Molecular Imaging Probes

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Cell Mechanics: Basic Questions

• How mechanical forces are distributed inside a living cell

• How forces applied to a cell, either directly or through the FAC, induce reorganization of the cytoskeleton (including microtubules, actin filaments and stress fibers), thus changing its mechanical properties

• How the dynamics of the cytoskeleton affect cell spreading, rounding, crawling and adhesion

• If, and how, the interaction between ECM and FAC transduces a mechanical signal (force or deformation) into cells

• How to model the constitutive behavior of a living cell, especially its active behavior

• How to analyze the contributions of subcellular structures, including cytoskeleton, cell nucleus, mitochondria, endoplasmic reticulum, and Golgi, to the mechanical behavior of a cell
Recent studies confirm that mechanical forces can alter almost all cellular processes, including cell growth, differentiation, movement, signal transduction, protein production/secretion and gene expression.

An example is altered gene expression in endothelial cells under shear flow. We found that eNOS mRNA increased its level by ~4.5 fold under laminar shear for 24 hr, while Klf2 mRNA level increased more than 25 fold with just 2 hr of laminar shear. No change in eNOS and Klf2 mRNA level under oscillatory shear.
Mechanosensing and Mechanotransduction

- To establish the molecular mechanism(s) of how cells sense mechanical force and/or deformation, and transduce them into biological response
- Although mechanotransduction may have many mechanisms, it is likely that the most important one is protein deformation, or protein conformational change under force

- Major issues:
  - To identify stress sensitive genes and their regulators
  - To observe force-induced protein conformational changes in live cells
  - To understand the effect of forces on the dynamics of protein-protein interactions in living cells

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Mechanosensing and Mechanotransduction

What Protein Deformation Could Do?
Molecular Biomechanics

To understand the molecular basis of mechanotransduction, how mechanical forces affect molecular interactions in a living cell, and the mechanochemical coupling in protein machines

*Mechanotransduction* The molecular mechanisms of how cells sense mechanical force/deformation

*Mechanical behavior of biomolecules* How the structural rigidity of DNA, RNA and proteins under stretching, twisting, bending and shear alters DNA condensation, gene replication and transcription, DNA-protein/RNA-protein interactions, protein function, protein-protein interactions

*Proteins as molecular machines* To study mechanochemical coupling in, and mechanical functions of proteins as nano machines, including molecular motors (linear and rotary motors), molecular scissors (proteases), molecular connectors and switches (adhesion molecules)

*Biomolecular assembly* How mechanical forces affect macromolecular assemblies, including filaments, stress fibers, focal adhesion complexes, membrane rafts, subcellular networks …
Molecular Imaging Applications in Biomechanics

- To quantify changes in gene expression in living cells
- To visualize the dynamics of proteins and protein complexes in living cells
- Molecular imaging in vivo (in animals)
Molecular Imaging Probes

- Probes for live cell imaging of proteins
- Probes for live cell imaging of RNA
- In vivo imaging probes
Live-cell Imaging Methods

Optical microscopy is one of the most widely used imaging methods in biomedical research due to its molecular specificity and fast image acquisition. Optical microscopy methods include Bright field, Dark field, Phase contrast, Differential interference contrast (DIC), Fluorescence, Confocal laser scanning, and Deconvolution microscopy.

However, the spatial resolution of conventional optical microscopy, classically limited by the diffraction of light to ~250 nm, is substantially larger than typical molecular length scales in cells.

Advanced optical microscope technologies, including STED-4pi, stochastic optical reconstruction microscopy (STORM), photo-activated localization microscopy (PALM), and structured illumination (SI) have been developed recently to overcome the diffraction limit.
How to ‘See’ Proteins in Living Cells

- Protein tagging using fluorescent proteins, FLAsH or ReAsH, SNAP, HaLo, etc
  - add a tag (recombinant protein or peptide) to the target proteins
  - high specificity
  - altered gene expression (and thus biology)
  - large size of the label (thus interference)

- Direct labeling using organic fluorophores or quantum dots
  - best for labeling cell surface proteins or biomembranes
  - lack of specificity in labeling intracellular proteins

- No existing method for specific labeling of unmodified proteins in living cells
Fluorescent Proteins

- GFP isolated from the jellyfish; DsRed (RFP) from coral
- β-barrel with buried fluorophore
- Different colored FPs were generated using mutagenesis (Roger Tsien’s fruit yard)
- Proteins expressed in *E.coli* and purified using chromatography

GFP PDB 1EMA

2004 palette of nonoligomerizing fluorescent proteins. Nathan Shaner, Lei Wang, Paul Steinback

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SNAP tag and Tetra-cysteine Tag

**SNAP tag**

**Benefits:**
1. Stable covalent labeling
2. One tag, multiple substrates, flexible color choice
3. Higher signal intensity
4. Better photostability

**ReAsH-EDT2 tag**

ReAsH Labeling Molecule (Non-Fluorescent)  ReAsH – TC complex (Fluorescent)
Simultaneous labeling and imaging of two receptors in HEK cells. HEK cells coexpressing a LAP-LDLR fusion and either AP-EGFR (a) or AP-EphA3 (b) were labeled by first treating with LplA, biotin ligase, azide 7 and biotin, followed by cyclooctyne-Cy3 to derivatize the azide, followed by monovalent streptavidin-Alexa Fluor 488 to detect the biotin.
Semiconductor Quantum Dots

- CdSe/ZnS and other core-shell nanocrystals
- Up to 1000x brighter than organic fluorophores
- Resistant to photobleaching
- Chemically robust
- Broad, overlapping absorption
- Narrow, tunable emission

Fluorophores for Intracellular Protein Tagging

- **Organic fluorophores**
  - Pros: small, many choices, easy to color-multiplex
  - Cons: photobleaching, variable signal level, hydrophobicity

- **Fluorescence Proteins**
  - Pros: Highly specific, easy to color-multiplex, versatile
  - Cons: photobleaching, low signal level, possible interference

- **Quantum Dots**
  - Pros: high signal intensity and photostability, easy to color multiplex, can excite with a single wavelength
  - Cons: large size (~20 nm), interference, low signal-to-noise ratio
Fluorescent Probes for Live-cell RNA Detection
Cellular Delivery of Probes

SLO

CPP

Molecular Beacon

peptide

thiol group

maleimide

carbon linker

SLO + NLS

NLS-peptide

cysteine

maleimide

carbon linker

Molecular Beacon

reversible membrane permeabilization

detecting nuclear RNA

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How to Design a Molecular Beacon

Survivin cDNA: total 429

- tgggtgcctcc gacgttgccc cctgcttgac ggcctttct caaggaccac gcacatctca
cattcagaa ctggcccttc ttggagggct gcgcctgcac cccggagcgg atggccgagg
cctggcttcct ccactgcccc actgagaacg agccagactt ggcccaggtt ttctcttgct
tcaaggacgct gagaaggctgg
gagccagatg acgaccccat agaggaacat aaaaagcatt cgctcgggta cgctttctt
tctgtcaaga agcagtttga agaattaacc ctgggtgaat tttgaaact ggacagagaa
tagcaagaaga acaaaaattgc aaaggaacc aacaataaga agaaagaatt tgaggaaact
gcgaagaaag tgcgccgtgc
catcgagcag ctggctgcca tggattga

- Select sequences of 15-20 bases that are unique to the target gene
- Add stem sequences and determine the melting temperature
- Perform solution studies to determine signal-to-noise ratio
- Perform cellular studies to check the target accessibility
Different mRNA localization in Living Cells

Survivin mRNA in MiapaCa-2  \(\beta_1\)-integrin mRNA in MG63  AR mRNA in LNCaP
In designing molecular beacons it is necessary to avoid targeting sequences that are double-stranded, or occupied by RNA binding proteins. It is often necessary to select multiple (3-12) unique sequences along the target mRNA, and have corresponding molecular beacons synthesized, tested and validated.

Rhee et al, NAR, 2008
A New Model of Cancer Development

Tumour cells are heterogeneous, but most cells can proliferate extensively and form new tumours.

Tumour cells are heterogeneous and only the cancer stem cell subset (CSC; yellow) has the ability to proliferate extensively and form new tumours.

Need to isolate cancer stem cells from tumor and analyze them in order to develop new drug molecules.

Detection & Isolation of Cancer Stem Cells

- Typically only 1-10 cancer stem cells per 1,000,000 cancer cells. To have very sensitive detection, we need to target multiple cancer stem cell markers.
- Stem cell surface markers (e.g. SSEA-1) can be used to detect stem cells but they are very limited.
- Need to keep cells alive and minimize perturbation to cell biology.
- Many genes are highly expressed in stem cells, including Oct3/4, Sox2 and Nanog. Therefore, targeting mRNAs of these genes is very attractive.
- We target both mRNA and protein markers and use FACS to process cells.
Targeting Oct 4 mRNA in Cancer Stem Cells Using Molecular Beacons

PCR results of Oct4 mRNA expression 4 days after retinoic acid treatment in mouse P19 embryonal carcinoma cells

Immunocytochemistry of Oct-4 protein (Red) before (UD) and after (RA) differentiation

WJ Rhee & G Bao, In press
Detection of Oct-4 mRNA and SSEA-1 Protein in Live P19 Stem Cells

Flow Cytometry Assay

no probe with probes

Oct-4 MB

Random MB

UD

Differentiated UD

Mixed population

SSEA-1

Oct-4 mRNA

Sig Oct4 mRNA (red) and SSEA-1 protein (green)
Safety of Using Molecular Beacons

Probes (molecular beacon) should not affect and disturb stem cell physiology for the further use of the cells after detection and isolation.

In normal embryonal carcinomas, Oct-4 mRNA decreases and IGF-2 increased after differentiation (RA treatment).
QD-FP FRET Probes
Applications of QD-FP FRET Probes

- Sensitive enzymatic activity assay using cleavable linkers
- Utilize changes in FP optical properties in response to environment
  - **pH sensitivity** (mOrange; YFP; pHluorins)
  - Chloride sensitivity (YFP variants)
  - Copper sensitivity (DsRed; hcRed)
QD-FP FRET Pairs

- **QD donors:**
  Carboxyl-functionalized EviTags, QD520, QD540, QD560

- **Fluorescent protein acceptors:**
  mCherry, mOrange, tdTomato

<table>
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<th>acceptor</th>
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<th>peak emission (nm)</th>
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<tr>
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<td>548</td>
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<tr>
<td>mCherry</td>
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FRET results using FPs mOrange and tdTomato as acceptors and QD as donors. A, C, and E: Spectra of QD520 + His6-mOrange, QD520 + His6-tdTomato, and QD540 + His6-tdTomato, respectively. N is the average number of fluorescent proteins per QD. B, D, and F: Relative area under the QD emission peak of background-subtracted spectra. The non-his-tagged FP control is represented as a circle (●), the His10-only (no fluorescent protein) control is a diamond (♦), and the His6-FP is represented by a square (■).
Why Nanoparticles for In Vivo Imaging

- Nano size helps better penetrating through the leak vessels in tumor
- Targeting specific cells (such as cancer cells) and organs effectively
- The ability to tailor pharmacokinetics and biodistribution
- Increased S/N and combining imaging with targeted therapy