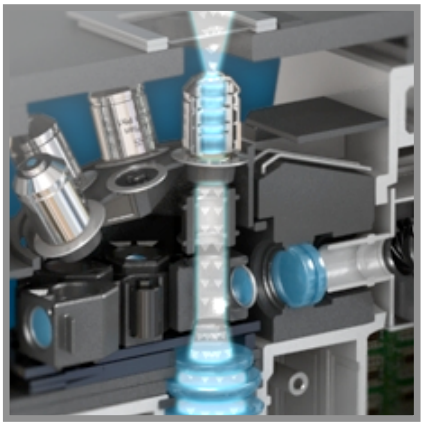
Bioanalytical chemistry

6. Immunohistochemistry and fluorescence imaging

Primary Source Material

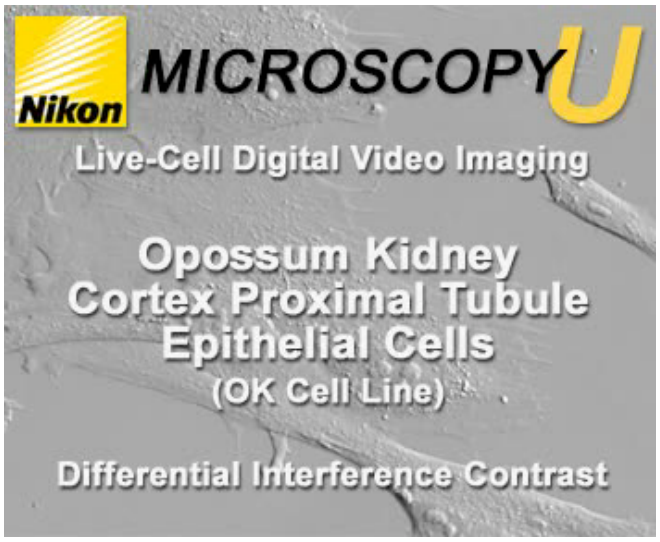
- Chapters 5 & 10 of Mikkelsen, S.R. and Corton, E., Bioanalytical Chemistry (2004).
- Chapter 6 of Goldsby Immunology 5th edition (WH Freeman)
- Appendix 1 of Immunobiology 5th edition. (NCBI bookshelf).
- http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?cmd=Search&db=books&doptcmdl=GenBookHL&term=diagnostic+AND+imm%5Bbook%5D+AND+125975%5Buid%5D&rid=imm.section.2405

Transmitted light microscopy



http://zeiss-campus.magnet.fsu.edu/tutorials/axioobserver/index.html

Transmitted light differential interference contrast (DIC) imaging of mammalian cells



principles of DIC are beyond scope of lecture - essentially contrast is generated at edges where refractive index is rapidly changing with location

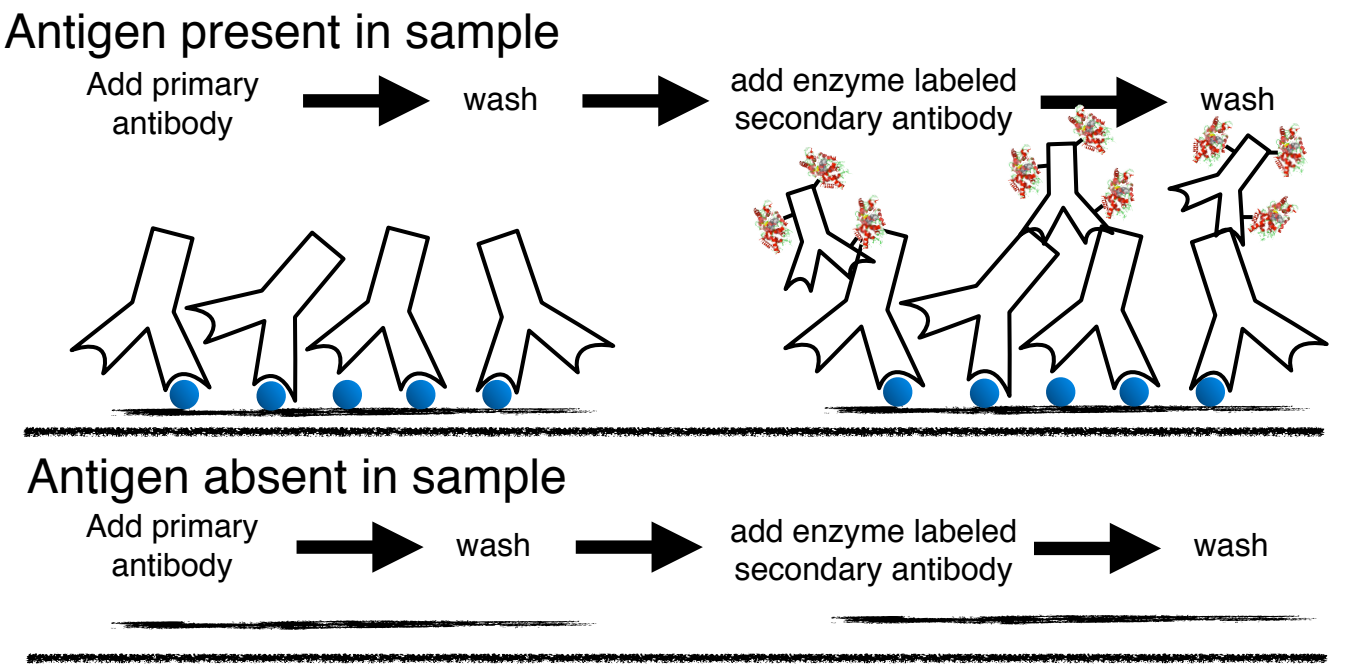
Opossum Kidney Cortex Proximal Tubule Epithelial Cells (OK Line)
The OK cell line was initiated from the kidney of an adult female North American opossum (***Didelphis marsupialis virginiana***) and was originally intended for use as a source of X chromosomes for studies of X inactivation. The line was soon discovered, however, to display many characteristics of kidney proximal tubular epithelial cells and has since been commonly utilized as a cell culture model for the cell type.

http://www.microscopyu.com/moviegallery/livecellimaging/index.html

- Take away message: transmitted light microscopy can be used to visualize some structures in cells, but there is not a lot of contrast. Essentially no molecular information can be extracted by simply imaging cells. To get molecular information about what proteins (i.e., antigens) are present, we need to use a probe of some sort.

Immunohistochemistry

Immunohistochemistry (*histo* means tissue) is used for the detection of specific antigens in a tissue sample. For example a pathologist uses immunohistochemistry to detect cancer biomarkers in tissue that is suspected of being cancerous.



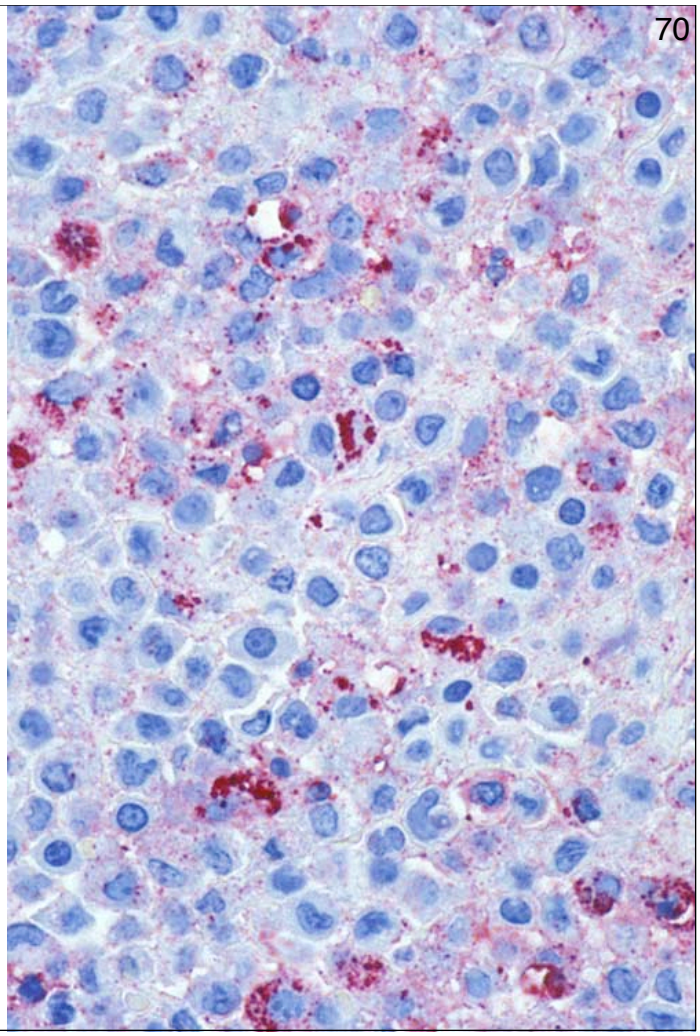
Add **substrate that forms a colored precipitate**, allow to react for a while, image sample with a microscope. Presence of colored precipitate indicates presence and localization of the antigen in original sample

- The basic principle of immunohistochemistry is the use of enzyme-linked antibodies to detect tissue antigens. The colorless substrate is converted by the enzyme into a colored product that precipitates on the slide at the site of the reaction. Thus, immunohistochemistry localizes antigens in a tissue section.
- Immunohistochemistry is often used for diagnosis of tumors, especially to distinguish among tumors that appear similar on standard histologic stains.
- Q. In immunohistochemistry (immunostaining). Why, in your written notes) do you mention secondary Ab use when we can just use a primary Ab linked to AP or HRP to detect the presence of an Ag in or on a monolayer sample of cell culture? It seems like a waste of reagents and money to use both a primary and secondary Ab for this simple job. If we are looking to use fluorescence then why not just use a primary Ab conjugated to a fluorophore? Can you explain please?
- A. The reason we use a secondary is to minimize the waste of reagents and money. You could label your primary (say from mouse), but then you would have to go through the effort of chemically labelling every antibody that you want to use. This consumes time and money. It is more efficient to purchase a secondary antibody (say goat anti-mouse) that is already labeled with an antibody or fluorophore and use it to label any mouse primary. You can think of secondary Abs as just easy and convenient ways of adding a fluorophore or enzyme to an otherwise unlabeled primary.

Example of immunohistochemical detection of *Bacillus anthracis*

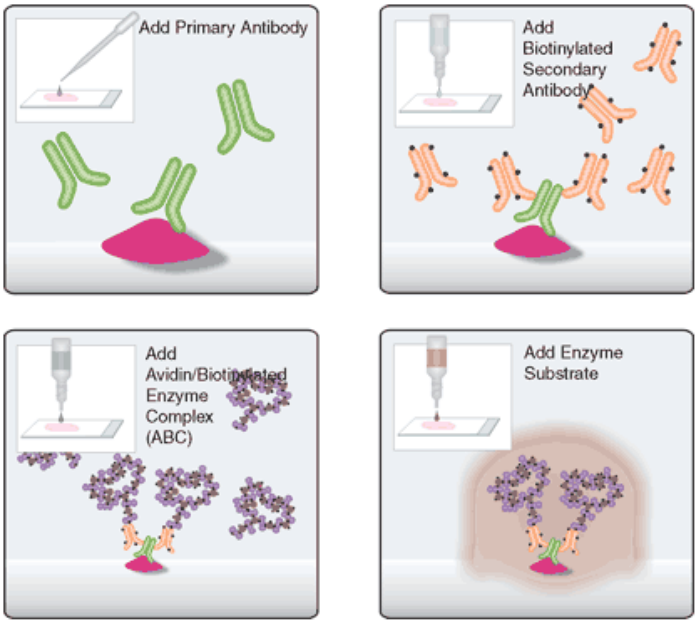
- Pleural fluid cell block from a nonfatal case showing abundant *Bacillus anthracis* granular antigen staining inside mononuclear inflammatory cells.
- Immunohistochemical assay with a mouse monoclonal anti-*B. anthracis* capsule antibody and detection with alkaline phosphatase and naphthol fast red, original magnification 158X.
- Jernigan et al. Bioterrorism-Related Inhalational Anthrax: The First 10 Cases Reported in the United States, *Emerging Infectious Diseases*. Vol. 7, No. 6 Nov–Dec 2001

http://www.cdc.gov/ncidod/EID/vol7no6/jerniganG7A.htm



- *Q: You mention some commercial tests that are used today, I was wondering for the detection of Hep B are they testing for the antigen instead of looking for anti Hep B antibodies?*
- *A: The three choices for any diagnostic test for a virus are: 1) Detect the viral proteins; 2) Detect the viral DNA (or RNA); and 3) Detect antibodies against the virus. I suspect that for every virus that infects humans (Hep B included), examples of each of these 3 types of tests have been developed. Generally speaking, I would expect the detection of antibodies to be the easiest and most rapid sort of test to carry out. Detection of DNA is the most sensitive, but will typically require a PCR amplification step that increases the time and instrumentation required to carry out the test. Detection of viral proteins could be fast, like with antibody detection, but the viral proteins are likely at a relatively low concentration so detection might be more challenging. On the other hand, you have a better idea of your target (relative to antibodies, which could bind to a variety of possible epitopes) so it might be possible to develop more specific detection strategies.*

Using biotin/avidin complexes to amplify an immunohistochemical signal



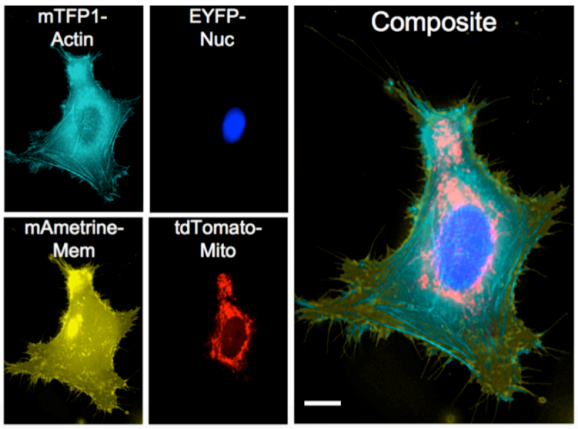
- the signal is ultimately proportional to the number of copies of the enzyme that are immobilized for each copy of the antigen
- by adding a biotinylated secondary antibody, followed by a complex of avidin and biotinylated reporter enzyme, many copies of enzyme can be immobilized

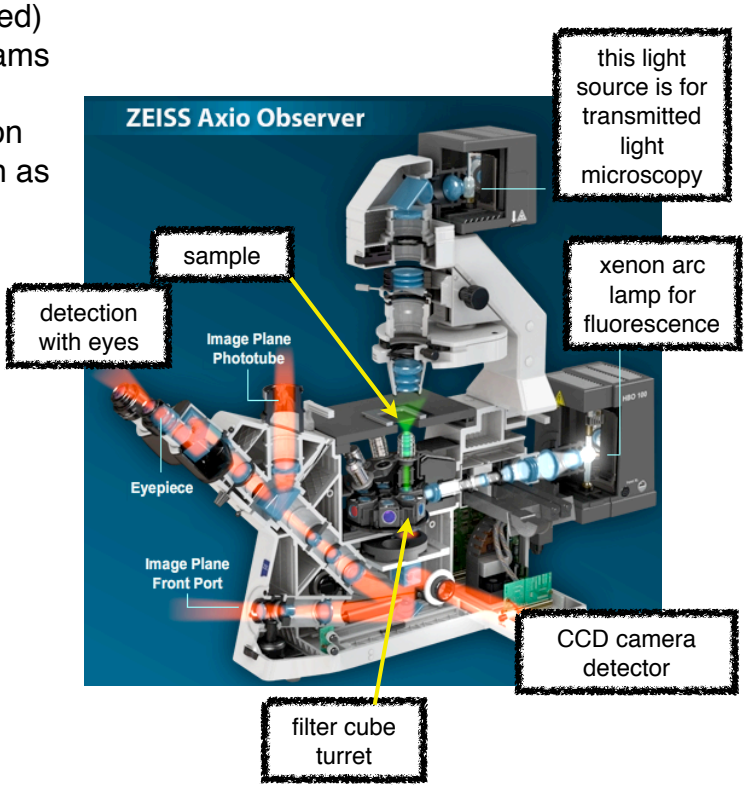
http://www.vectorlabs.com

- You might think that there will be no binding sites for biotin left, following mixture of avidin and biotinylated enzyme
- However, this can be adjusted by changing the ratio of avidin to biotinylated enzyme
- Note that this sort of approach takes advantage of the tetrameric structure of avidin. If avidin was a monomer, this wouldn't work.
- *Q: Would you please explain all steps of biotin/avidin complexes again? What is attached to primary and secondary antibodies?*
- *A: The primary antibody (generated in species 1) is the one that actually binds to the antigen. The secondary antibody is from a different species (species 2) and would bind to any species 1 antibody. That is, the secondary antibody is 'species 2 anti-species 1' (e.g., goat anti-chicken). The secondary antibody has many copies of biotin covalently attached to it. After washing away unbound secondary antibody, a mixture of (strept)avidin and biotinylated reporter enzyme is added. The (strept)avidin serves as a cross linker which will link reporter enzymes to reporter enzymes and reporter enzymes to the secondary antibody. Accordingly, each secondary antibody will end up with many copies of reporter enzyme linked to it. Once substrate is added, a much larger amount of product (a precipitating chromophore or fluorophore) will be generated for each copy of antigen that is present in the sample, compared to the situation where the secondary has just a few copies of reporter enzyme linked directly to it.*

Fluorescence microscopy

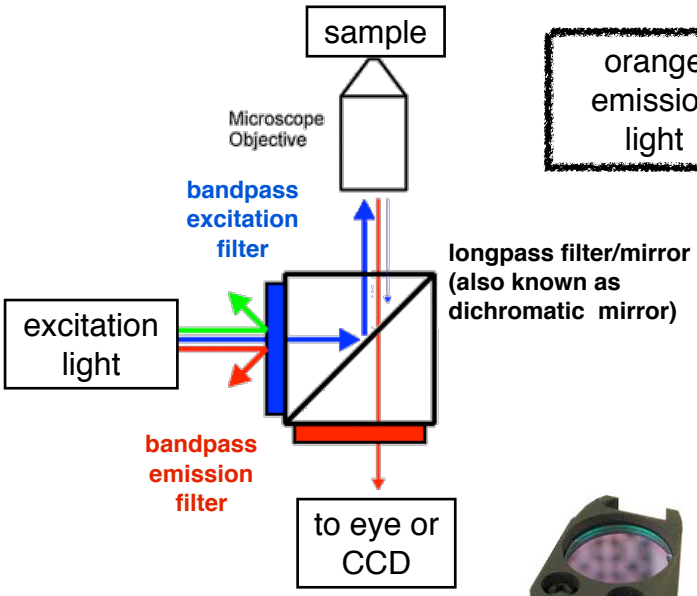
- an **epifluorescence** microscope is basically a fluorometer (i.e., it is filter based) that transmits images rather than just beams of light
- epifluorescence means that the emission path is measured from the same direction as excitation.
- this is made possible by filter cubes

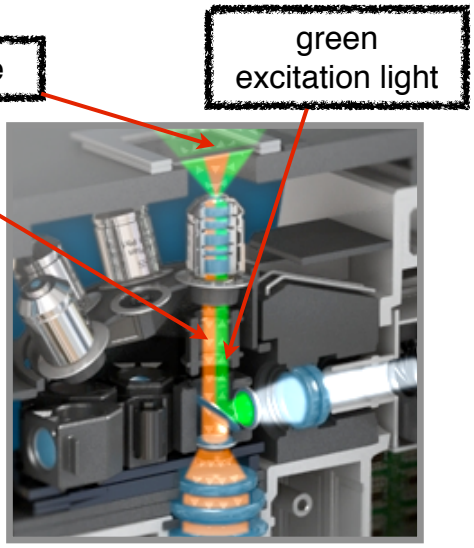


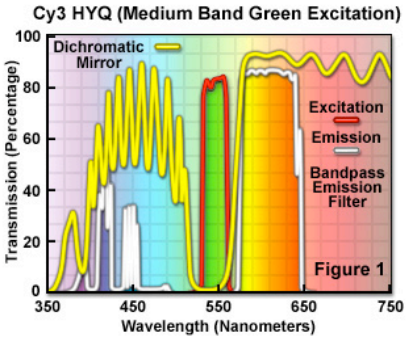


http://zeiss-campus.magnet.fsu.edu/tutorials/axioobserver/index.html

Filter cubes for fluorescence imaging







http://www.fluorescence.com/tutorial/fm-optic.htm

Immunofluorescence assay

An immunofluorescence assay is basically the same thing as immunohistochemistry, but using fluorophore-labeled antibodies. The advantages are that higher resolution imaging can be achieved and multiple antigens can be detected at once using different antibodies labeled with different colors of fluorophore.

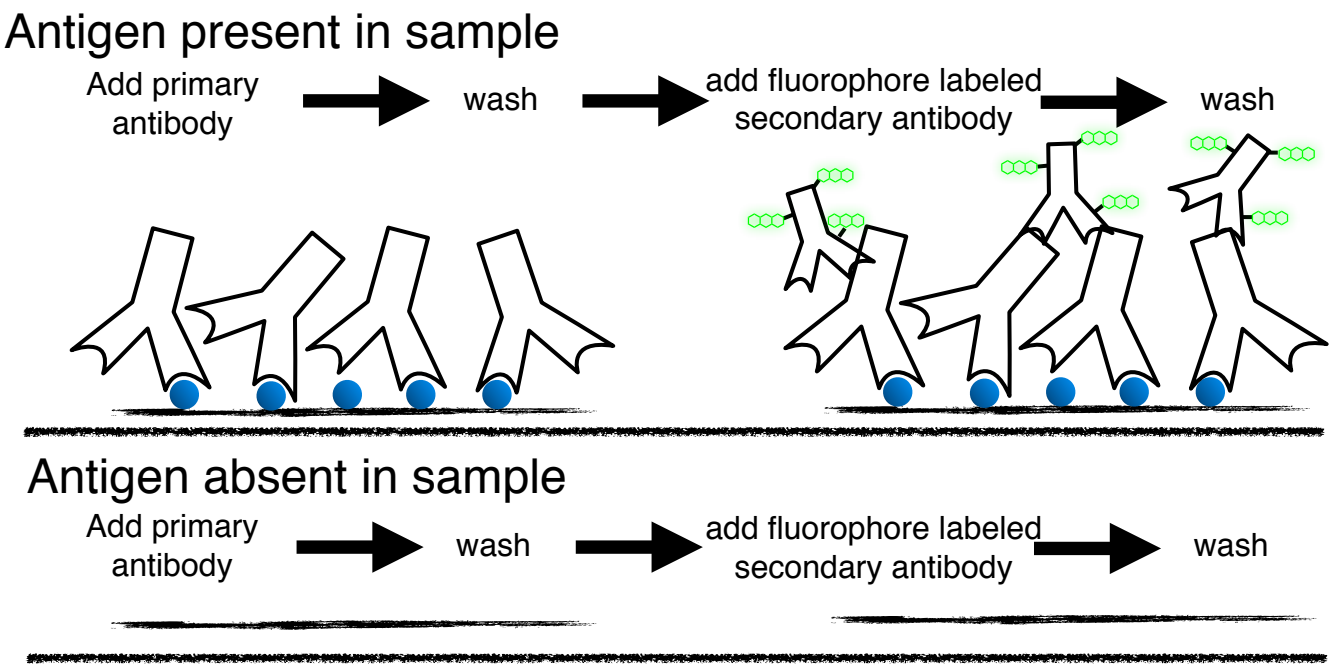
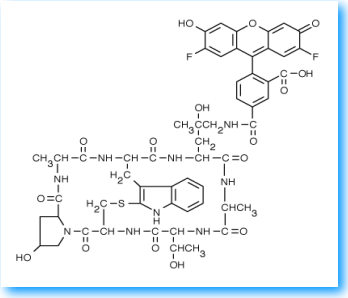


Image sample with a microscope. Localization of fluorescent signal indicates location of the antigen in original sample

- The basic principle of the immunofluorescence assay (IFA) is to use a fluorescent compound (often fluorescein) to detect the binding of Ag and Ab. This approach is also known as immunocytochemistry (when the sample is individual cells) or immunohistochemistry (when the sample is a tissue).
- In the example shown, the Ab is labeled with the fluorescent compound and its presence is detected using a fluorescence microscope. Under a fluorescence microscope, the sample will fluoresce bright green wherever the binding occurred.
- It is more common to use an unlabelled primary antibody and a fluorescently labeled secondary antibody that binds to the primary antibody. This approach provides some amplification of the signal, since many secondary antibodies could bind to each primary antibody. It is also more convenient since the fluorescent labelling reaction does not have to be performed for every antibody of interest.

Immunofluorescence imaging of fixed cells

cells stained with two different fluorophore labeled antibodies and an actin cytoskeleton probe

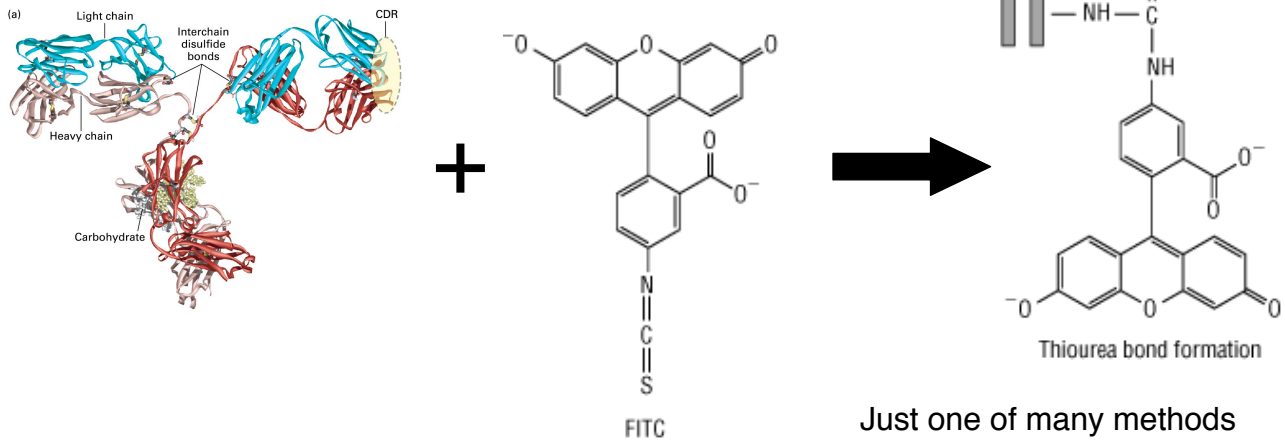


F-actin specific probe
Oregon Green 488
phalloidin
(www.probes.com)

http://micro.magnet.fsu.edu/primer/techniques/fluorescence/gallery/cells/u2/u2cellsexlarge17.html

- *Description from http://micro.magnet.fsu.edu/primer/techniques/fluorescence/gallery/cells/u2/u2cellsexlarge17.html:*
- *"The generalized procedure for immunofluorescence labeling of adherent cell cultures involves fixation of the cells with a suitable reagent, such as paraformaldehyde, glutaraldehyde, acetone, or methanol. The organic solvents serve double duty by also removing most of the plasma membrane components, thus exposing the interior of the cell to antibodies and other non-permeant reagents. Cell cultures fixed with aldehydes require a permeabilization step with a detergent. Triton X-100 is a popular permeabilizing agent, as are saponin, Tween, and sodium dodecyl sulfate (SDS). After permeabilization, cells are treated with a primary antibody (or a mixture of two different antibodies from different hosts) followed by a fluorophore conjugated secondary antibody. In some cases, the primary antibody is labeled with a fluorophore. After immunostaining, the cells can be counterstained with other reagents to visual structural details not revealed by the fluorescent antibodies."*
- *"In a double immunofluorescence labeling protocol, the culture of human bone cancer (U-2 OS) cells presented above was treated with a cocktail of mouse anti-histone (pan) and rabbit anti-PMP 70 (peroxisomal membrane protein) primary antibodies. The target proteins were subsequently visualized with goat anti-mouse and anti-rabbit secondary antibodies conjugated to Texas Red and Alexa Fluor 488, respectively. The filamentous actin cytoskeletal network was counterstained with Alexa Fluor 350 conjugated to phalloidin, a bicyclic peptide isolated from the death cap mushroom (Amanita phalloides). Images were recorded in grayscale with a QImaging Retiga Fast-EXi camera system coupled to an Olympus BX-51 microscope equipped with bandpass emission fluorescence filter optical blocks provided by Omega Optical. During the processing stage, individual image channels were pseudocolored with RGB values corresponding to each of the fluorophore emission spectral profiles."*

Preparing a fluorescently labeled antibody



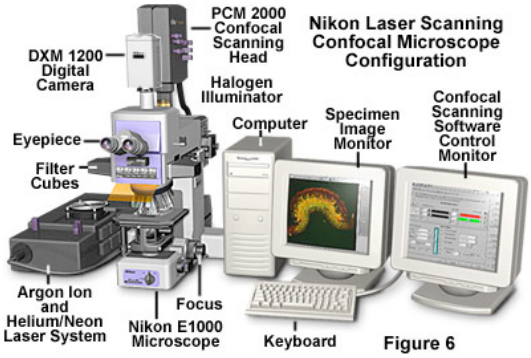
Just one of many methods

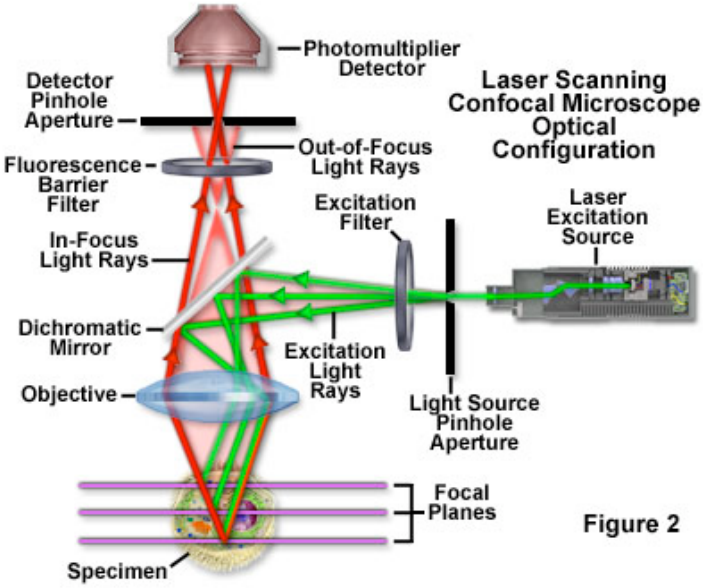
- as with enzyme labelling, there are many commercially available kits that make this process very simple to carry out

http://www.piercenet.com/

- Antibody molecules can be labeled with any of a number of different fluorescent probes currently available from commercial sources. Each probe option has its own characteristic spectral signals of excitation (or absorption) and emission (or fluorescence). Many derivatives of these fluorescent probes possess reactive functional groups convenient for covalently linking to antibodies and other molecules.
- Each of the main fluorophore families contains at least a few different choices in coupling chemistry to direct the modification reaction to selected functional groups on the molecule to be labeled. These choices include amine-reactive, sulfhydryl-reactive and aldehyde-reactive.
- Perhaps the most common fluorescent tags with application to immunoassays include derivatives of fluorescein, rhodamine, aminomethylcoumarin (AMCA) and phycoerythrin. Instrumentation is widely available for measuring the fluorescent response of any of these probes, including standard filter selections that match their excitation and emission patterns. Such fluorescently labeled antibodies can be used in immunohistochemical staining, in flow cytometry or cell sorting techniques, for tracking and localization of antigens and in various double-staining methods.
- In choosing a fluorescent tag, the most important factors to consider are good absorption, stable excitation, and efficient, high-quantum yield of fluorescence. All fluorophores, to various extents, exhibit photobleaching which lowers the quantum yield over time. Up to 50% of the fluorescent intensity observed on a fluorescein-stained slide can be lost within 1 month of storage.

Confocal fluorescence microscopy





•laser excitation instead of arc lamp
•beam is rastered over specimen
•out of focus fluorescence is rejected by a pinhole

- tends to give much ‘sharper’ in focus images (and movies) than widefield imaging.
- technically more complex and higher light levels may cause photodamage
- generally slower than widefield due to need to raster beam over specimen

Two-photon excitation only excites fluorophores at a precise focal plane

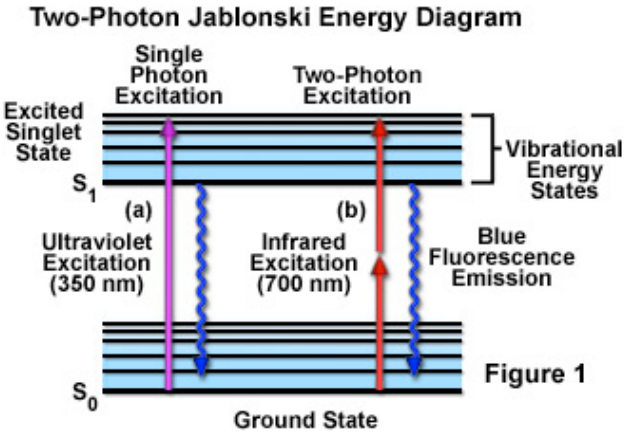


Figure 1

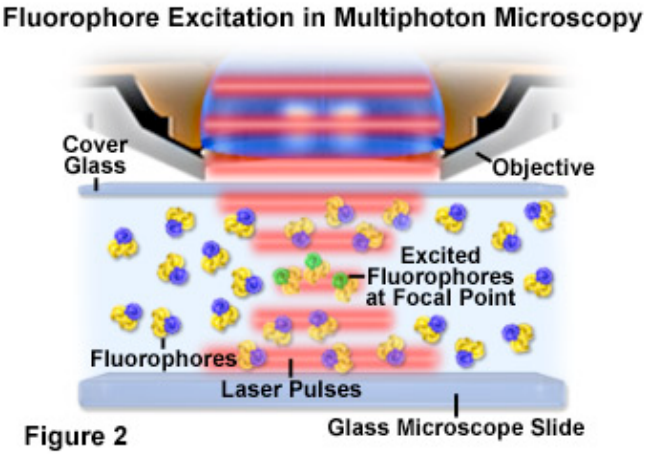
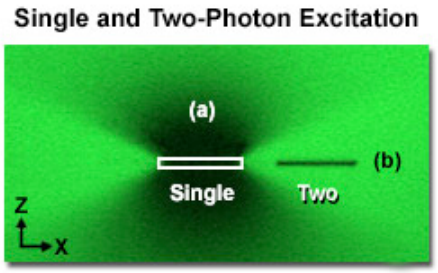


Figure 2

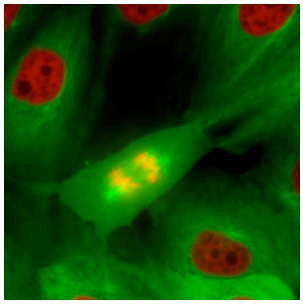


http://www.microscopyu.com/articles/fluorescence/multiphoton/multiphotonintro.html

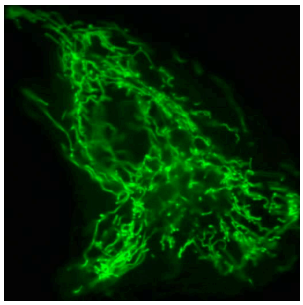
- The major advantage of 2-photon imaging is that it used near-infrared light to excite visible wavelength fluorophores. This light penetrates deeper into tissue (i.e., near-infrared window where hemoglobin absorbance is at a minimum) and is less damaging to cells.
- 2-photon excitation is inherently ‘confocal’ in the sense that only fluorophores in a very thin confocal plane are excited. Fluorophores that are above and below the focal plane are not excited since the photon flux is not high enough to get 2 photons simultaneously absorbed by the fluorophore.

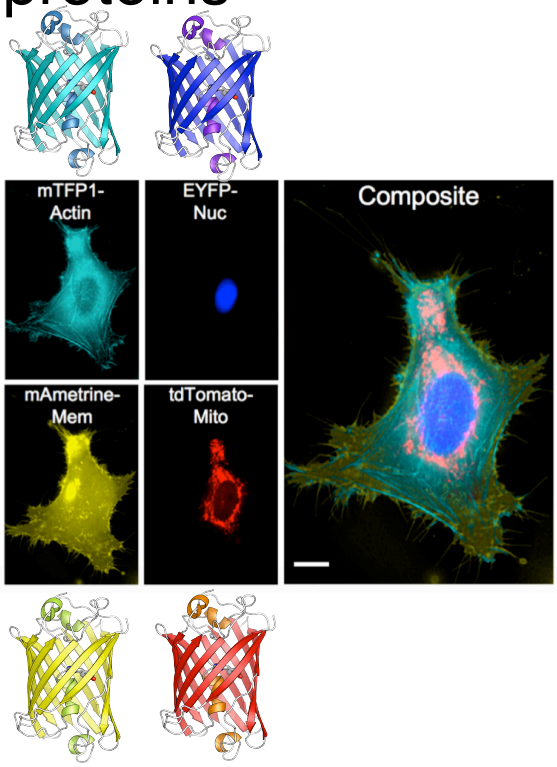
Live cell fluorescence imaging using fluorescent proteins

mammalian cell with red histones (mCherry) and green EB3 (mEmerald)



human cervical carcinoma cells (HeLa line) expressing enhanced yellow fluorescent protein (EYFP) fused to a mitochondrial targeting signal.





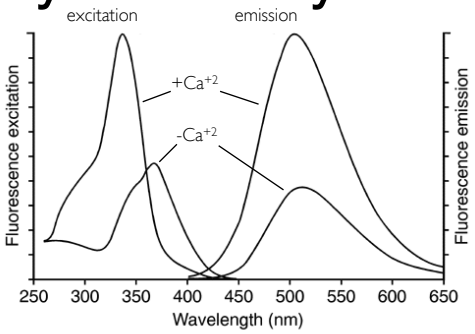
Hui-wang Ai, Kristin L. Hazelwood, Michael W. Davidson, and Robert E. Campbell*, "Fluorescent protein FRET pairs for ratiometric imaging of dual biosensors", *Nature Methods*, 2008, **5**: 401 - 403.

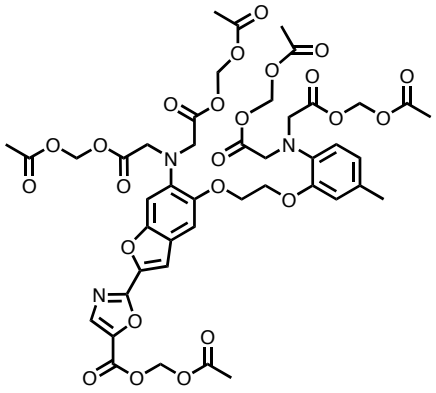
http://learn.hamamatsu.com/galleries/digitalvideo/index.html
http://www.microscopyu.com/moviegallery/sweptfield/hela-eyfp-mito-sfc/

- images acquired with a monochrome camera
- 4 different filter sets (cubes), 1 for each color
- composite is made digitally

Live cell fluorescence imaging of Ca^{2+} dynamics using a synthetic dye

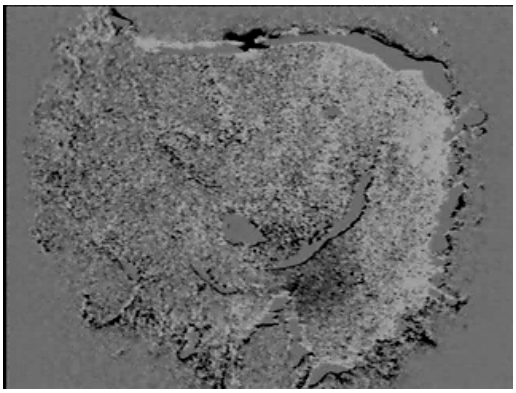
- Fluorescent proteins are just one type of fluorophore used for fluorescence imaging.
- Synthetic dyes are also widely used
- One of the most famous, and most useful, fluorescent dyes is fura-2, a Ca^{2+} indicator





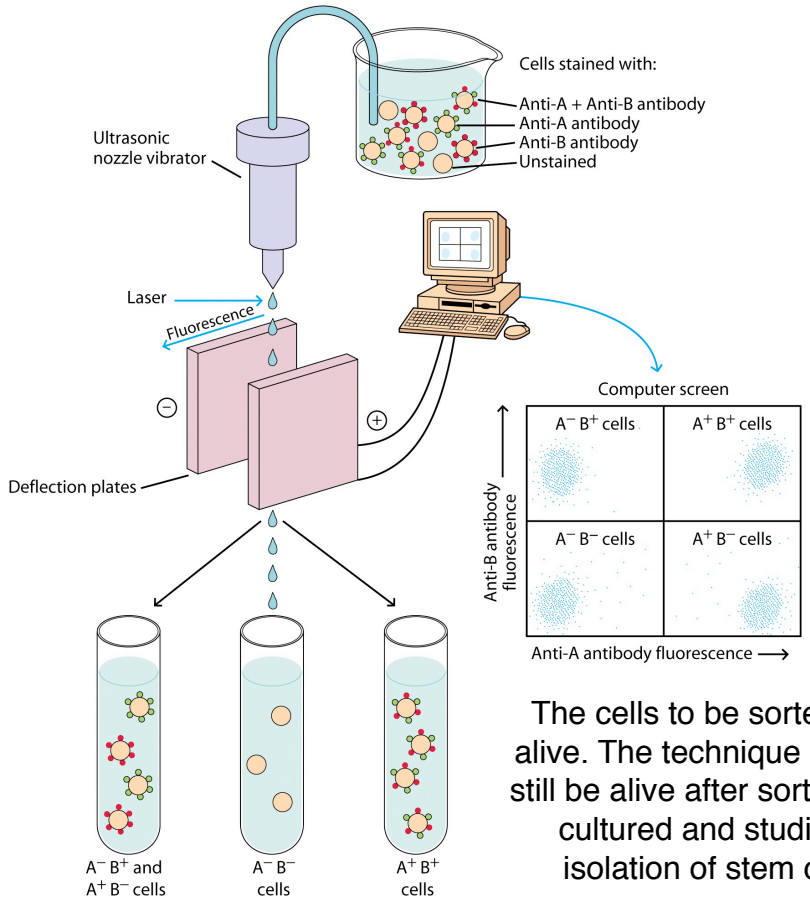
fura-2, AM

Grynkiewicz, Poenie, & Tsien *JBC*, **1985**
~20,000 citations (ISI Web of Science)



Ca^{2+} oscillations in mouse retina
Singer JH, Mirotznik RR, Feller MB *J. Neurosci.* 2001, **21**:8514-22.

Fluorescence activated cell sorting (FACS)



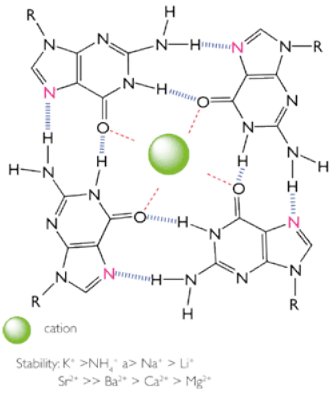
Flow cytometry and FACS are immunofluorescence assays applied to suspended cells. That is, they are not fixed on a surface.

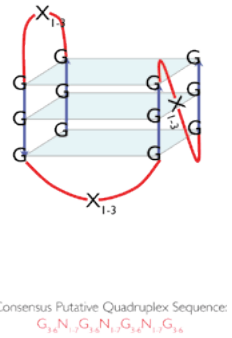
Flow cytometry and FACS are essentially the same technique. The difference is that a FACS instrument allows cells to be sorted into separate tubes, whereas flow cytometry is only for analysis (not sorting)

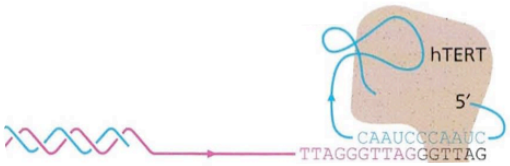
The cells to be sorted in a FACS experiment are alive. The technique is gentle enough that they will still be alive after sorting so that they can be further cultured and studied. One example could be isolation of stem cells from a tissue sample.

- FACS instruments and Flow cytometers are expensive instruments that involve sophisticated fluidics, laser optics, electronic detectors, analog to digital converters, and computers.
- Cells in a monodisperse suspension are treated with an antibody against a surface antigen. The cells could be alive or dead for flow cytometry, but FACS only makes sense if the cells are alive.
- The cells travel through a thin stream of liquid which passes at high speed through a nozzle that causes the stream to break into drops. The cells are dilute enough that only some of the drops have cells in them (maybe 1 in 10). It is unlikely that one drop would have 2 or more cells in them.
- The drops pass through a laser beam and some of the light is scattered. If the cell is labeled with a fluorophore, there will also be a fluorescence signal. The signal from scattering and from fluorescence is collected at distinct photomultiplier tube detectors. The 3 main measurements are:
    - low angle forward scatter intensity, approximately proportional to cell diameter
    - orthogonal (90 degree) scatter intensity, approximately proportional to the quantity of granular structures within the cell
    - fluorescence intensities at several wavelengths
- Light scatter alone is often quite useful. It is commonly used to exclude dead cells, cell aggregates, and cell debris from the fluorescence data.
- The instrument can records data for up to 100,000 cells per second, and displays the data graphically on a 2-dimensional chart of fluorescence intensities in one emission channel vs. fluorescence intensity in a second emission channel. Alternatively amount of side or forward scatter could be represented on one of the axis.
- In a FACS instrument, each droplet is given an electrostatic charge that allows it to be deflected to the left or to the right as it passes between two metal plates. By interactively drawing a region of interest around cells on the 2-dimensional chart, the computer can select cells that fall into this region of interest. That is, a subset of the cells can be deflected so that they go into one tube or the other. Cells that don't meet the selection criteria pass straight into the waste stream.

G-quadraplexes: Do they occur in cells?







Recall that the telomeres have a repeating sequence of $(TTAGGG)_n$

ARTICLES
PUBLISHED ONLINE: 20 JANUARY 2013 | DOI: 10.1038/NCHEM.1548
nature chemistry

Nature Chemistry **5**, 182–186 (2013)

Quantitative visualization of DNA G-quadruplex structures in human cells

Giulia Biffi[1], David Tannahill[1], John McCafferty[2] and Shankar Balasubramanian[1,3]*

Four-stranded G-quadruplex nucleic acid structures are of great interest as their high thermodynamic stability under near-physiological conditions suggests that they could form in cells. Here we report the generation and application of an engineered, structure-specific antibody employed to quantitatively visualize DNA G-quadruplex structures in human cells. We show explicitly that G-quadruplex formation in DNA is modulated during cell-cycle progression and that endogenous G-quadruplex DNA structures can be stabilized by a small-molecule ligand. Together these findings provide substantive evidence for the formation of G-quadruplex structures in the genome of mammalian cells and corroborate the application of stabilizing ligands in a cellular context to target G-quadruplexes and intervene with their function.

http://www.cambridgecancer.org.uk/research-groups/balasubramanian-group/research

G-quadraplexes: Do they occur in cells?

Generate anti-G-quadraplex antibody by phage display of library of 2.3 x 10^{10} scFv

Characterize binding of highest affinity binder by ELISA

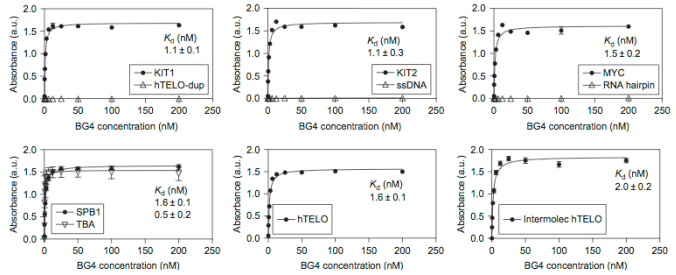
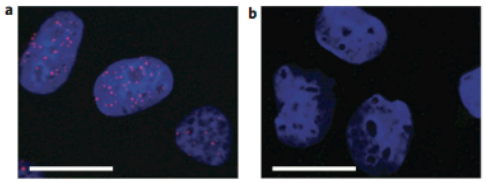


Figure 1 | Structure specificity of the BG4 antibody for G-quadruplex structures. Binding curves as determined by ELISA show that the BG4 antibody has a high affinity for intramolecular and intermolecular DNA G-quadruplex structures with negligible binding to a RNA hairpin, to double-stranded DNA (hTELO-dup) and to single-stranded DNA (ssDNA). BG4 does not show a preference for any particular G-quadruplex conformation as it binds with similar affinity to parallel propeller (KIT1, KIT2 and MYC), anti-parallel propeller (SPB1 and TBA), mixed parallel/anti-parallel propeller (hTELO) and intermolecular (intermolec hTELO) G-quadruplex structures. Dissociation constants (K_d) are indicated. Error bars represent the s.e.m. calculated from three replicates. a.u. = arbitrary units.



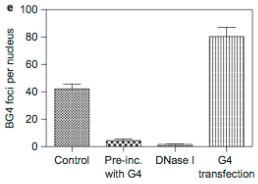


Figure 2 | Visualization of DNA G-quadruplex structures in nuclei of human cancer cells. a, Immunofluorescence showing BG4 foci (red) in U2OS osteosarcoma cell nuclei. **b**, Loss of BG4 foci in U2OS cells after pre-incubation of the antibody with pre-folded G-quadruplex oligonucleotides. **c**, Loss of BG4 foci in U2OS cells after DNase I treatment. The dotted lines show the boundary of the nuclei. **d**, Increase in BG4 foci number after transfection with pre-folded G-quadruplex oligonucleotides. The nuclei are counterstained with DAPI (blue). **e**, The quantification of BG4 foci number per nucleus for **a**–**d**. 100–200 nuclei were counted per condition and the s.e.m. was calculated from a set of three replicates. These observations support the targeting and visualization of DNA G-quadruplex structures in human cells by the BG4 antibody. Scale bars, 20 μm.

Nature Chemistry **5**, 182–186 (2013)