss of motor function

Suman Nag,1 Ruth F. Sommese,1 Zoltan Ujfalusi,2 Ariana Combs,3 Stephen Langer,3 Shirley Sutton,1 Leslie A. Leinwand,3 Michael A. Geeves,2 Kathleen M. Ruppel,1,* James A. Spudich1

Single Molecule Force Spectroscopy on Titin Implicates Immunoglobulin Domain Stability as a Cardiac Disease Mechanism*

Received for publication, July 16, 2012, and in revised form, December 10, 2012. Published, JBC Papers in Press, January 6, 2013, DOI 10.1074/jbc.M112.401372

Brian R. Anderson4,5, Julius Bogomolovas6, Siegfried Labeit7, and Henk Granzier5,1
More reading...


For any question or feedback: jalegre@cnic.es
Some of the world-leading single-molecule laboratories

- **Optical Tweezers**
  - Carlos Bustamante (UC-Berkeley)
  - Steve Block (UC-Stanford)

- **Fluorescence**
  - Xiaowei Zhuang (Harvard)
  - Sunney Xie (Harvard)

- **AFM**
  - Julio Fernández (Columbia)

- **Magnetic Tweezers**
  - Vincent Croquette (ENS-Paris)
  - Nynke Dekker (Delft)

- **AFM**
  - Hermann Gaub (U. Munich)
Single-molecule in Madrid

Optical Tweezers
J. Ricardo Arias-González (IMDEA-Nanociencia)
Borja Ibarra (IMDEA-Nanociencia)

AFM
Mariano Carrión-Vázquez (I. Cajal)
Jorge Alegre-Cebollada (CNIC)

Magnetic Tweezers
Fernando Moreno-Herrero (CNB)

Fluorescence

Also Félix Ritort (U. Barcelona, Optical Tweezers), Raúl Pérez-Jiménez (Nanogune, AFM)…
Mechanobiology Seminar Series at CNIC. If interested, send an e-mail to jalegre@cnic.es
From single molecules to heart disease