

Fluorochromes in selective cell analysis

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The immunofluorescent approach introduced by COONS AH and co-workers (1941) opened specific investigations on cellular structure and function at the light microscopic level. Since then, the immunofluorescent antibody technique (FITC, fluorochromes other than FITC and fluorescent double staining methods) has been employed in numerous research and diagnostic areas, and considerable progress could be achieved in understanding biomedical phenomena.

The original COONS' immunofluorescence has been refined during the last two decades in a manifold way. Developments such as confocal, deconvolution, ratio-imaging, total internal reflection and applications of fluorescent reporter molecules (such as green fluorescent protein [GFP] and variants, briefly called FP for fluorescent proteins) have now become integral tools in functional genomics and cell development. In functional genomics, transgenic approaches using FPs might well be exploited to examine promoter activity and to clone regulatory elements (PRASHER DC et al., 1992; CHALFIE M et al., 1994; STEWART CN, 2006).

Fluorescent dyes and fluorochromes for labeling purposes

Fluorochromes can be divided into at least three groups: (1) one large group including FITC that require labeling to other molecules such as antibodies to bind specifically to cellular target molecules; (2) another group is represented by fluorochromes that have inherent binding capacities such as the DNA-intercalating DAPI (4',6-diamidino-2-phenylindole) stain which can be used directly and need not to be labeled to antibodies or other transportation systems; (3) the third group of fluorochromes are the natural fluorescent proteins being produced by the cells themselves such as GFP (green fluorescent protein as reporter in recombinant DNA studies) and variants thereof which have become integral tools in functional genomics and development.

Until the 1990's, the number of fluorochromes was very restricted. Fluorescein isothiocyanate (FITC) is still one of the most employed fluorescent labels. The synthesis of FITC and similar fluorescein derived reagents yields a mixture of isomers at the 5- and 6-positions of fluorescein's "bottom" ring. The isomers may differ in the geometry of their binding with proteins. Hence, the conjugates may elute under different chromatographic conditions or migrate differently in an electrophoretic gel, and certain applications may require single-isomer preparations. Many fluorescein probes are commercially available either as a mixture of isomers or as purified single isomers. The 5-isomer (Isomer I) of FITC is the most widely used FITC isomer, probably because it is easier to isolate this product in pure form. In addition to its widespread use for preparing immunoreagents, FITC has a multitude of other applications (for more details refer to Invitrogen Life Science, <http://www.probes.invitrogen.com/handbook/>).

Unfortunately, fluorescein based dyes and their conjugates have several drawbacks.

- Rate of photobleaching is quite high.
- Fluorescence is pH sensitive; fluorescence is significantly reduced below pH 7.
- Fluorescence emission spectrum is relatively broad, thus, limiting their utility in multicolor applications.
- Tendency toward quenching of fluorescence on conjugation to biomolecules, at least at high degree of substitution.

These limitations have led to the development of alternative fluorochromes. In the meantime, their number has grown rapidly and are currently available from commercial sources. Also, with modern developments in fluorescence microscopy and filter techniques, several fluorochromes can be easily combined for multicolor analysis.

Many of the new fluorochromes can be purchased with reactive functional groups for direct labeling. These reactive derivatives of fluorescent dyes are especially convenient for their conjugation with antibodies and other biomolecules. The functional groups on the molecules enable choices in coupling chemistry and include *amine-reactive*, *sulfhydryl-reactive* and *aldehyde-reactive* groups. Popular reactive groups are the N-Hydroxysuccinimide esters and the isothiocyanates that are easy to couple to amino groups on lysine side chains or to N-terminal amines, and which form covalent bonds after reaction. Generalized protocols for the conjugation of fluorochromes to protein molecules can be found in the literature.

Table 1: Selection of fluorochromes for labeling purposes in microscopy

Fluorochromes ¹⁾	Excitation peak ²⁾	Emission peak ²⁾	Fluorescent light
Alexa Fluor 350	346	442	Blue (< 499 nm)
Alexa Fluor 405	402	421	Blue
AMCA	349	448	Blue
Cascade Blue	400	420	Blue
Methoxycoumarin	340	405	Blue
Pacific Blue	410	455	Blue
Alexa Fluor 430	434	539	Green (500-549 nm)
Alexa Fluor 488	495	519	Green
Alexa Fluor 500	503	525	Green
Alexa Fluor 514	518	540	Green
BODYPY FL	505	513	Green
Cyanine 2	492	510	Green
DTAF	494	520	Green
Fluorescein, FITC	494	518	Green

Oregon green 488	496	524	Green
Oregon green 514	511	530	Green
Rhodamine green	502	527	Green
Alexa Fluor 532	531	554	Yellow (550-584 nm)
Alexa Fluor 546	556	573	Yellow
Alexa Fluor 555	555	565	Yellow
R-phycoerythrin	565	575	Yellow
Alexa Fluor 568	578	603	Orange (585-615 nm)
Cyanine 3.5	581	596	Orange
Lissamine Rhodamine B	570	590	Orange
Tetramethylrhodamine, Rhodamine	555	580	Orange
Texas Red	595	615	Orange
Alexa Fluor 594	594	617	Red (616-700 nm)
Alexa Fluor 610	612	628	Red
Alexa Fluor 633	632	647	Red
Alexa Fluor 635	633	647	Red
Alexa Fluor 647	650	668	Red
Alexa Fluor 660	663	690	Red
Allophycocyanin	650	660	Red
Alexa Fluor 680	679	702	Far red (> 700 nm)
Alexa Fluor 700	702	723	Far red
Alexa Fluor 750	749	775	Far red
Alexa Fluor 790	782	805	Far red

1) For overview of fluorochromes, appropriate filter sets and microscopical objective choices, see http://www.micro-shop.zeiss.com/de/de_de/spektral.php?cp_sid. For fluorochromes, reactive derivatives see <http://www.probes.invitrogen.com/handbook/>.

2) Spectral characteristics of absorption and emission (nm) for different fluorophores; excitation and emission maxima may vary upon conjugation with antibodies and may depend on the light source used.

A novel approach is the use of long-lived luminescent labels such as lanthanide chelates with long decay times in fluorescence microscopy. Time-gated detection allows efficient suppression of scattered light and autofluorescence from both biological specimens and optical components. Several applications of europium and other lanthanide chelates could prove the high sensitivity of time-resolved fluorescence in immunohistology and in situ

hybridization (SOINI EJ et al. 1988; SEVEUS L et al. 1992; VEREB G et al. 1998; SCORILAS A et al. 2000).

The advantage of europium chelates in comparison to conventional fluorochromes is the large Stokes shifts (~ 290 nm) with no overlap between the excitation and emission spectra. Moreover, europium chelates have very narrow (10 nm bandwidth) emission spectra at 615 nm which do not overlap with serum native fluorescence. Their long fluorescence lifetimes (600-1000 μ s) allow the use of microsecond time-resolved fluorescence measurements which further reduce background signals.

Apart from fluorophore labeling of antibodies or other molecular ligands for fluorescence microscopy, a variety of fluorescent molecules can be also used for photoconversion of diaminobenzidine (DAB) which is known to be a powerful substrate in peroxidase cytochemistry. The process of fluorescence photooxidation of DAB involves the use of fluorescent dyes to oxidize DAB giving an insoluble, colored and electron dense product. By this technology, one of the disadvantages of fluorescent labeling (i.e. the inability for correlated light and electron microscopic resolution) can be overcome.

AR MARANTO (1982) was the first to use a fluorophore (lucifer yellow) for DAB photoconversion. When a fluorophore is exposed to high-intensity photon illumination, excitation from the electronic ground state to a higher singlet state occurs. Instead of emitting a photon, the excited state of the fluorophore can generate highly reactive singlet oxygen ($^1\text{O}_2$) by energy transfer. The reactive potential of $^1\text{O}_2$ can oxidize DAB into an insoluble, colored and electron dense compound. The usefulness of fluorescence photooxidation was shown in a number of publications (DEERINCK TJ et al., 1994; HUANG S et al., 1994; DANTUMA NP et al., 1998; CAPANI F et al., 2001).

Multicolor labeling

Two or more probes can be used to monitor simultaneously different functions. For this purpose, fluorophores with narrow spectral bandwidths are needed. An ideal combination of dyes means strong absorption at a coincident excitation wavelength and well-separated emission spectra. For example, excitation in overlapping absorption bands of two fluorochromes (A_1 and A_2) will produce fluorescent species with two spectra (E_1 and E_2). Modern microscopes are equipped with optical filters which are adapted to isolate the emission signals S_1 and S_2 .

Selected publications for further readings

Coons AH *et al.* (1941)
Coons AH and Kaplan MH (1950)
Marshall JM (1951)
Coons AH (1954, 1958)
Silverstein AM (1957)
Chadwick CS *et al.* (1958)
Goldwasser RA and Shepard CC (1958)
Hiramoto R *et al.* (1958)
Riggs JL *et al.* (1958)
Borek F and Silverstein AM (1960)

Beutner EH (1961)
Borek F 1961)
Curtain CC (1961)
Goldstein G *et al.* (1961)
Mc Devitt HO *et al.* (1963)
Wellensiek HJ and Coons AH (1964)
Johnson GD *et al.* (1982)
Maranto AR (1982)
Soini EJ *et al.* (1988)
Prasher DC *et al.* (1992)
Seveus L *et al.* (1992)
Chalfie M *et al.* (1994)
Deerinck TJ *et al.* (1994)
Huang S *et al.* (1994)
Nairn RC 1976)
Mason WT (1997)
Dantuma NP *et al.* (1998)
Vereb G *et al.* (1998)
Scorilas A *et al.* (2000)
Capani F *et al.* (2001)
Coling D and Kachar B (2001a, 2001b)
Stewart CN (2006)

Full citation of publications is given in chapter *References*

link: <https://www.kuhlmann-biomed.de/wp-content/uploads/2020/12/References.pdf>