Single cell molecular profiling using Quantum Dots

Technical Journal Club
01.10.2013
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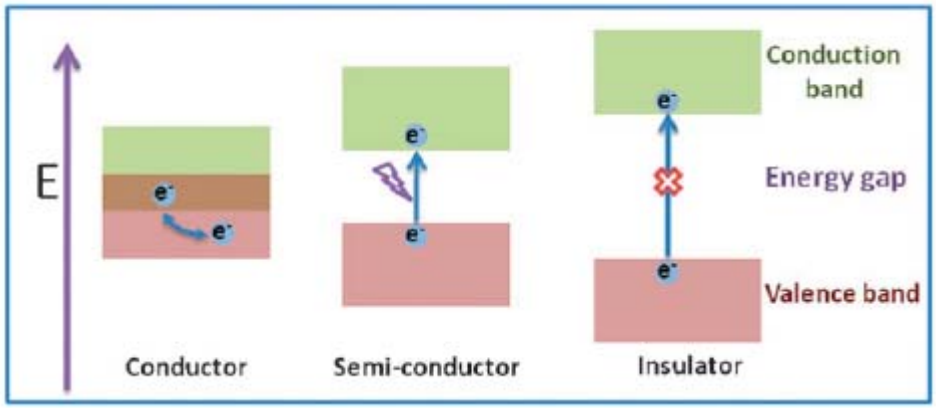
Molecular Profiling

- Powerful technique to study complex molecular networks underlying physiological and pathological processes
- Useful in biomedical research, clinical diagnostics and targeted therapy
- Fluorescence microscopy widely used for evaluation of phenotypes of healthy cells as well as for detection of molecular signatures of diseases
- Organic fluorophores widely used in these applications, highlighting cell structures or for specific labeling biomarkers
- Use of organic fluorophores for molecular profiling is limited by the quick photobleaching, spectral overlap between probes, and the need to excite fluorophores at unique wavelengths
- Other tools for molecular imaging → visualization, characterization and quantification of biological processs at the molecular level ????

Quantum Dots

- Quantum Dots are nanocrystals made of semiconductor materials
- Nanocrystals are nanoparticals with a crystalline structure
- A semiconductor is a material which has electrical conductivity to a degree between that of a metal and that of an insulator → movement of electrons inside a crystalline structure of atoms
- In certain semiconductors, excited electrons can relax by emitting light instead of producing heat → these semiconductors are used in the construction of fluorescent quantum dots (QDs)
- QD probes have become an essential tool for quantitative multiplexed studies → enabling simultaneous detection and analysis of multiple targets within a single specimen
- QD photostability is essential for robust image acquisition and accurate quantitative analysis of staining intensity
- Multiplexed quantitative analysis of cellular phenotypes, real-time monitoring of intracellular processes, and in vivo molecular imaging are possible using QDs

Quantum Dots - Functionality



Zrazhevskiy P et al., Chem. Soc. Rev., 2009

Valence band:

- Ground state
- Electrons are localized to individual atoms

Conduction band

- Excited state
- Electrons move freely throuought the material

Band gap

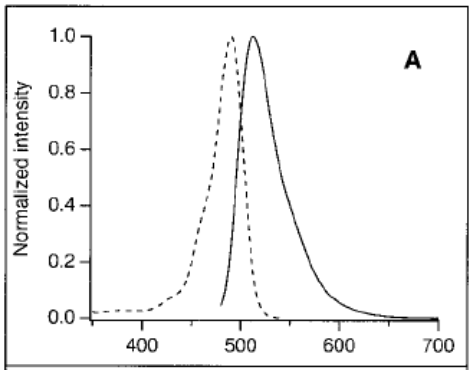
- Energy range in a solid where no electron states can exist
- Energy difference between the top of the valence band and the bottom of the conduction band
- Equivalent to the energy required to free an electron from its orbit to become a mobile charge carrier, able to move freely within the solid material
- So the band gap is a major factor determining the electrical conductivity of a solid

- Electrons can be promoted to higher energy levels by supplying an amount of energy (e.g. a photon) that exceeds the band gap
- One way for the system to relax is to emit a photon, thus losing its energy (fluorescence)
- So fluorescence is the re-emission of longer wavelength photons (lower frequency or energy) by a molecule that has absorbed photons of shorter wavelengths (higher frequency or energy)

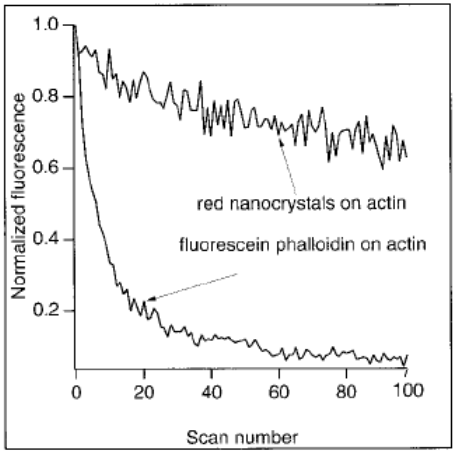
Organic Fluoresent Probes

- Narrow excitation spectrum
  → simultaneous excitation difficult
- Broad emission spectrum
  → long tail at red wavelengths

Fluorescein



- Photobleaching

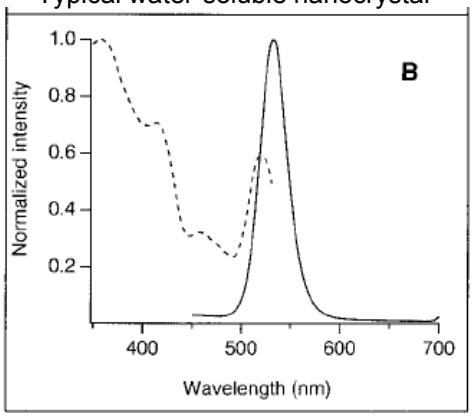


Bruchez M. Jr. et al., Science, 1998

Quantum Dots

- Broad, continuous excitation spectrum
  → simultaneous excitation possible
- Narrower emission
  → no red tail

Typical water-soluble nanocrystal



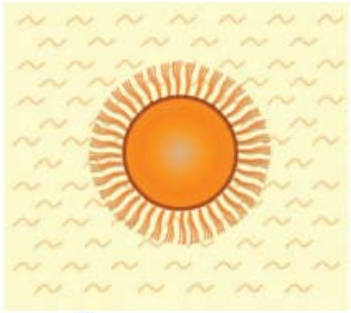
- Photochemical stable
- Excitation and emission are tunable

Engineering of QD probes

- QDs are semiconductor nanoparticles often made from hundreds to thousands of atoms of group II and VI elements (e.g. CdSe and CdTe) or group III and V elements (e.g. InP and InAs)
- QDs are crystalline particles that range from 2 to 10 nanometers in diameter
- Preparation of QD-based probes represents a multi-step process → each step aimed at controlling optical, physical and chemical properties of the final probe

1. Inorganic nanoparticle core → manipulation of the core chemical composition, size, and structure controls the photo-physical properties of the probe

1.



Design of QD core

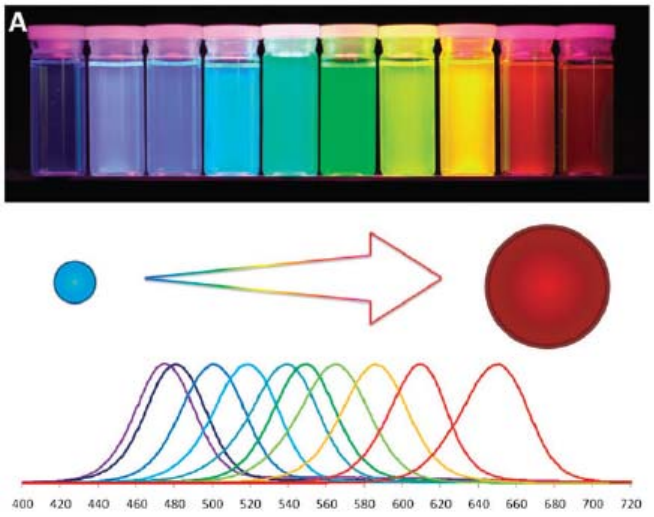
Size / distribution
Geometry
Chemical composition
Surface architecture

⇩

Optimization of photo-physical properties

Zrazhevskiy P et al., Chem. Soc. Rev., 2009

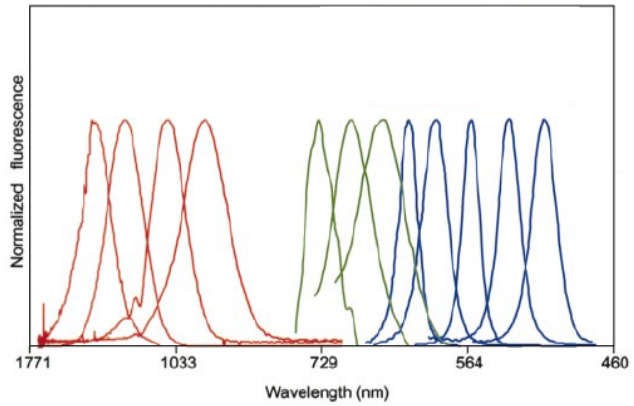
Design of the QD core



Zrazhevskiy P et al., Chem. Soc. Rev., 2009

Nanocrystal probes in aqueous buffer, all illuminated simultaneously with a handheld ultraviolet lamp

- The QD core defines optical properties of the probe
- The color of emitted light can be fine-tuned by adjusting the QD size
- QDs emitting light from the UV, throughout the visible, and into the infrared spectra (400–4000 nm)
- Little or no cross-talk between adjacent colors enables simultaneous detection and quantification of multiple fluorescence signals



Bruchez M. Jr. et al., Science, 1998

- Blue series represents different sizes of CdSe nanocrystals with diameters of 2.1, 2.4, 3.1, 3.6, and 4.6 nm
- Green series is of InP nanocrystals with diameters of 3.0, 3.5, and 4.6 nm
- Red series is of InAs nanocrystals with diameters of 2.8, 3.6, 4.6, and 6.0 nm.

Engineering of QD probes

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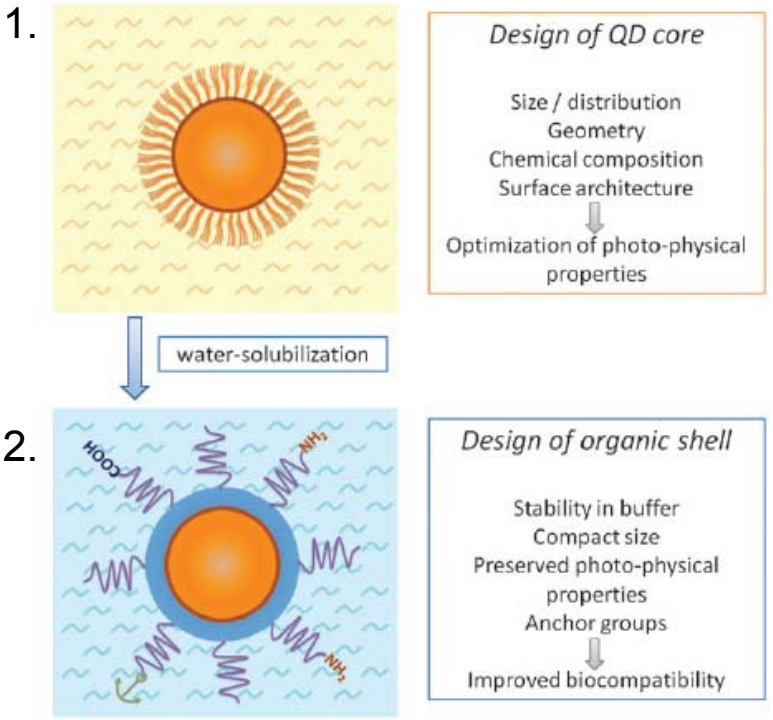
- QDs are crystalline particles that range from 2 to 10 nanometers in diameter
- Preparation of QD-based probes represents a multi-step process → each step aimed at controlling optical, physical and chemical properties of the final probe

1. Inorganic nanoparticle core → manipulation of the core chemical composition, size, and structure controls the photo-physical properties of the probe
2. Design of coating materials that can encapsulate the QD core and shield it from the environment → water soluble → biocompatible

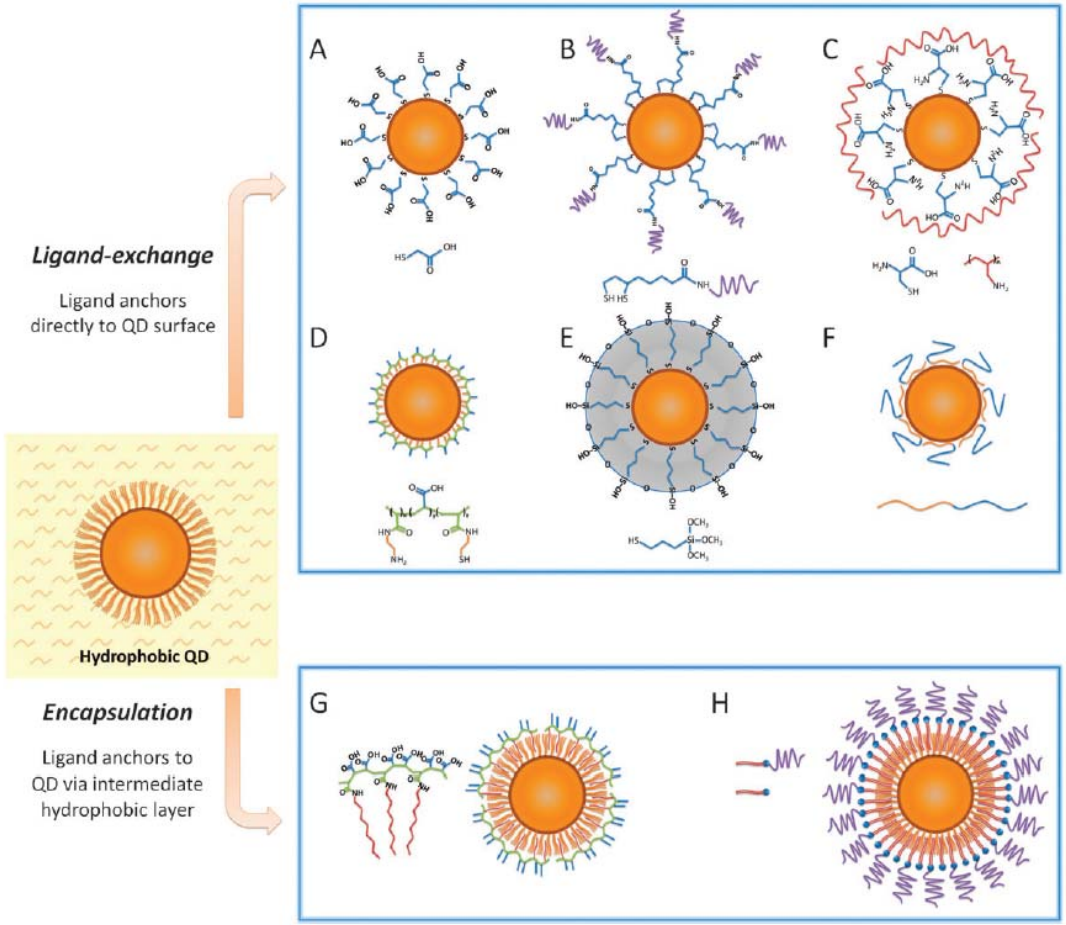


Zrazhevskiy P et al., Chem. Soc. Rev., 2009

Design of an organic shell

- Synthesized QDs are hydrophobic and soluble only in nonpolar organic solvents, such as chloroform and hexane
- To be useful for biological applications QDs must be made water-soluble
- QDs should be soluble and stable in biological buffers, preserve the original photo-physical properties, retain relatively small particle size, and provide reactive groups for subsequent conjugation to biomolecules
- Methods used are:
  - Ligand-exchange
  - Encapsulation

Design of an organic shell



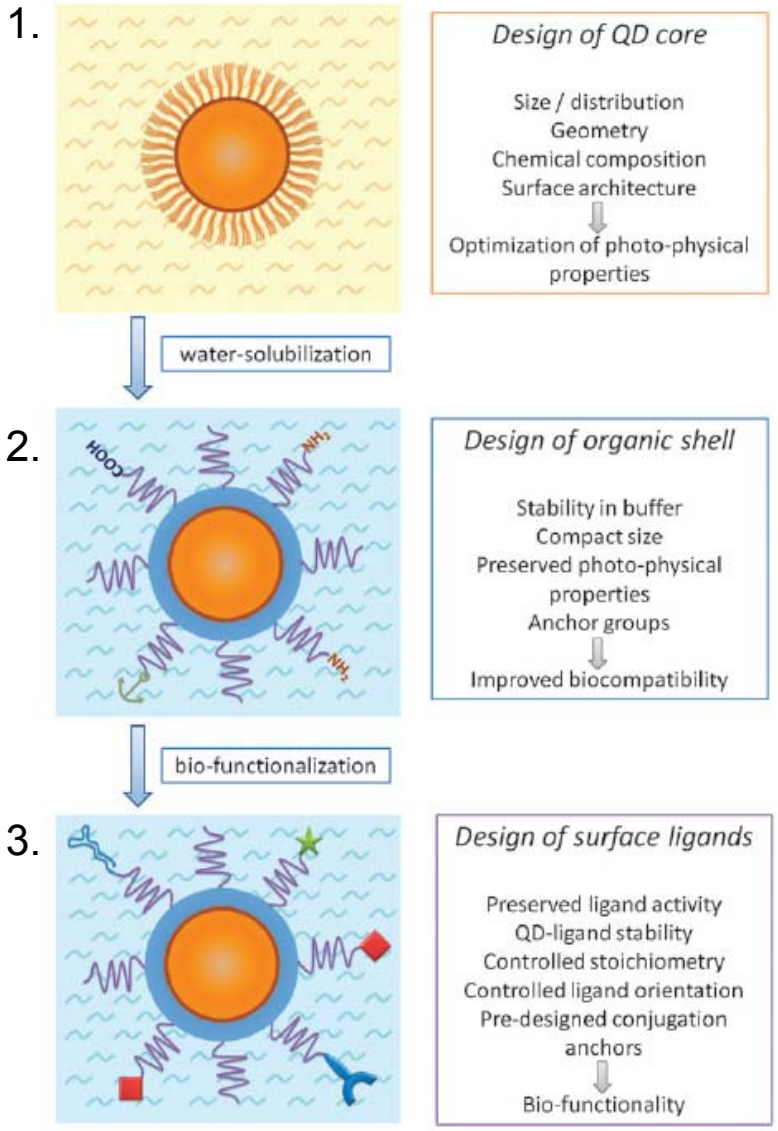
Zrazhevskiy P et al., Chem. Soc. Rev., 2009

- **Ligand exchange**: replacing hydrophobic surface ligands with hydrophilic ones by direct anchoring of ligands to the QD surface
- **Encapsulation**: encapsulate the hydrophobic QDs with amphiphilic molecules such as polymers (G) or phospholipids (H)
- Ligand-exchange approaches often yield compact probes at an expense of reduced stability and fluorescence efficiency
- Encapsulation produces exceptionally stable and bright particles at an expense of increased size

Engineering of QD probes

- QDs are semiconductor nanoparticles often made from hundreds to thousands of atoms of group II and VI elements (e.g. CdSe and CdTe) or group III and V elements (e.g. InP and InAs)
- QDs are crystalline particles that range from 2 to 10 nanometers in diameter
- Preparation of QD-based probes represents a multi-step process → each step aimed at controlling optical, physical and chemical properties of the final probe

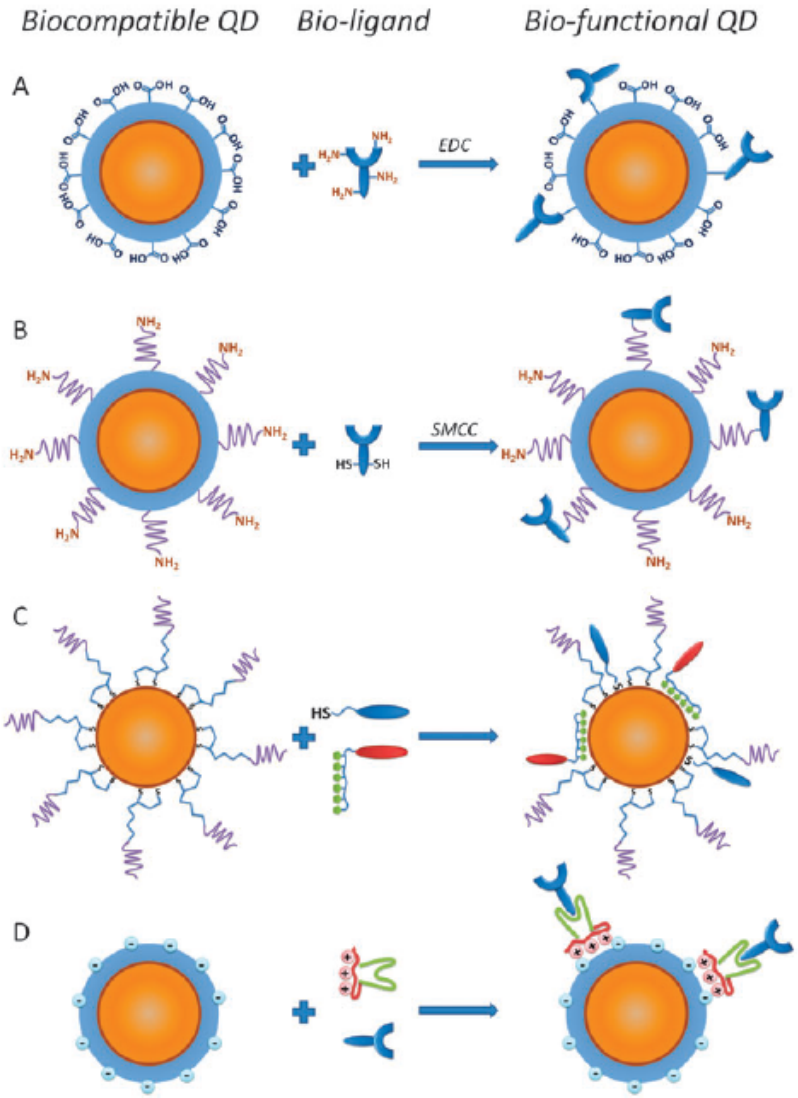
1. Inorganic nanoparticle core → manipulation of the core chemical composition, size, and structure controls the photo-physical properties of the probe
2. Design of coating materials that can encapsulate the QD core and shield it from the environment → water soluble → biocompatible
3. Further decoration of the QDs with biomolecules give biofunctionality and enables probe interaction with biological systems



Zrazhevskiy P et al., Chem. Soc. Rev., 2009

Design of surface ligands

- Bio-functionality has to be added to otherwise inert nanoparticles
- Usually achieved by decorating QDs with proteins, peptides, nucleic acids, or other biomolecules that mediate specific interactions with living systems
- Decoration of QD surface with bio-ligands can be achieved via covalent conjugation (A, B), non-covalent coordination of thiol groups or polyhistidine tags with the QD surface metal atoms (C), or electrostatic deposition of charged molecules on the QD organic shell (D).



Zrazhevskiy P et al., Chem. Soc. Rev., 2009

Semiconductor Nanocrystals as Fluorescent Biological Labels

Marcel Bruchez Jr., Mario Moronne, Peter Gin, Shimon Weiss,*
A. Paul Alivisatos*

Science
AAAS
25 SEPTEMBER 1998

- Biological applications require water-soluble nanocrystals
- First time it was demonstrated that semiconductor nanoparticles could be made water-soluble and used as biological imaging probes
- Extended the chemistry of the core-shell systems by adding a third layer of silica that makes the core-shell water soluble
- The core-shell nanocrystals prepared in this manner are soluble and stable in water or buffered solution

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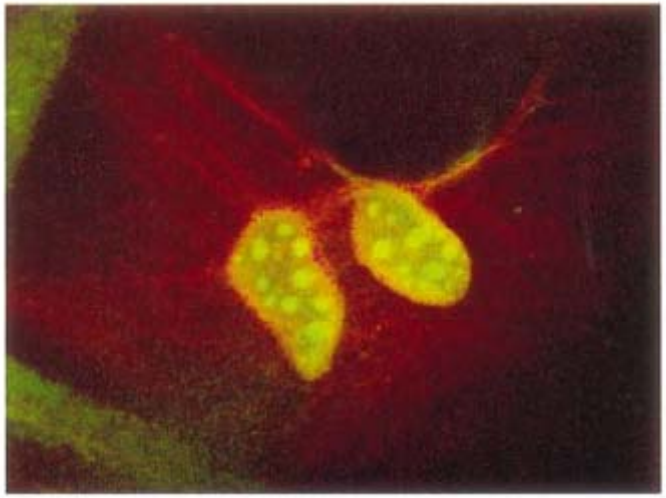

- Fluorescently labeled 3T3 mouse fibroblast cells using two different size CdSe-CdS core-shell nanocrystals enclosed in a silica shell
- The smaller nanocrystals (2-nm core) emitted green fluorescence the larger (4-nm core) red fluorescence
- Nanocrystals coated with trimethoxysilylpropyl urea and acetate groups were found to bind with high affinity in the cell nucleus → property was used to “stain” the nucleus with the green-colored nanocrystals
- Avidin-biotin interaction, a model system for ligand-receptor binding, was used here to specifically label the F-actin filaments with red nanocrystal probes
- Biotin was covalently bound to the nanocrystal surface → fibroblasts had been incubated in phalloidin-biotin and streptavidin
- Samples were imaged with both conventional wide-field and laser-scanning confocal fluorescence microscopes
- Green and red labels were clearly spectrally resolved to the eye and to a color Polaroid camera
- Nonspecific labeling of the nuclear membrane by both the red and the green probes resulted in a yellow color
- Red actin filaments, however, were specifically stained

nature COMMUNICATIONS

ARTICLE

Received 20 Nov 2012 | Accepted 21 Feb 2013 | Published 19 Mar 2013

DOI: 10.1038/ncomms2635 OPEN

Quantum dot imaging platform for single-cell molecular profiling

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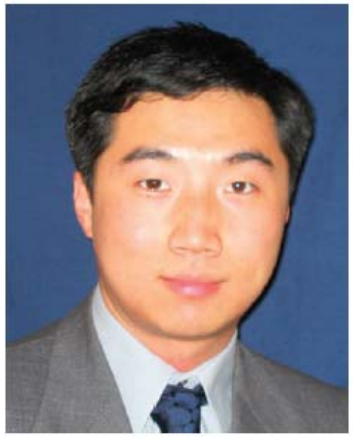

Xiaohu Gao

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Multicolor multicycle molecular profiling with quantum dots for single-cell analysis

Pavel Zrazhevskiy[1], Lawrence D True[2] & Xiaohu Gao[1]

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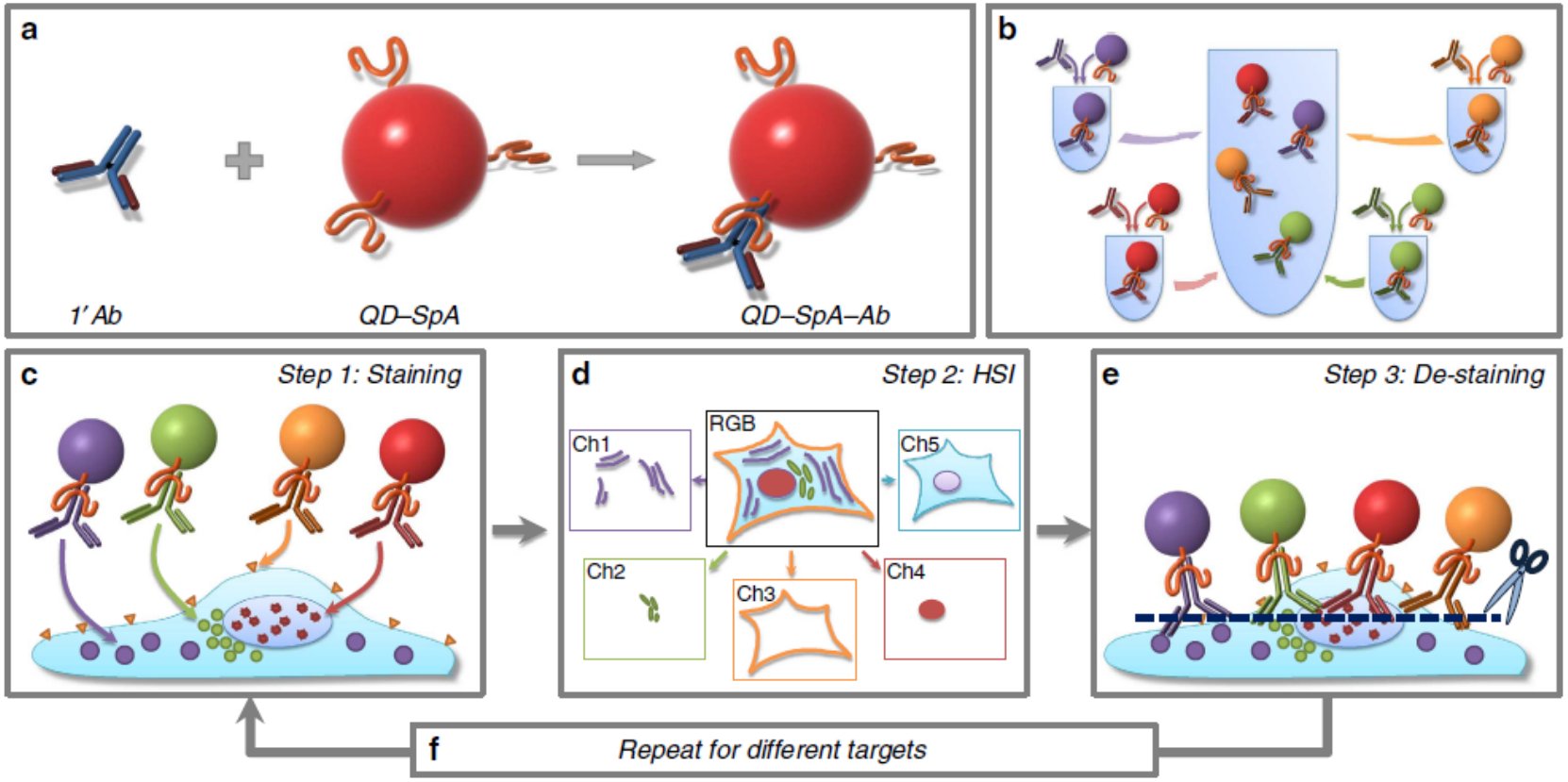
Introduction

- The state of the art is imaging of 5–6 molecular targets with custom-designed QD probes
- Use the extensive multiplexing potential of QDs
- Development of a multicolour multicycle molecular profiling (M3P) technology potentially capable of examining over 100 molecular targets in single cells at subcellular resolution

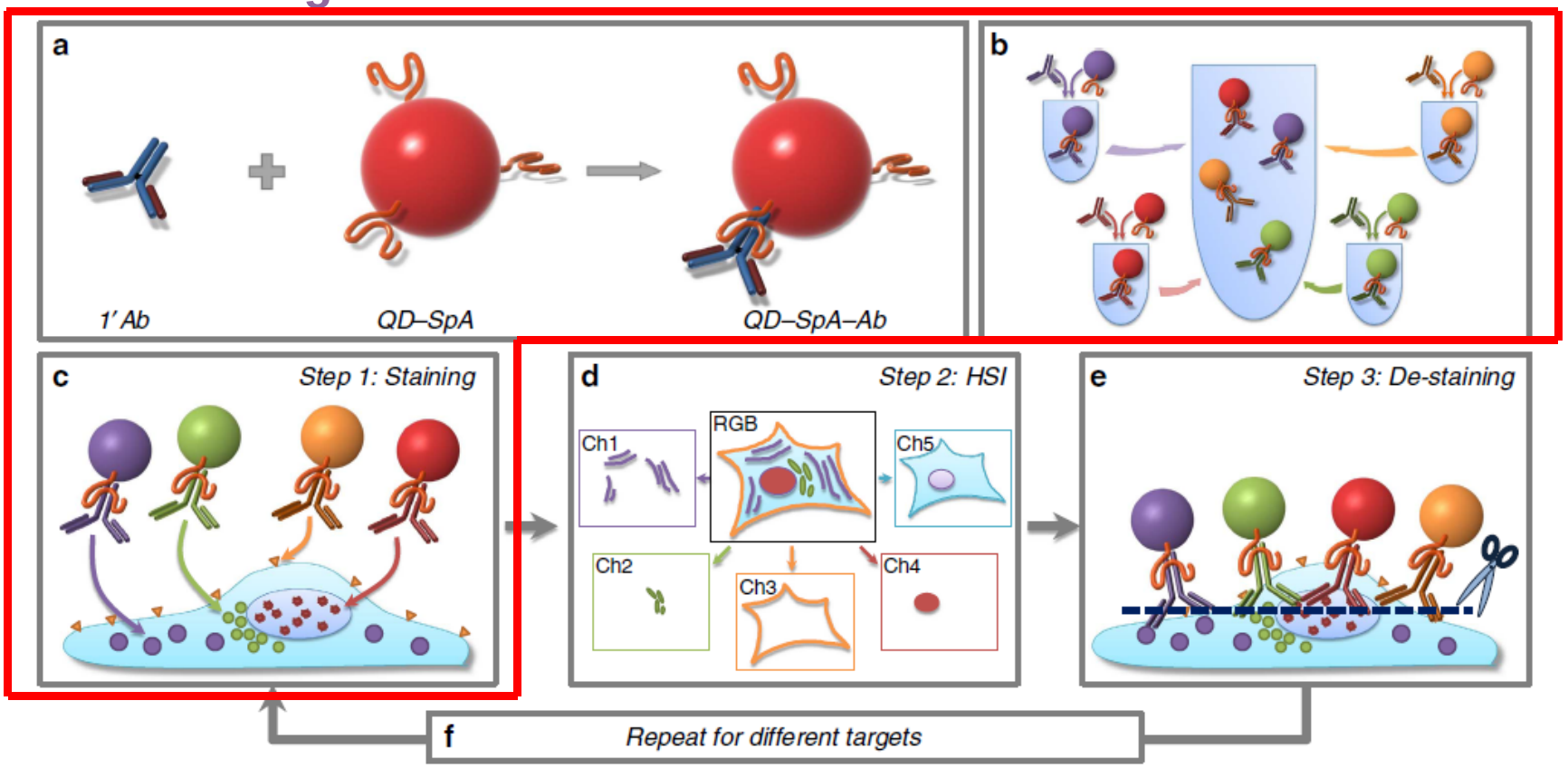
The M3P Technology



The M3P Technology consists of three main steps

The M3P Technology

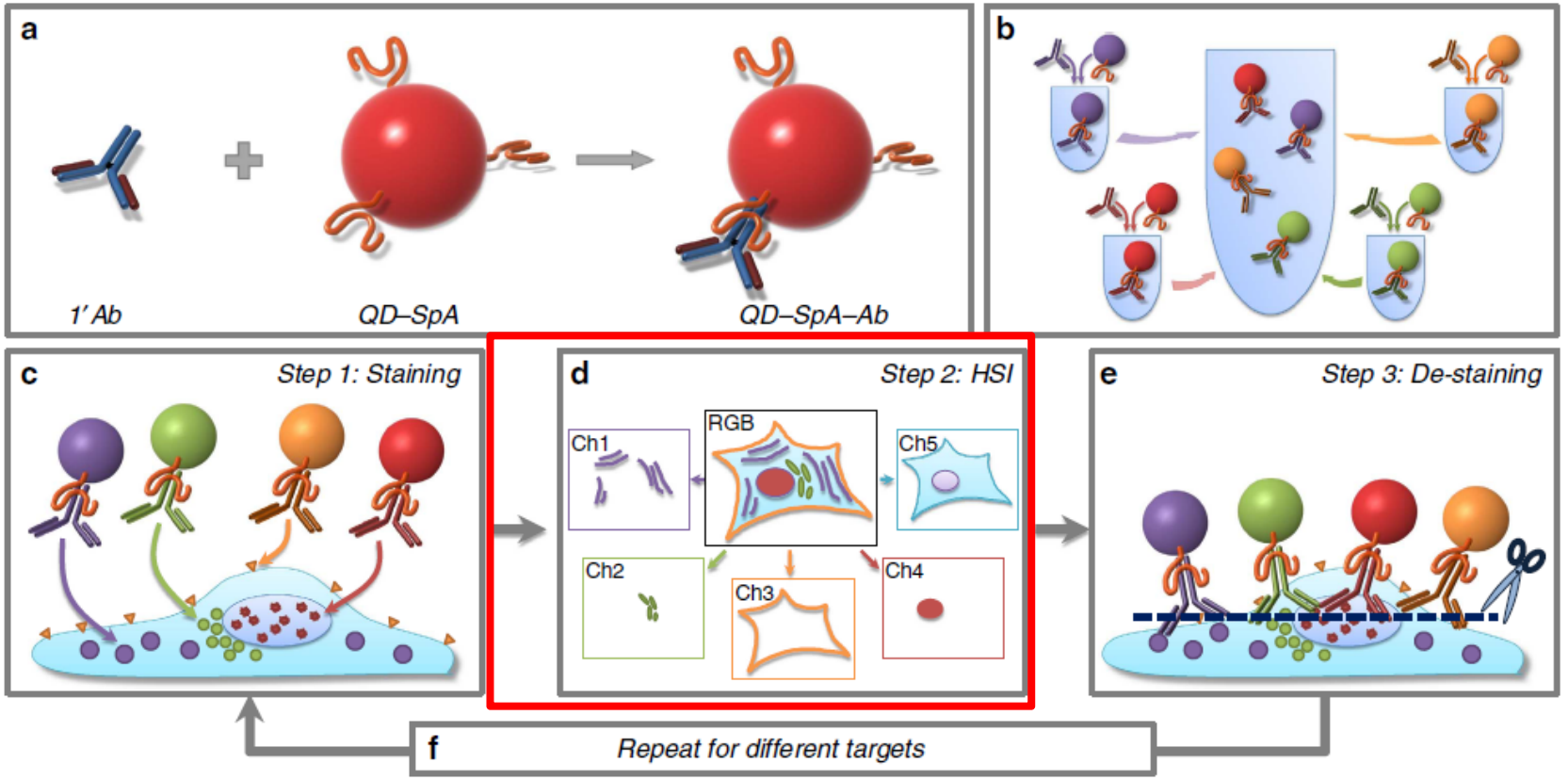
1. Staining



- Universal QD–SpA bioconjugates are used to capture intact Abs in solution during a pre-staining step to form functional QD-SpA-Ab fluorescent probes
- Different probes are pooled in a single cocktail
- Cocktail is incubated with cells for parallel multiplexed staining

The M3P Technology

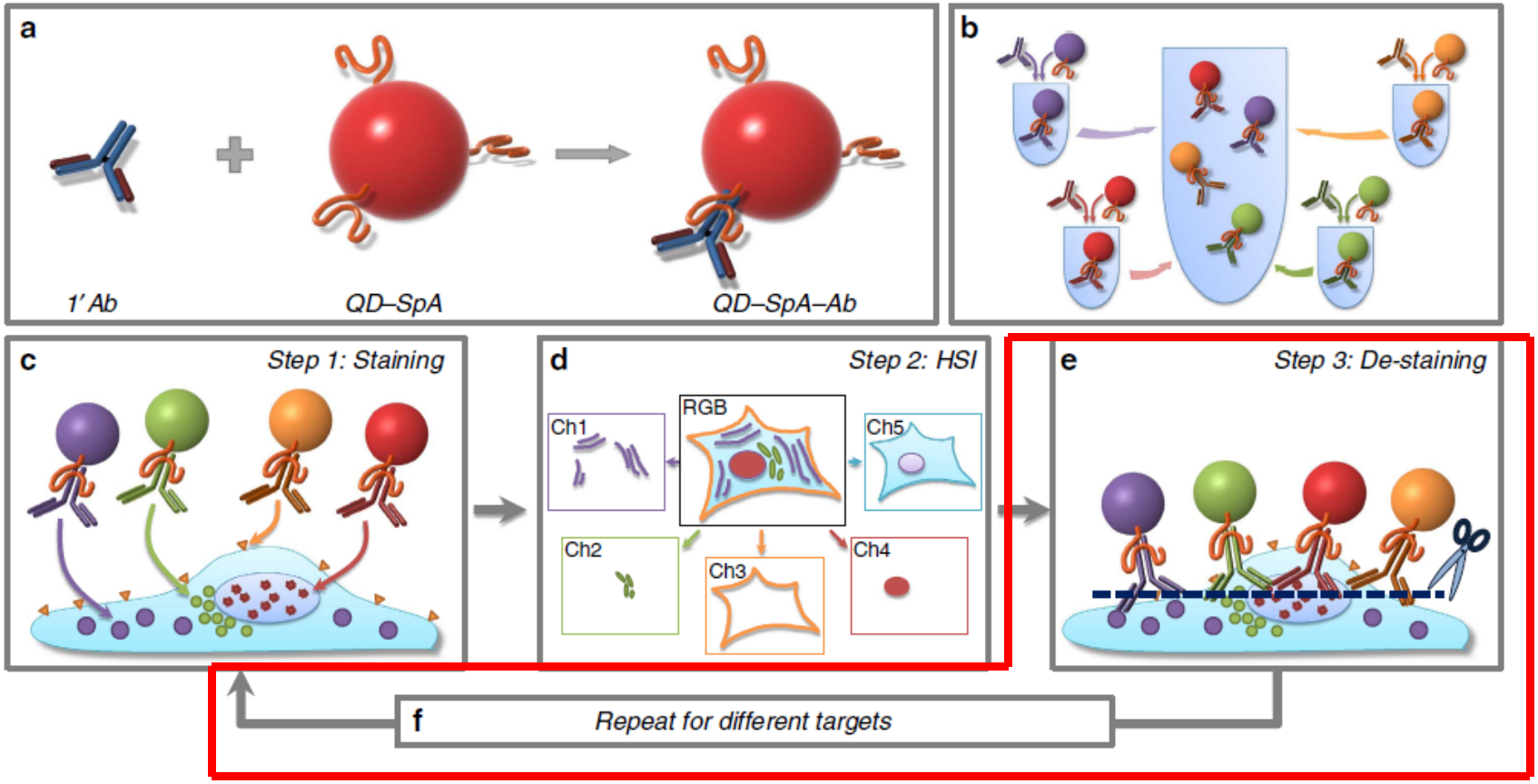
2. Spectral imaging



- Fluorescence microscopy with HSI capability is utilized to acquire and unmix signals from each QD color to generate quantitative antigen expression profiles in separate channels
- **HSI=Hyperspectral imaging** divides the spectrum into many bands. This technique of dividing images into bands can be extended beyond the visible

The M3P Technology

3. De-staining



- Complete de-staining of the specimen is done by brief washing with a regeneration buffer, enabling the next full cycle of IF staining for a different subset of targets

Used QDs

In general

- Ten spectrally distinct QDs with at least 20nm separation between fluorescence maxima are commercially available currently and can be linked to ten Abs
- This multiplexing capability is complemented by the spectral unmixing power of HSI (also ten channels at this time) for separation and quantification of individual colors from multicolor images

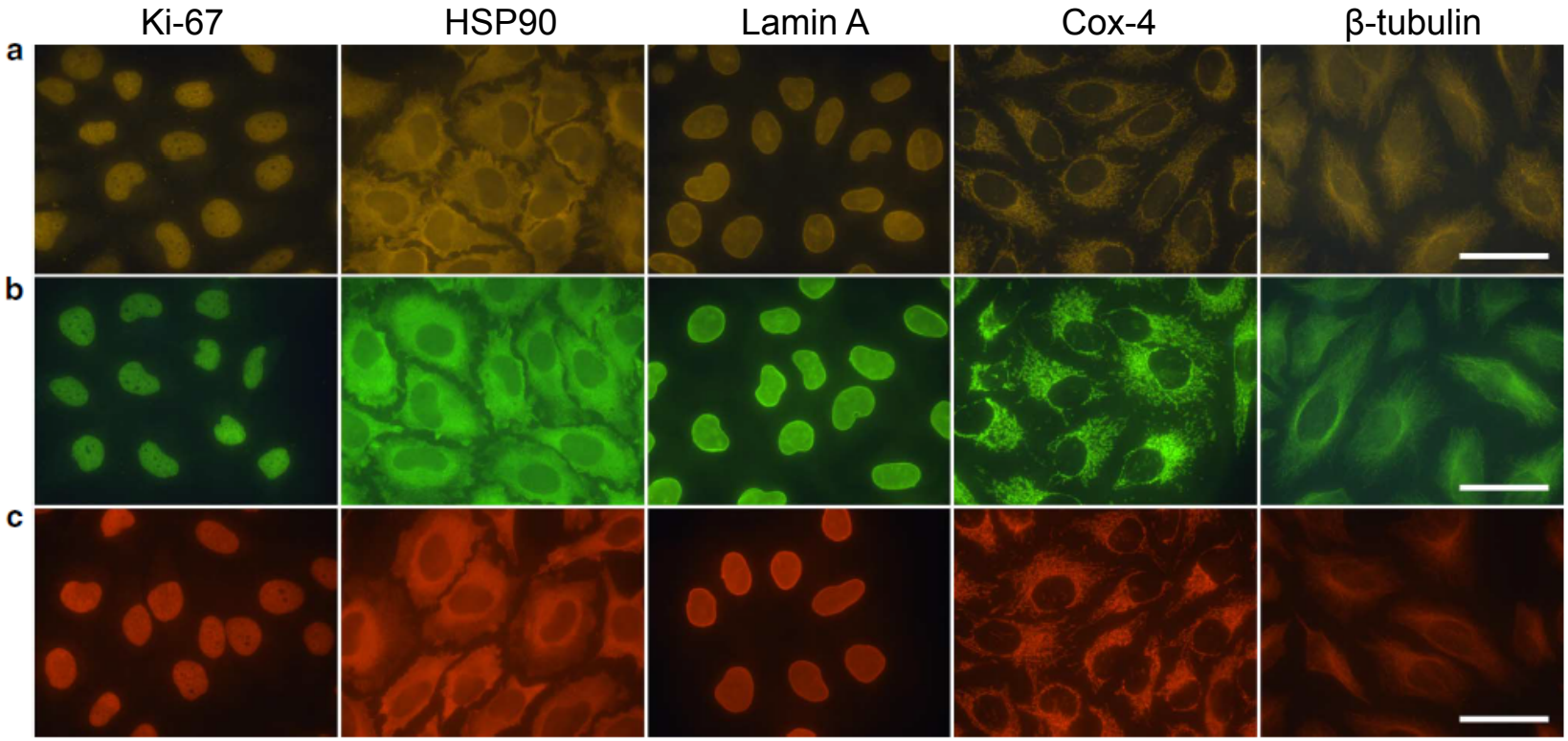
In this study

- As a platform for M3P technology, use of highly stable and bright QDs coated with a non-fouling layer of polyethylene glycol (PEG) → water-soluble
- Protein A from Staphylococcus aureus (SpA) is covalently linked to QDs as an adaptor for orientation-controlled IgG immobilization via binding to the Fc region of IgG
- Engineering a universal QD-protein A (QD-SpA) platform for flexible and fast preparation of a library of functional QD-antibody (QD-SpA-Ab) probes and utilizing such probes in a multicycle staining procedure

- QD-based multicolour multicycle technology heavily depends on several key challenges:
    1. Each QD probe must be uniquely matched to a specific target, exhibiting no crosstalk between different probes within a staining cocktail
    2. QD fluorescence must be completely removed after each imaging cycle to prevent signal carry-over to the next staining cycle
    3. Target antigenicity and specimen integrity must be retained throughout multiple staining/imaging/destaining cycles

QD-Ab probe specificity

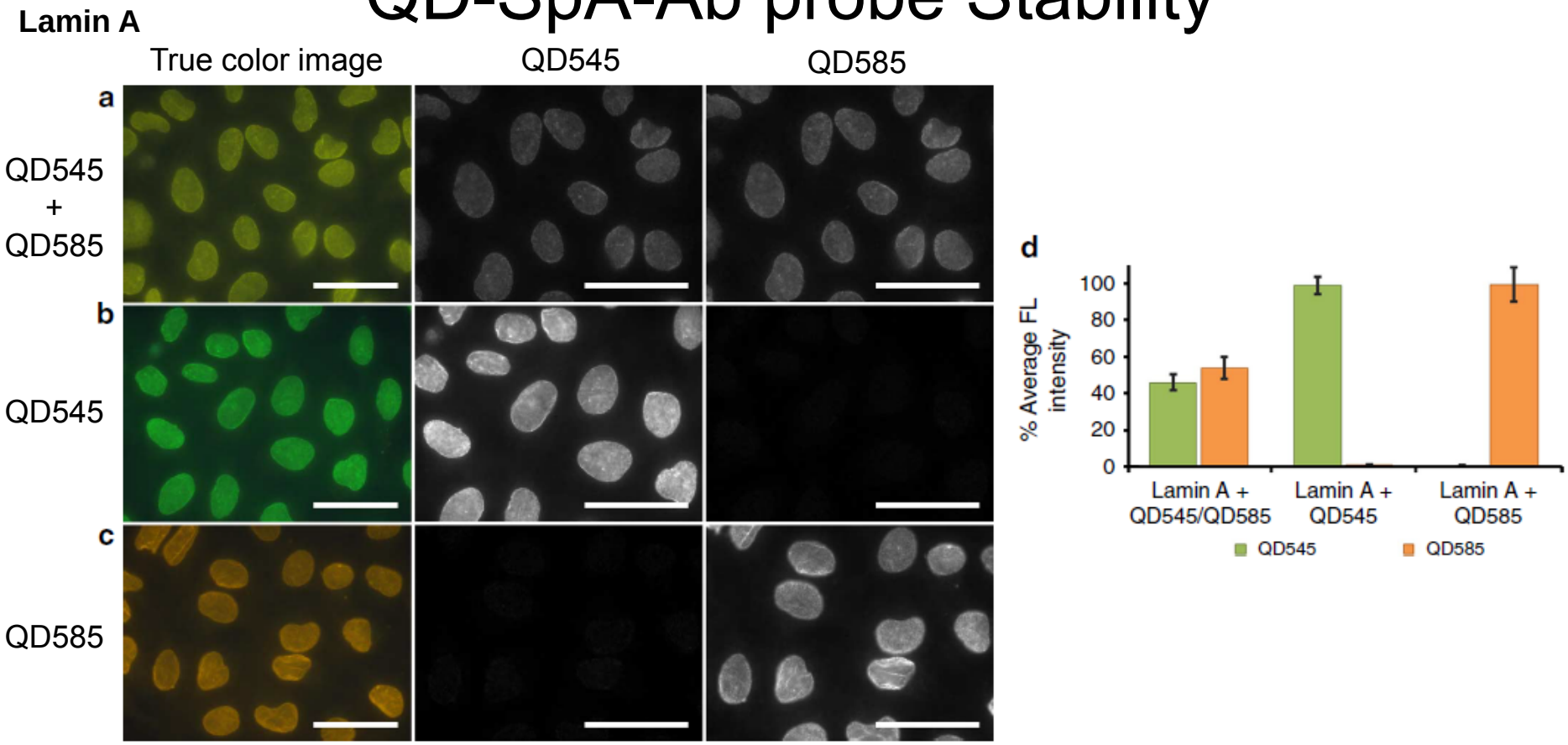
- Staining 5 molecular targets separately in formalin-fixed HeLa cells
- The five model targets used represent a spectrum of cell compartment localizations and expression levels
- Comparing the relative target distribution patterns with those obtained by conventional two-step IF using commercially available QD-labelled 2°Ab and Alexa Fluor 568-labelled 2°Ab



a) QD585-Spa-Ab labelled in a single step procedure
b) QD585 2° Ab labelled in a conventional two-step staining
c) Alexa Fluor 568-labelled 2° Ab in a conventional two-step staining

Staining patterns are consistent throughout all three procedures, indicating preserved specificity and affinity of antibodies in the QD–SpA–Ab complex.

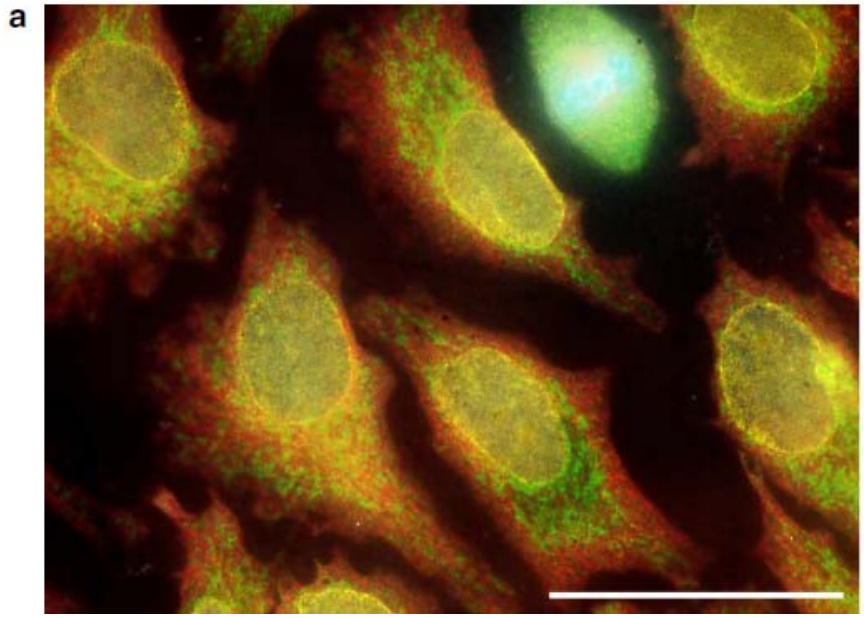
QD-SpA-Ab probe Stability



- QD545-SpA and QD585-SpA probes mixed together with Ab and incubated with cells efficiently captured free Ab from solution and produced mixed-color Lamin A staining with nearly 50% contribution each
- Mixed fully assembled QDSpA- Ab probes with counterpart non-complexed QD-SpA (for example, QD545-SpA-Ab mixed with QD585-SpA, or QD585- SpA-Ab with QD545-SpA), and incubated cells with this solution
- Did not observe any crosstalk or interference with either QD545 or QD585 probes
- HSI used to unmix true-colour images (left panels) into individual QD545 (middle panels) and QD585 (right panels) channels

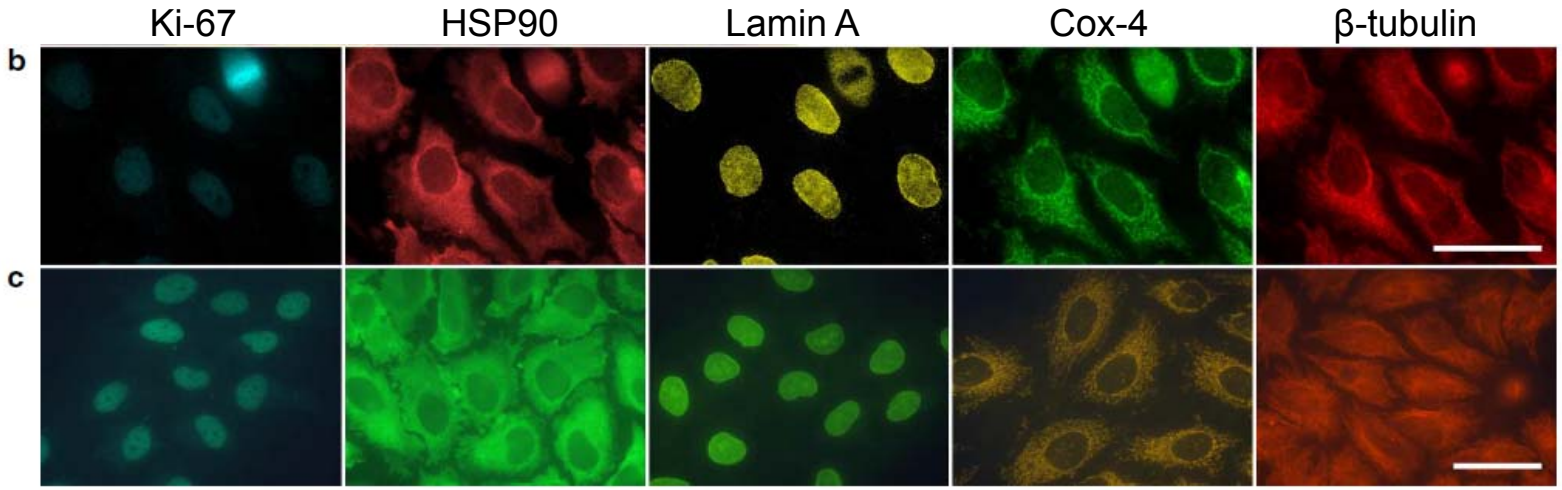
Parallel multiplex staining with five pre-assambled QD-SpA-Ab probes

- Demonstrate the utility of the new probes for multicolour imaging:
  - parallel staining of five model molecular targets is performed
- Ki-67, HSP90, Lamin A, Cox-4 and β-tubulin are simultaneously stained with QD-SpA-Ab probes emitting at 525, 545, 565, 585 and 605 nm, respectively(false color image)



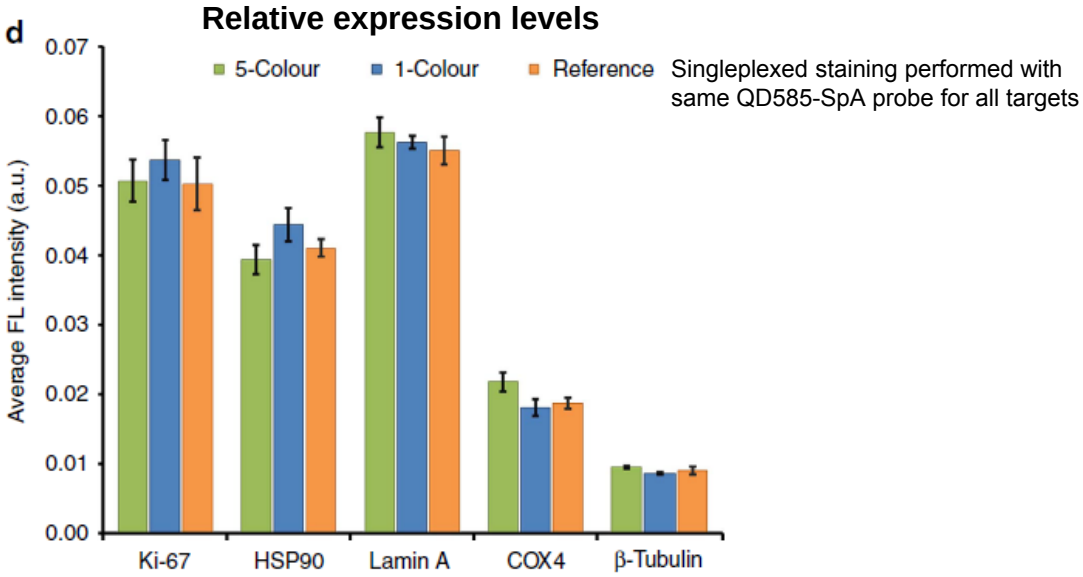
Ki-67	Nucleus	525 nm
HSP90	Cytoplasm	545 nm
Lamin A	Nuclear membrane	565 nm
Cox-4	Mitochondria	585 nm
β-tubulin	Microtubules	605 nm

Parallel multiplex staining with five pre-assambled QD-SpA-Ab probes



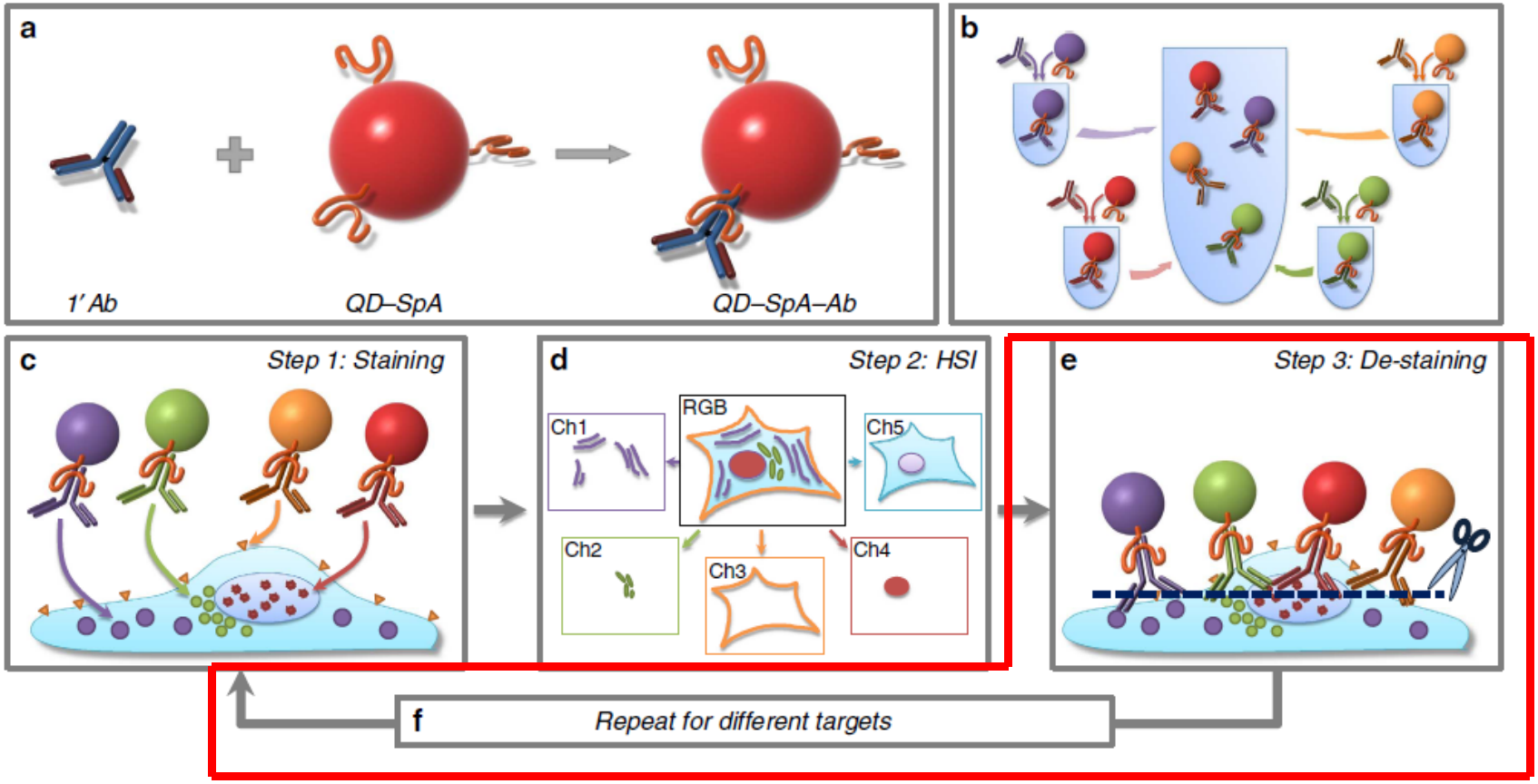
b) HSI and spectral unmixing are used to extract individual QD
c) Single-color staining performed with colour-matched QD-SpA-Ab probes

Quantitative analysis of staining intensity



The M3P Technology

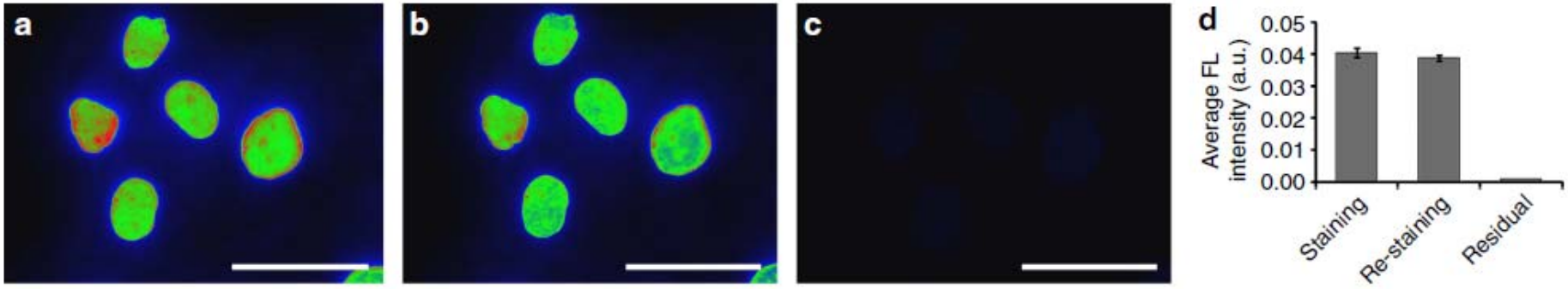
3. De-staining



- Complete de-staining of the specimen is done by brief washing with a regeneration buffer, enabling the next full cycle of IF staining for a different subset of targets

Specimen regeneration and target re-staining with multicycle staining procedure

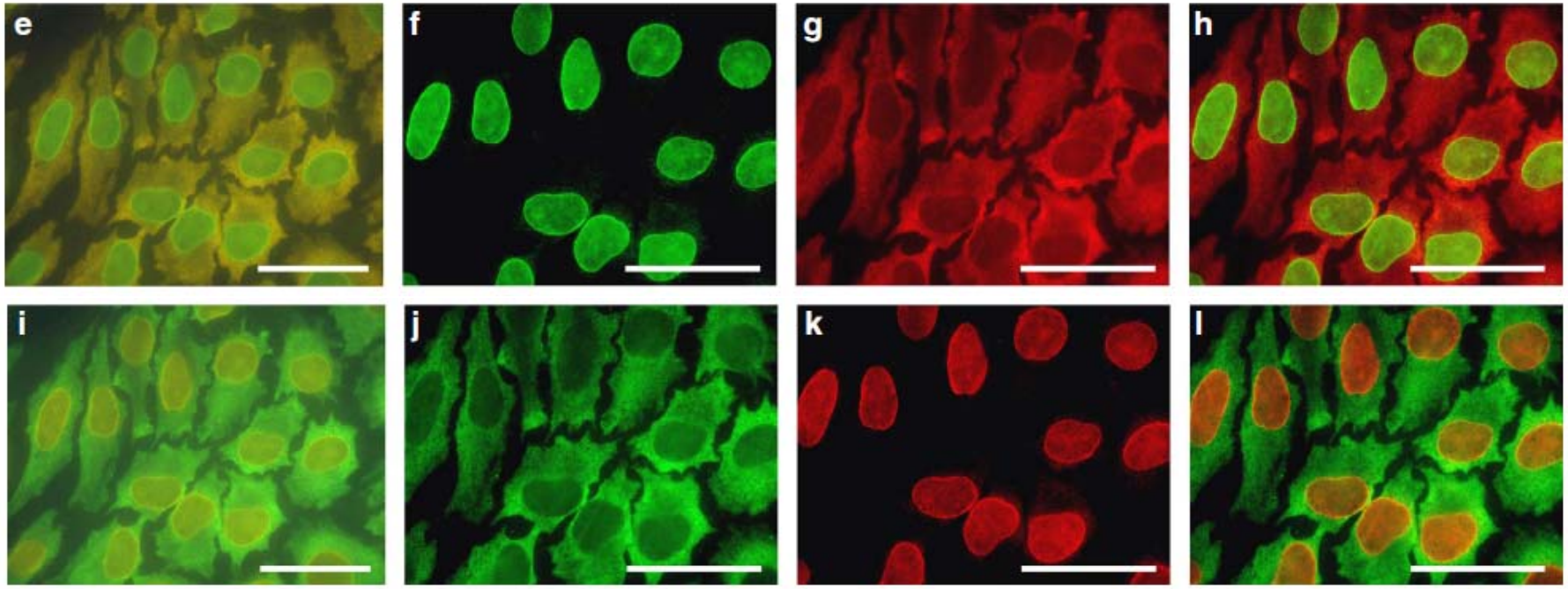
- New QD-SpA-Ab probes, quick and efficient de-staining by brief exposure to low-pH/detergent-based regeneration buffer because



a) Characteristic nuclear envelope staining is obtained with QD545-SpA probes pre-assembled with anti-Lamin A antibody
b) Brief incubation with regeneration buffer removes QD-SpA-Ab probes, achieving specimen restoration to pre-staining condition and enabling nearly complete target re-staining
c) Incubation of regenerated specimen with 'blank' QD545-SpA probes (lacking target-specific Abs) fails to produce any residual staining

→ confirming complete removal of 1°Abs during de-staining

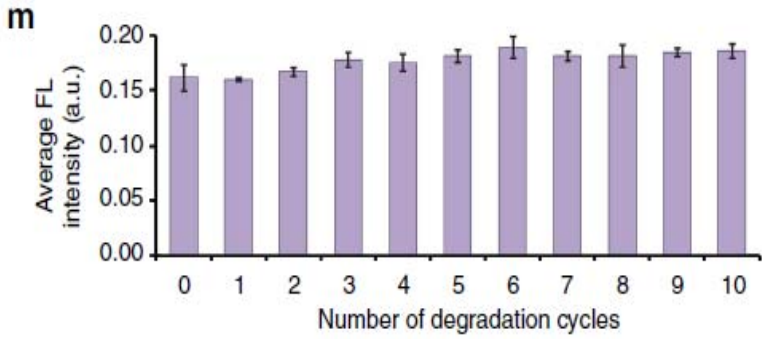
Specimen regeneration and target re-staining with multicycle staining procedure



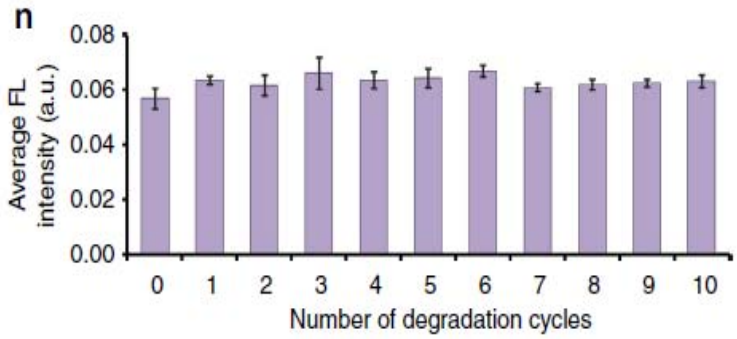
- Similarly, in a dual colour staining of Lamin A and HSP90 with QD545 and QD585 probes complete exchange of fluorescent reporters during the second cycle
- HSI reveals distinct Lamin A staining pattern in QD545 channel (f) and HSP90 pattern in QD585 channel (g)
- Following de-staining, during the second cycle the same targets are stained with the counterpart QD probes (i–l), yielding clear HSP90 pattern in QD545 channel (j) and Lamin A pattern in QD585 channel (k)
- (e) and (i) are true-colour images and (h) and (l) are false-colour composite images.

Specimen regeneration and target re-staining with multicycle staining procedure

Lamin A

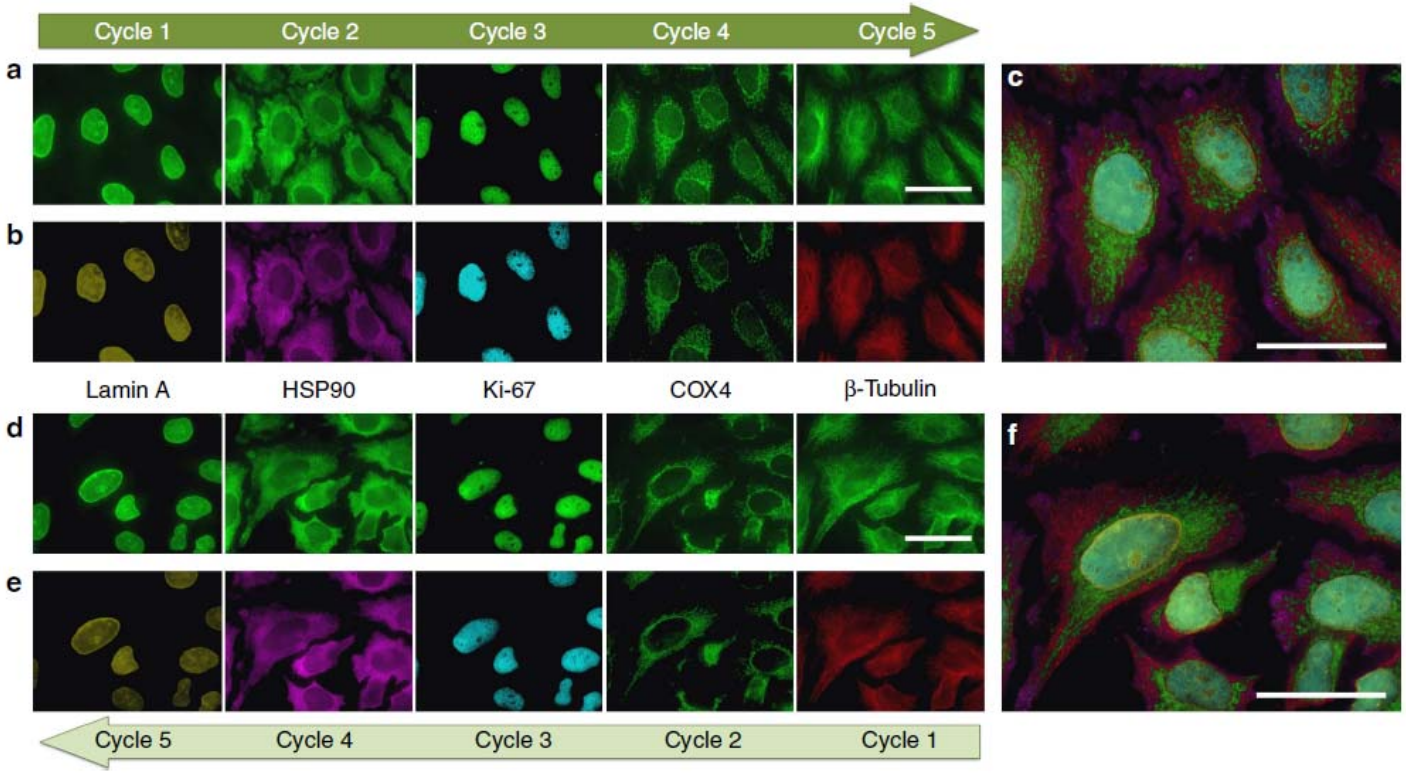


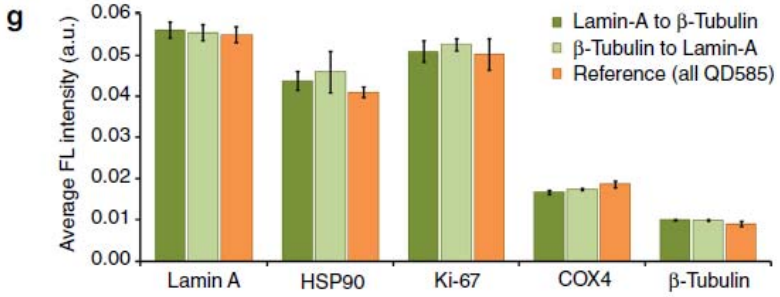
HSP90



- To evaluate effect of repeated regeneration procedure on specimen stability and staining intensity, up to 10 ‘degradation’ cycles are performed on separate specimens before cells are stained for Lamin A (m) or HSP90 (n)
- HSI was used for quantitative analysis of average staining intensities
- Measured average fluorescence staining intensity does not vary significantly regardless of the number of ‘degradation’ cycles performed
- Demonstrating sufficient preservation of specimen morphology and target antigenicity required for staining

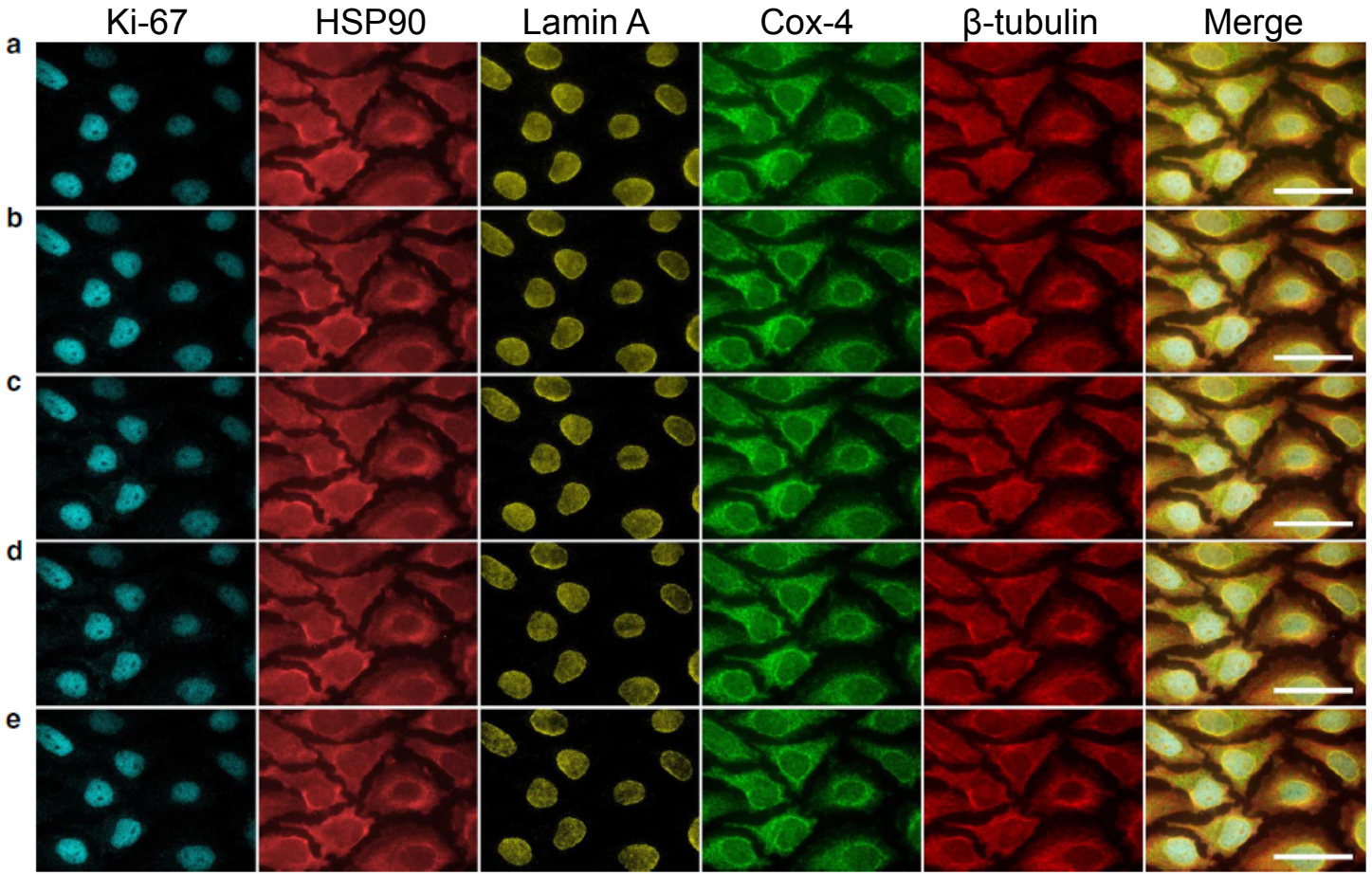
Evaluation of the robustness of sequential staining procedure

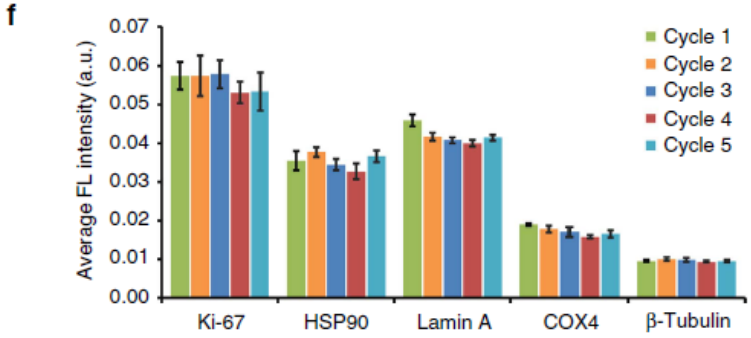




- Complete specimen regeneration and lack of specimen degradation enables staining of the molecular targets
- Correct staining pattern and target expression profile can be obtained for staining sequence from highly condensed Lamin A down to diffusely distributed β-tubulin (a–c) and in reverse (d–f)
- Matching relative profile obtained from reference single-cycle staining with QD585-SpA (g).
- No carry-over or residual staining is detectable throughout the five cycles

Validation of M3P technology with 25-target staining





- 5 colors x 5 cycles
- Re-stain the same model 5-target panel with multicolor QD-SpA-Ab probes for five cycles and perform quantitative analysis of staining intensity after each cycle
- All five targets are consistently stained through five cycles yielding accurate staining patterns and identical average staining intensity profiles (no carry over)

Summary

- M3P of single cells and tissue specimens is based on production of QD-Ab libraries via simple, fast and inexpensive methods
- The universal QD-SpA platform features on-demand, flexible, single-step, purification-free QD-Ab assembly, along with high probe specificity
- Demonstrated that the SpA–Ab bond is sufficiently stable to avoid QD-SpA-Ab probe crosstalk
- Used QDs coated with a non-fouling layer of PEG, which resists nonspecific binding even after bioconjugation with SpA and Ab
- With this probe design, fully assembled functional QD-SpA-Ab allows direct target labelling while the high brightness of QD probes makes imaging without signal amplification possible
- Demonstrated that at least ten staining cycles can be performed without loss of specimen antigenicity and cell morphology
- Brief treatment with regeneration buffer leads to effective removal of QD–SpA–Ab complex and thus completely regenerates specimens for next-cycle staining enabling re-staining of the same or probing of a different panel of molecular targets

Conclusion

- Developed a technically straightforward, yet analytically powerful, imaging platform for comprehensive in situ single-cell molecular profiling

- Overcoming the fundamental limitations of conventional and nanoparticle-based methods, the functionality of this technology comes from the unique combination of favorable optical properties of QDs, flexible QD-Ab probe preparation and the ability to perform multiple cycles of staining/de-staining without affecting specimen antigenicity and cell morphology

- Further development of M3P technology, especially by integrating it with automated staining–imaging instruments, will offer exciting opportunities in systems biology, signalling pathway analysis, gene expression studies, molecular diagnostics and drug discovery


THANKS
FOR YOUR
ATTENTION