

Fluorochromes and their applications

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Fluorochromes and their applications

... in Immunophenotyping

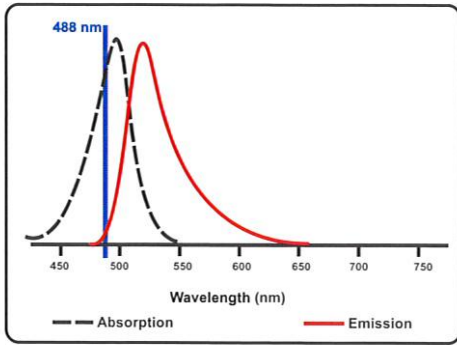
Fluorochrome	Laser (nm)	Emission (nm)	Emission dye
Alexa Fluor [®] 405	360, 405, 407	421	blue
Pacific Blue [®]	360, 405, 407	455	blue
Alexa Fluor [®] 488	488	519	green
FITC	488	525	green
Cy3 [™]	488	570	yellow
R-PE	488	575	yellow
PE-Texas Red [®]	488	615	orange
PE-Alexa Fluor [®] 610	488	628	orange
PE-Cy5 (TC [′])	488	670	red
PerCP [™]	488	678	red
PE-Cy5.5	488	694	far red
PerCP-Cy5.5 [™]	488	695	far red
PE-Alexa Fluor [®] 700	488	723	far red
PE-Cy7	488	767	infrared
Texas Red [®]	595	615	orange
APC	633 / 635	660	red
Alexa Fluor [®] 647	633 / 635	668	red
Cy5 [™]	633 / 635	670	red
APC-Cy5.5	633 / 635	694	far red
APC-Cy7	633 / 635	767	infrared
APC-Alexa Fluor [®] 750	633 / 635	775	infrared

... in Viability Tests and DNA-Staining

Fluorochrome	Laser (nm)	Emission (nm)	Application
DAPI	351-364	455	DNA
Hoechst 33258	351-364	478	DNA
Hoechst 33342	351-364	483	DNA
Propidiumiodid (PI)	488	617	DNA, Viability
7AAD	488	650	DNA, Viability
TOTO-3, TO-PRO-3	633 / 635	661	DNA, Viability

... in Functional Tests

Fluorochrome	Laser (nm)	Emission (nm)	Application
Indo 1	351-364	400 / 490	Calcium
Fluo-3	488	526	Calcium
Dihydrorhodamin 123	488	534	Oxidative burst
Fura Red [™]	488	672	Calcium
CFDA-SE	488	518	Proliferation



Fluorescein isothiocyanat (FITC)

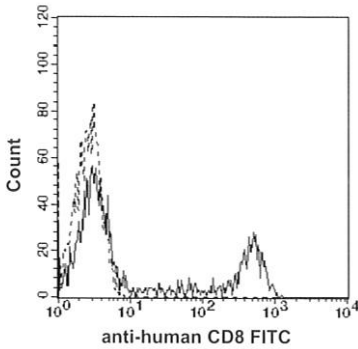
(Xanthene dye)

Excitation: 488 nm
Emission max.: 525 nm
Molecular weight: 389 Da

General remarks

FITC has a good quantum yield. Almost half of the absorbed photons are emitted in the form of fluorescent light. Compared to Alexa Fluor® 488, a fluorochrome with similar spectral characteristics, however, FITC exhibits weaker intensity of fluorescence and less photostability. The intensity of fluorescence of the fluorochrome FITC is also affected by the pH value. These characteristics must be taken into account in each respective application.

Flow Cytometry:

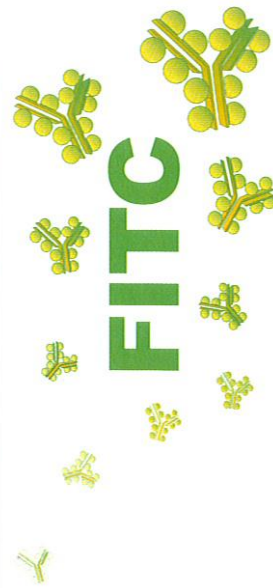


Human peripheral blood lymphocytes were marked with FITC-conjugated anti-human CD8 antibodies as indicated by the manufacturer (Caltag). The analysis was carried out on a FACSCaliber (BD).

FITC is widely used in the area of flow cytometry. This fluorochrome can usually be measured with any standard commercial flow cytometer and sorter. Compensation between FITC and other fluorochromes needs to be taken into consideration only in the case of phycoerythrin (E_{max} : 575 nm) due to the overlapping emission spectra. However, compensation of the two fluorochromes is usually unproblematic. FITC is therefore often used in multicolor flow cytometry. The low fluorescent intensity of the FITC fluorochrome should be taken into account when considering its usage. It is a good idea to detect strongly expressed antigens with FITC-conjugated antibodies and to demonstrate the presence of weakly expressed markers with the help of stronger fluorochromes such as Alexa Fluor® 488 or phycoerythrin (R-PE, E_{max} : 575nm).

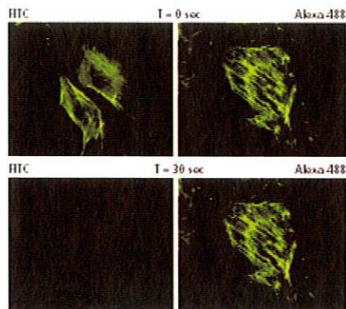
FC	EPICS™ XL /MCL	Cytomics™ FC500	EPICS™ Altra	BD FACScan™	BD FACSCalibur™	BD LSR II™	BD FACSCanto™	BD Vantage™ SE	BD FACSAria™	CyAn™ MLE	CyAn™ LX	MoFlow™	CyFlow™ SL	CyFlow™ space	CyFlow™ ML	PAS™	PAS III™
Laser	488	488	488	488	488	488	488	488	488	488	488	488	488	488	488	488	488
Channel*	FI1	✓	FI1	FI1	FI1	✓	✓	FI1	✓	✓	✓	✓	✓	✓	✓	✓	✓
Filter optimization																	

FC: Flow cytometer; * Standard filter configuration of the manufacturer



Microscopic applications

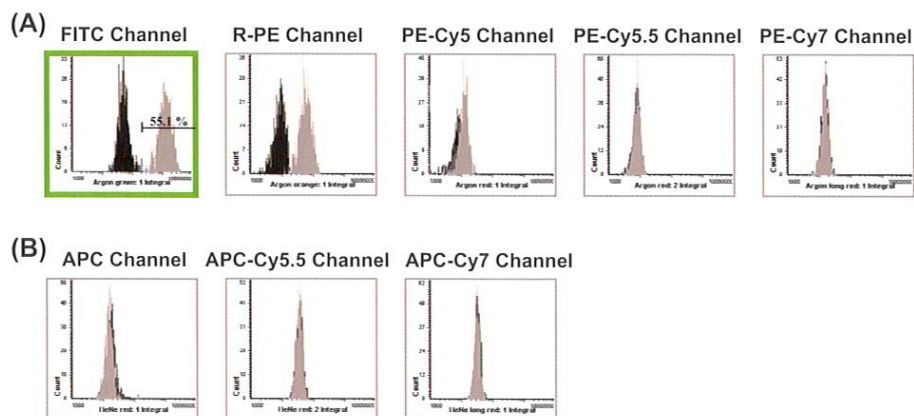
Laser Scanning Microscopy (LSM)/ Immunofluorescence (IF) Microscopy:



Actin filaments of bovine endothelial cells were marked with FITC or Alexa Fluor[®] 488-coupled phalloidin. After exposure to light for 30 seconds, FITC has a residual intensity of ca. 20%, in contrast to the fluorochrome Alexa Fluor[®] 488, which is nearly photostable. (Figure from Molecular Probes, Eugene, OR)

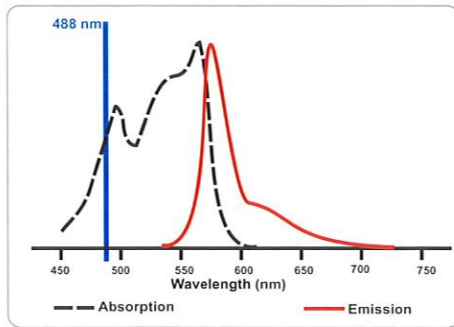
FITC-conjugated antibodies are used very widely in Laser Scanning Microscopy (LSM) and in Immunofluorescence (IF) Microscopy. However, the low photostability of the fluorochrome FITC becomes evident in these applications. In particular, high-energy excitation with a laser (LSM) results in rapid photo bleaching of the FITC fluorochrome. With special covering media, however, results can be notably improved. Nowadays more photostable alternatives to the fluorochrome FITC are available, such as Alexa Fluor[®] 488 or the Cyano dye Cy2.

Laser Scanning Cytometry (LSC):



Human leukocytes were marked with a biotinylated anti-human CD3 antibody and detected with streptavidin-FITC. Then the lymphocytes were analyzed on the LSC (CompuCyte). The figure shows the intensity of fluorescence measured in the fluorochrome channels named above. (A): fluorochromes excitable at 488 nm. (B): fluorochromes excitable at 633nm. (The method has been described by Lenz *et al.*, *Proc. of SPIE Vol. 4962, 2003*). Our thanks to Dr. Tarnok of the Leipzig Heart Center for making these data available.

FITC is often used in Laser Scanning Cytometry. The spectral characteristics of the FITC fluorochrome cause it to cross-radiate only into the R-PE channel. This must be taken into account accordingly. The use of FITC-conjugated antibodies in multicolor- analysis is therefore possible without difficulty. The intensity of the FITC fluorochrome is sufficient for the detection of antigens expressed at high concentrations. Reanalysis of FITC-marked samples is nearly impossible due to the low degree of photostability. However, photo bleaching of the samples can be delayed by the use of proper covering media.



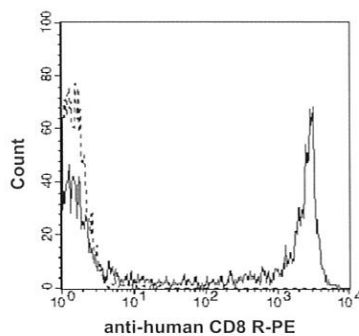
R-Phycoerythrin (R-PE)* (phycobiliprotein of red algae)

Excitation: 488 nm
Emission max.: 575 nm
Molecular weight: > 240 kDa

General remarks

Phycoerythrin (R-PE) is a constituent part of the photosynthesis apparatus of red algae. It consists of a protein and 34 phycoerythrobilin fluorochromes. This fluorochrome/protein ratio is the main reason for the bright intensity of R-PE. However, the low degree of photostability limits its range of application. Its high molecular weight and its tendency to agglomeration must be taken into consideration during the use of R-PE conjugates. Non-specific binding of the fluorochrome to cells is occasionally observed. R-PE-conjugated antibodies should not be stored at -20 °C, as freezing damages the fluorochrome.

Flow Cytometry:



Human peripheral blood lymphocytes were marked with R-PE-conjugated anti-human CD8 antibodies as indicated by the manufacturer (Caltag). The analysis was carried out on a FACSCaliber (BD).

R-PE can be measured on all standard commercial flow cytometers and sorters. Its high light intensity makes it the fluorochrome of choice in the detection of antigens expressed in low concentrations. The R-PE fluorochrome is also often used in multicolor analysis. However, the spectral characteristics of R-PE require compensation with FITC (E_{max} : 525 nm) and fluorochromes in the long-wave emission range (E_{max} : 550 to ca. 680 nm). For intracellular stainings the size of the fluorochrome should be weighed against its bright intensity. Under certain circumstances it may be advantageous to use Alexa Fluor® conjugates, or FITC conjugates for intracellular applications.

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Lasers	488	488	488	488	488	488	488	488	488	488	488	488	488	488	488	488	488
Channel*	F12	✓	F12	F12	F12	✓	✓	F12	✓	✓	✓	✓	✓	✓	✓	✓	✓
Filter optimization																	

FC: flow cytometer; * Standard filter configuration of the manufacturer

* US Patent Nr. 4.520.110, Kanad. Patent Nr.: 1.779.942, Europ. Patent Nr. 76.695

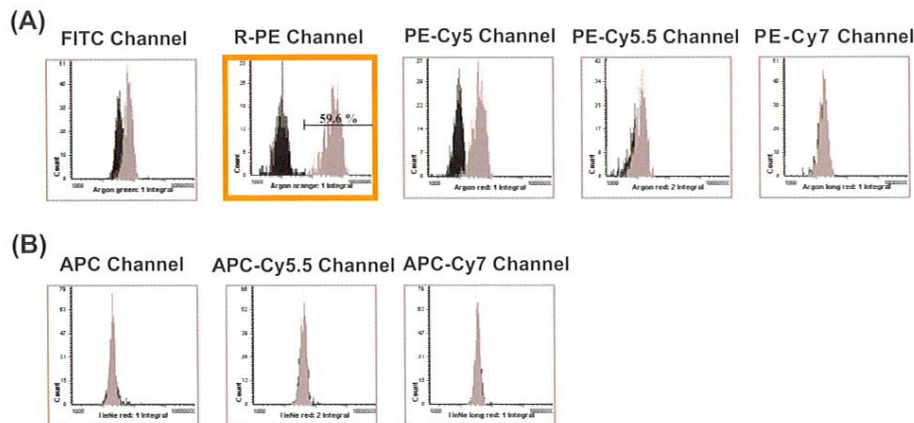


Microscopic applications

Laser Scanning Microscopy (LSM)/ Immunofluorescence (IF) Microscopy:

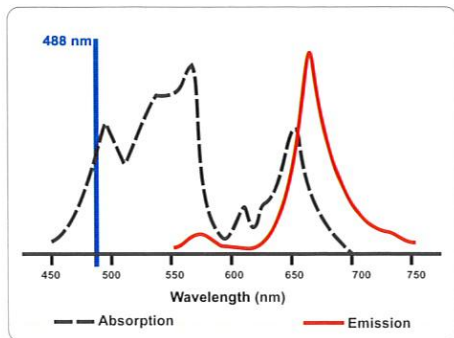
R-PE-conjugated antibodies are not usually used in Laser Scanning Microscopy (LSM), as the repeated high-energy excitation by the laser causes the dye to fade quickly. Similarly, R-PE is not often used in immunofluorescence microscopy. Under certain circumstances, special covering media can delay the photo bleaching of R-PE and improve the results. Generally, however, other red dyes are used in IF such as Cyano dye Cy3 (E_m : 565 nm) or Alexa fluorochromes.

Laser Scanning Cytometry (LSC):



Human leukocytes were marked with a biotinylated anti-human CD3 antibody and detected with streptavidin-R-PE. Then the lymphocytes were analyzed with the LSC (CompuCyte). The figure shows the intensity of fluorescence measured in the fluorochrome channels named above. (A): fluorochromes excitable at 488 nm. (B): fluorochromes excitable at 633 nm. (The method has been described by *Lenz et al., Proc. of SPIE Vol. 4962, 2003*). Our thanks to Dr. Tarnok of the Leipzig Heart Center for making these data available.

R-PE is regularly used in Laser Scanning Cytometry (LSC). Its high intensity of fluorescence permits the detection of antigens that are expressed in low concentrations. Depending on the instrument used, however, the signal intensity is somewhat less than that generated on the flow cytometer. As the above histograms show, some parts of the R-PE signal are also detectable on the FITC and PE-Cy5 channels. This makes compensation necessary. However, use in multicolor analysis is possible without difficulty.



PE-Cy5

(Tandem conjugate: R-PE und Cy5)

Other designations: Tricolor¹, Cy-Chrome², PC5³

Excitation: 488 nm

Emission max.: 670 nm

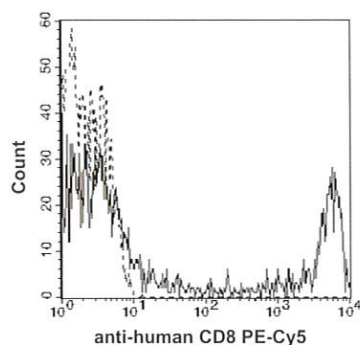
Molecular weight: R-PE + X Cy5 >242 kDa

1) Caltag Laboratories 2) BD Biosciences 3) Immunotech

General remarks

PE-Cy5 is a tandem conjugate. The R-PE part of the tandem is excited at 488 nm and functions as an energy donor. The Cyano dye Cy5, which is inadequately excited at 488 nm, is the energy acceptor. Excitation energy is transferred from R-PE to Cy5 via energy resonance transfer. Cy5 emits the energy received in the form of long-wave light (679 nm). Since the Cy5-part of the tandem conjugates can also be directly excited at 633 nm, the use of the PE-Cy5-conjugate in conjunction with the second excitation wavelengths (488 nm; 633 nm) is only possible to a certain degree. Cyano dyes like those used in PE-Cy5, among others, can bind non-specifically under certain circumstances to monocytes. However, this characteristic of the Cyano dyes has been eliminated in tandem conjugates like Tricolor by means of special production processes. Like R-PE-conjugates, PE-tandem dyes can form agglomerations.

Flow Cytometry:



Human peripheral blood lymphocytes were marked with PE-Cy5-conjugated anti-human CD8 antibodies according to the manufacturer's instructions (Caltag). The analysis was carried out on a FACSCaliber (BD).

As a rule, PE-Cy5 can be measured with all standard commercial flow cytometers and sorters. Depending on the antibody conjugation, PE-Cy5 provides bright fluorescence signals, in comparison to the FITC conjugates. However, R-PE conjugates are preferred for antigens present at very low concentrations. PE-Cy5 is often used in combination with FITC, R-PE and PE-TR. However, since the transfer of energy from R-PE to the Cy5 is incomplete, the PE-part also emits light (ca. 5%). This "lost light" is detected on the PE-channel and makes slight compensation necessary. The Cy5-part of the tandems can be excited with a 633 nm laser (E_{max} : 670 nm). A combination of PE-Cy5 and APC (E_{max} : 660 nm) is not recommended due to the very high compensation levels with instruments lacking "cross-beam compensation".

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Laser	488	488	488	488	488	488	488	488	488	488	488	488	488	488	488	488	488
Channel*	FI4	✓	✓	FI3	FI3	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Filter optimization																	

FC: flow cytometer; * Standard filter configuration of the manufacturer

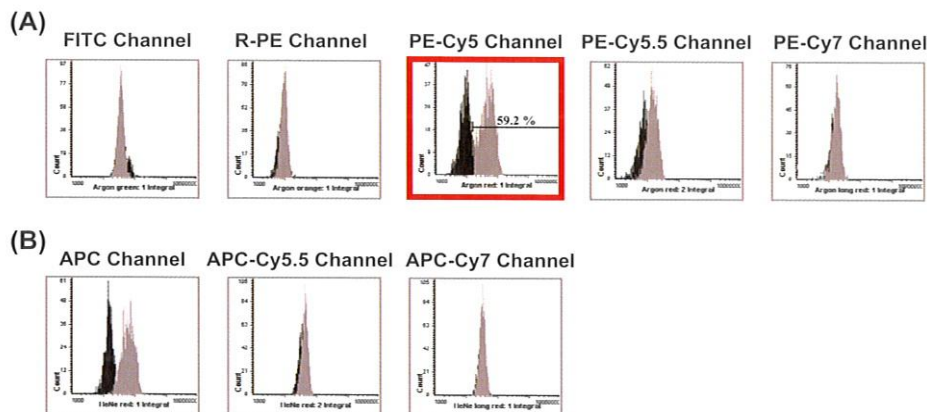


Microscopic applications

Laser Scanning Microscopy (LSM)/ Immunofluorescence (IF) Microscopy:

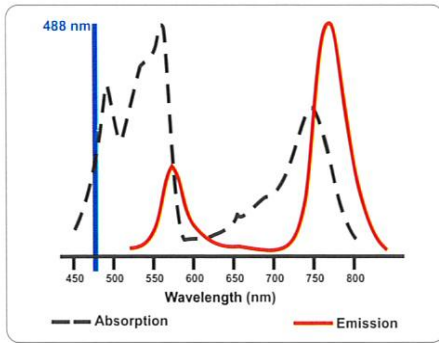
Tandem conjugates such as PE-Cy5 are not usually used in Laser Scanning Microscopy (LSM), as the repeated high-energy excitation causes the R-PE part of the conjugate to fade very quickly, so that the PE-fluorochrome can no longer function as an energy donor. One possibility, however, would be direct excitation of the Cyano dye Cy5 at 633 nm. As a rule, PE-tandem conjugates are not used in Immunofluorescence Microscopy. Conventional mercury vapour lamps can directly excite the various Cyano dyes, in contrast to the monochromatic lasers (488 nm or 633 nm excitation) used in flow cytometry. A tandem construction is therefore unnecessary. For example, Cy5 conjugates or Alexa Fluor[®] 647-conjugates are used for the emission range around 670 nm. In this emission range, however, a corresponding camera system may be required for visualization.

Laser Scanning Cytometry (LSC):



Human leukocytes were marked with a biotinylated anti-human CD3 antibody and detected with streptavidin-PE-Cy5. Then the lymphocytes were analyzed with the LSC (CompuCyte). The figure shows the intensity of fluorescence measured in the fluorochrome channels named above. (A): fluorochromes excitable at 488 nm. (B): fluorochromes excitable at 633 nm. Our thanks to Dr. Tarnok of the Leipzig Heart Center for making these data available.

PE-Cy5 is often used in Laser Scanning Cytometry (LSC). The incomplete transfer of energy from R-PE to the Cy5-dye results in a signal in the R-PE-channel which must be compensated. However, it can be used without difficulty in multicolor analysis. With optimized filter configurations and sequential re-analysis, PE-Cy5 can be used in combination with FITC, PE, APC, PE-Cy7, PE-Cy5.5, APC-Cy7, and APC-Cy5.5. The method and filter configuration have been described by *Lenz et al., in Proc. of SPIE, Vol. 4962, 2003.*



PE-Cy7

(Tandem conjugate: R-PE and Cy7)

Excitation: 488 nm

Emission max.: 767 nm

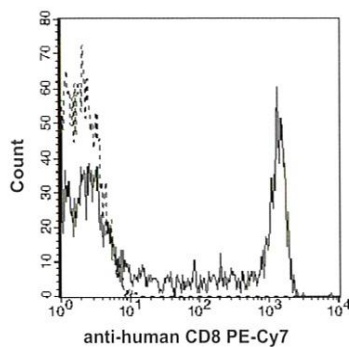
Molecular weight: R-PE + X Cy7 >242 kDa

General remarks

The PE-tandem conjugate PE-Cy7 is excited at 488 nm. Its maximum emission lies around 767 nm. Cy7 is the most photosensitive of the Cyano dyes. Unnecessary exposure to light should be avoided during work with PE-Cy7 in order to avoid photo-oxidation of the Cy7 part. For this reason, light-proof containers are used to package the conjugate in order to protect it. In some instances Cy7-conjugates are also sensitive to fixing solutions containing paraformaldehyde.



Flow Cytometry:



Human peripheral blood lymphocytes were marked with PE-Cy7-conjugated anti-human CD8 antibodies according to the manufacturer's instructions (Caltag). The analysis was carried out with an FACSCaliber (BD).

PE-Cy7-conjugates can be detected with many flow cytometers and sorters. Even though PE-Cy7 has a very long-wave emission maximum of 767 nm, it often emits brighter signals than the short-wave fluorochrome FITC (525 nm). Samples marked with PE-Cy7 should be analyzed immediately if possible in order to ensure optimum signal discrimination. PE-Cy7 can be used in multicolor analysis in combination with FITC, R-PE, PE-Cy5.5, APC and APC-Cy7. When the proper filter configuration is used, compensation of these fluorochromes is possible without difficulty.

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Laser	488	488	488	488	488	488	488	488	488	488	488	488	488	488	488	488	488
Channel*		✓		FL3	FI3	✓	✓	FI3	✓	✓	✓		✓	✓	✓	✓	✓
Filter optimization			✓									✓					

FC: flow cytometer; * Standard filter configuration of the manufacturer

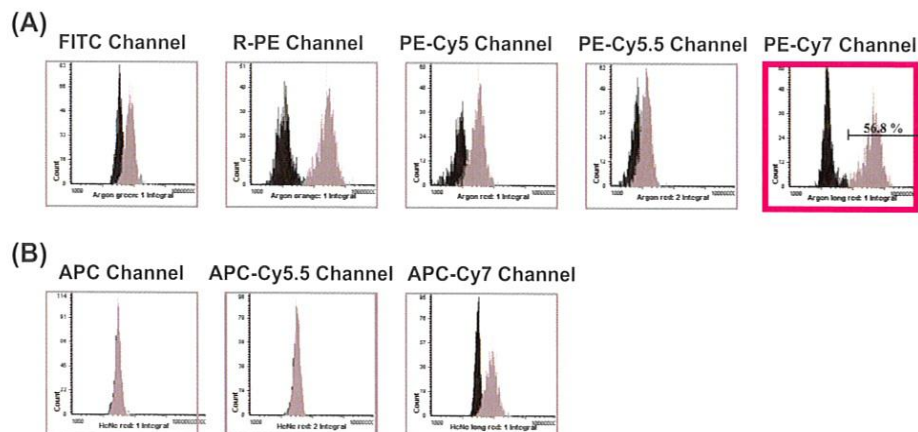
Microscopic applications

Laser Scanning Microscopy (LSM)/ Immunofluorescence (IF) Microscopy:

PE-Cy7 is not usually used in either Laser Scanning Microscopy (LSM) or in Immunofluorescence Microscopy.

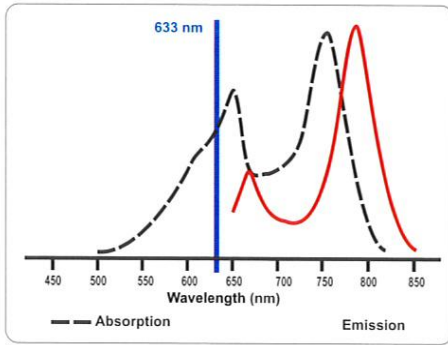
PE-Cy7 emits very long-wave and thus low-energy fluorescence signals which are very difficult to detect. The low degree of photostability of PE-Cy7 also makes it unsuitable for these applications.

Laser Scanning Cytometry (LSC):



Human leukocytes were marked with a biotinylated anti-human CD3 antibody and detected with streptavidin-PE-Cy7. Then the lymphocytes were analyzed on the LSC (CompuCyte). The figure shows the intensity of fluorescence measured in the fluorochrome channels named above. (A): fluorochromes excitable at 488 nm. (B): fluorochromes excitable at 633nm. Our thanks to Dr. Tarnok of the Leipzig Heart Center for making these data available.

PE-Cy7 can be used on the Laser Scanning Cytometer (LSC) following corresponding filter adjustment. The intensity of fluorescence of PE-Cy7 is often lower than that of the R-PE-marked antibodies, with their strong signals. After optimization of the filter configurations and sequential re-analysis, PE-Cy7 can be used in combination with FITC, PE, APC, PE-Cy5, PE-Cy5.5, APC-Cy7 and APC-Cy5.5. However, attention must be given to cross-radiation into other fluorescence channels such as R-PE, PE-Cy5 or PE-Cy5.5. The method and filter configuration have been described by *Lenz et al., Proc. of SPIE Vol. 4962, 2003.*



APC-Cy7

(Tandem conjugate: APC und Cy7)

Excitation: 633 nm

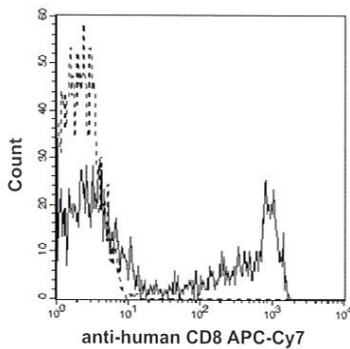
Emission max.: 767 nm

Molecular weight: APC + X Cy7 >105 kDa

General remarks

APC-Cy7 is an APC tandem conjugate. The APC part is excited at 633 nm. The energy of the donor (APC) is transferred to the acceptor (Cyano dye Cy7) by means of Fluorescence Resonance Energy Transfer (FRET); this acceptor in turn emits the energy received by it in the form of long-wave light (E_{max} : 767 nm). In order to avoid any possible photo-oxidation of the Cy7-part, unnecessary exposure to light should be avoided. In some instances Cy7-conjugates are also sensitive to fixing solutions containing paraformaldehyde.

Flow Cytometry:



Human peripheral blood lymphocytes were marked with APC-Cy7-conjugated anti-human CD8 antibodies according to the manufacturer's instructions (Caltag). Analysis was carried out with a modified FACScan after installation of a 633 nm laser.

The fluorochrome APC-Cy7 can be measured using flow cytometers and sorters equipped with a 633 nm laser. The signal intensity of APC-Cy7 is often lower than that of APC; this should be taken into account in the respective application. Samples marked with APC-Cy7 should be analyzed immediately after staining. This significantly reduces the possibility of light-induced oxidation of the Cy7 part. Now, however, more light-stable conjugates such as APC Alexa Fluor[®] 750 have also become available. Provided the necessary equipment is available, APC-Cy7 can be used with FITC, R-PE, PECy5.5, PE-Cy7 and APC without difficulty.

FC	EPICS [™] XL /-MCL	Cytomics [™] FC500	EPICS [™] Altra	BD FACScan [™]	BD FACSCalibur [™]	BD LSR II [™]	BD FACSCanto [™]	BD Vantage [™] SE	BD FACSAria [™]	CyAn [™] MLE	CyAn [™] LX	MoFlow [™]	CyFlow [™] SL	CyFlow [™] space	CyFlow [™] ML	PAS [™]	PAS III [™]
Laser	488	488	488	488	488	488	488	488	488	488	488	488	488	488	488	488	488
Channel*						✓	✓	F15	✓	✓	✓		✓	✓	✓	✓	✓
Filter optimization		✓	✓									✓					

FC: Flow cytometer; * Standard filter configuration of the manufacturer

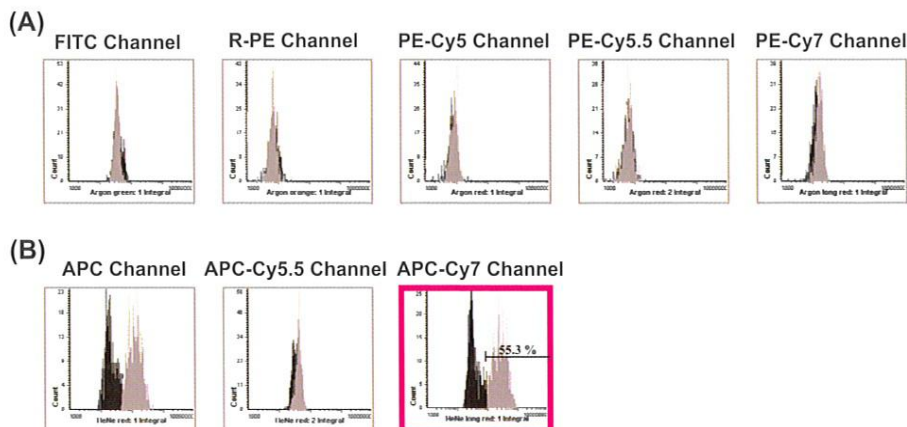


Microscopic applications

Laser Scanning Microscopy (LSM)/ Immunofluorescence (IF) Microscopy:

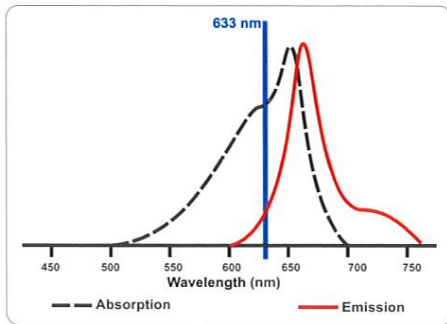
APC-Cy7 is hardly ever used in Laser Scanning Microscopy (LSM), as an adequate number of fluorochromes with greater photostability are available for this application. Fluorochromes in this wavelength range (767 nm) are not normally used in immunofluorescence microscopy, since the low-energy fluorescence signals (E_{max} : 767 nm) cannot be perceived by the human eye.

Laser Scanning Cytometry (LSC):



Human leukocytes were marked with a biotinylated anti-human CD3 antibody and detected with streptavidin-APC-Cy7. Then the lymphocytes were analyzed on the LSC (CompuCyte). The figure shows the intensity of fluorescence measured in the fluorochrome channels named above. (A): fluorochromes excitable at 488 nm. (B): fluorochromes excitable at 633nm. Our thanks to Dr. Tarnok of the Leipzig Heart Center for making these data available.

APC-Cy7 can be used with the Laser Scanning Cytometer (LSC) after optimizing filter settings. However, APC-Cy7's intensity of fluorescence is below the signal intensity of APC-marked antibodies. It can be used without difficulty in multicolor analysis, since there is no cross-radiation into other fluorescence channels (R-PE, PE-Cy5, PE-Cy5.5). A signal must be compensated for only in the APC channel. With optimized filter configurations and sequential re-analysis, APC-Cy7 can be used in combination with FITC, PE, APC, PE-Cy5, PE-Cy5.5, PE-Cy7 and APC-Cy5.5. The method and filter configuration have been described by Lenz *et al.*, in *Proc. of SPIE*, Vol. 4962, 2003.



Allophycocyanin (APC)* (phycobiliprotein of the cyanobacteria)

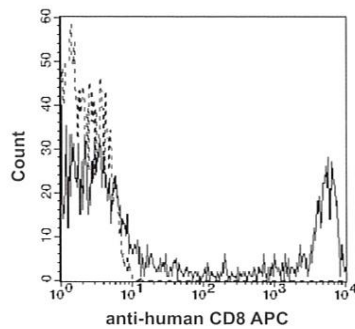
Excitation: 633/ 635 nm
Emission max.: 660 nm
Molecular weight: 105 kDa

General remarks

APC is an accessory photosynthesis pigment of the cyanobacteria/blue algae. It consists of a protein and 6 phycocyanobilin chromophores. APC is excited at 633/ 635 nm ("2nd laser"). It is used in various tandem conjugates such as APC-Cy5, APC-Cy5.5, and APC-Cy7 as an energy donor.



Flow Cytometry:



Human peripheral blood lymphocytes were marked with APC-conjugated anti-human CD8 antibodies as indicated by the manufacturer (Caltag). The analysis was carried out with a FACSCaliber (BD).

The fluorochrome APC can be measured on all standard commercial flow cytometers and sorters equipped with a 2nd laser (633 nm). The intensity of fluorescence is comparable to that of R-PE signals (although differences may be noticed depending on the antibody conjugation). APC is used widely in the area of multicolor flow cytometry, as it radiates only marginally into other fluorescence channels and is not excited at 488 nm ("1st laser"). When the proper instrument is used, APC can be combined with FITC, PE, PE-tandem conjugates (e.g. PE-Cy5.5, PECy7) and various APC-tandem conjugates. However, APC should not be combined with PE-Cy5 when flow cytometers without "cross-beam compensation" are used, since the spectral characteristics of the two fluorochromes require a high degree of compensation.

FC	EPICS™ XL /MCL	Cytomics™ FC500	EPICS™ Altra	BD FACScan™	BD FACSCalibur™	BD LSR II™	BD FACSCanto™	BD Vantage™ SE	BD FACSAria™	CyAn™ MLE	CyAn™ LX	MoFlow™	CyFlow™ SL	CyFlow™ space	CyFlow™ ML	PAS™	PAS III™
Laser	633	633	633	633	633	633	633	633	633	633	633	633	633	633	633	633	633
Channel*		✓	✓		F14	✓	✓	F14 (2)	✓	✓	✓	✓	✓	✓	✓	✓	✓
Filter optimization																	

FC: flow cytometer; * Standard filter configuration of the manufacturer

* US Patent Nr. 4.520.110, Kanad. Patent Nr.: 1.779.942, Europ. Patent Nr. 76.695

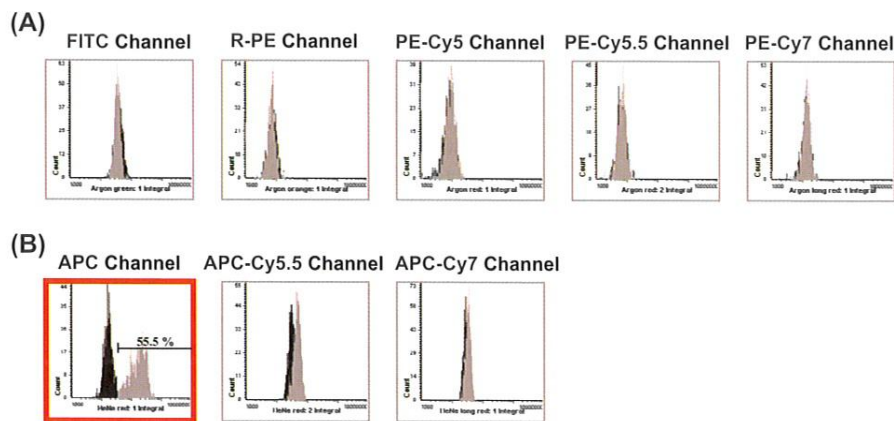
Microscopic applications

Laser Scanning Microscopy (LSM)/ Immunofluorescence (IF) Microscopy:

Given the proper instrument configuration, APC can also be used in Laser Scanning Microscopy (LSM). However, the use of Cy5 conjugates or Alexa Fluor® 647 conjugates is more common.

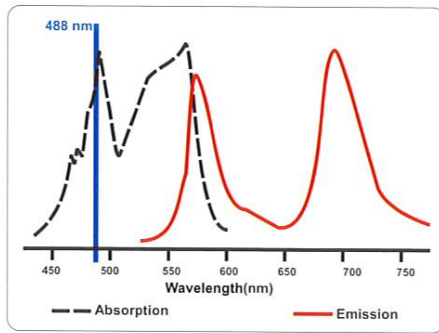
When APC is used in immunofluorescence microscopy, it must be remembered that the sensitivity of the human eye diminishes for wavelengths above 650 nm. Under certain circumstances, the use of corresponding camera systems may be required to visualize and detect fluorochromes with a maximum emission of more than 650 nm (e.g. APC).

Laser Scanning Cytometry (LSC):



Human leukocytes were marked with a biotinylated anti-human CD3 antibody and detected with streptavidin-APC. Then the lymphocytes were analyzed on the LSC (CompuCyte). The figure shows the intensity of fluorescence measured in the fluorochrome channels named above. (A): fluorochromes excitable at 488 nm. (B): fluorochromes excitable at 633 nm. Our thanks to Dr. Tarnok of the Leipzig Heart Center for making these data available.

Given the proper laser equipment (633 nm), APC can be used without difficulty in Laser Scanning Cytometry (LSC). This fluorochrome is often used in multicolor analysis since it provides bright signals and radiates only marginally into the other fluorescence channels. The literature describes the use of APC in combination with FITC, PE, PE-Cy5, PE-Cy7, PE-Cy5.5, APC-Cy7 and APC-Cy5.5 (Lenz *et al.*, *Proc. of SPIE Vol. 4962, 2003*).



PE-Cy5.5

(tandem conjugate: R-PE and Cy5.5)

Excitation: 488 nm

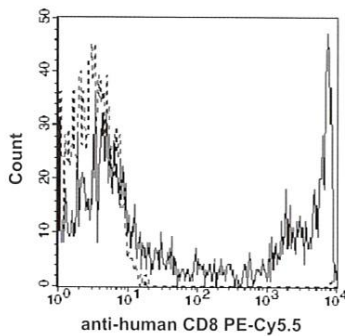
Emission max.: 694 nm

Molecular weight: R-PE + X Cy5.5 >242 kDa

General remarks

The tandem conjugate PE-Cy5.5 can be excited via its R-PE component at 488 nm. Via energy resonance transfer the energy is transferred to the Cyano dye Cy5.5, which then emits this energy in the form of long-wave light (E_{max} : 694 nm). The Cyano dye Cy5.5 is only weakly excited at 633 nm. In contrast to PE-Cy5, PE-Cy5.5 can therefore be combined with APC and other fluorochromes of this excitation wavelength without difficulty. Cyano dyes such as Cy5.5 can bind non-specifically to monocytes under certain circumstances. However, special production procedures have been developed which successfully minimize this non-specific binding in the tandem conjugates.

Flow Cytometry:



Human peripheral blood lymphocytes were marked with PE-Cy5.5-conjugated anti-human CD8 antibodies according to the manufacturer's instructions (Caltag). The analysis was carried out on an FACSCaliber (BD).

The fluorochrome PE-Cy5.5 can be measured with many flow cytometers and sorters. However, filter optimization is sometimes necessary (see the table). PE-Cy5.5 offers some advantages over PE-Cy5 and PerCP for flow cytometry. It is a very bright fluorochrome that also yields good results in sorting applications (high-energy excitation). In contrast to PE-Cy5, PE-Cy5.5 can also be used without "cross-beam compensation" in combination with APC. Generally, compensation between PECy5.5 and APC is below 1% (depending on instrument and settings). Compensation with the PE-channel is possible without difficulty. When certain instruments are used, PE-Cy5.5 can be combined with FITC, R-PE, PE-Cy7, APC and APC-Cy7 among others.

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Laser	488	488	488	488	488	488	488	488	488	488	488	488	488	488	488	488	488
Channel*				F13	F13	✓	✓	F16	✓	✓	✓		✓	✓	✓	✓	✓
Filter optimization	✓	✓	✓			✓	✓	✓	✓	✓	✓						

FC: flow cytometer; * Standard filter configuration of the manufacturer



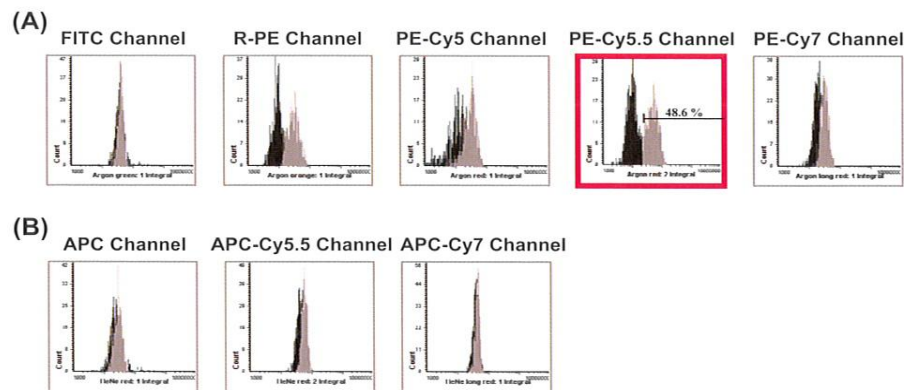
Microscopic applications

Laser Scanning Microscopy (LSM)/ Immunofluorescence (IF) Microscopy:

PE-Cy5-5 is only used under certain conditions in Laser Scanning Microscopy (LSM). This is because repeated high-energy excitation quickly causes the R-PE-part of the conjugate to fade, so that it can no longer exercise its function as an energy donor.

It is unnecessary to use PE-tandem conjugates in immunofluorescence microscopy, as monochromatic light is not used here for excitation (as it is in flow cytometry or LSM) but rather white light. The Cyano dyes can therefore be excited directly. However, the intensity of fluorescence in the longwave range decreases steadily, so that Cy5.5 (E_{\max} : 694 nm) would probably yield unsatisfactory results in microscopy.

Laser Scanning Cytometry (LSC):



Human leukocytes were marked with a biotinylated anti-human CD3 antibody and detected with streptavidin-PE-Cy5.5. Then the lymphocytes were analyzed on the LSC (CompuCyte). The figure shows the intensity of fluorescence measured in the fluorochrome channels named above. (A): fluorochromes excitable at 488 nm. (B): fluorochromes excitable at 633 nm. Our thanks to Dr. Tarnok of the Leipzig Heart Center for making these data available.

After corresponding filter adjustment, PE-Cy5.5 can also be used on the Laser Scanning Cytometer. The intensity of fluorescence is comparable to that of PE-Cy5, but lies below the signal strength of R-PE-marked antibodies. As the histograms show, compensation is required only for the R-PE and PE-Cy5 channels. Use in multicolor analysis is therefore possible. With optimized filter configurations and sequential re-analysis, PE-Cy5.5 can be used in combination with FITC, PE, APC, PE-Cy5, PE-Cy7, APC-Cy7 and APC-Cy5.5. The method and filter configuration have been described by *Lenz et al., in Proc. of SPIE, Vol. 4962, 2003.*