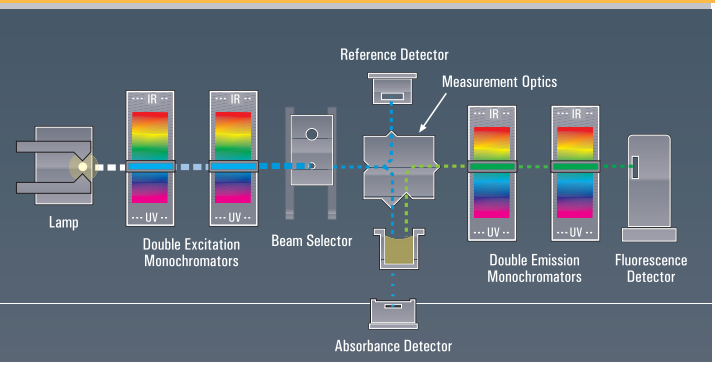


This application note describes optimization of fluorometric Caspase-3 assay performance with the spectral scanning feature of the Thermo Scientific Varioskan spectrophotometer and spectrofluorometer. Excitation and emission wavelengths producing the strongest dose response over Caspase-3 concentration range were considered to be the optimal for the assay. In this study, the optimal excitation wavelength was 370 nm and the emission wavelength was 490 nm when the commonly used wavelengths are 400 nm for excitation and 505 nm for emission.

Optimization of fluorometric Caspase-3 assay with Thermo Scientific Varioskan® spectral scanning

Jorma Lampinen, PhD, Senior Application Scientist, Thermo Fisher Scientific, Vantaa, Finland

Abstract

In this paper, we present the principle of the assay optimization for fluorometric Caspase-3 assays. It is based on the measurement of fluorescence spectra with the Thermo Scientific Varioskan spectral scanning fluorometer. From this spectral data, the optimal assay settings for the maximum sensitivity are determined, including excitation and emission wavelengths. The optimization procedure is shown with ApoTarget™ Caspase-3 assay (product number KHZ0012) from BioSource International Inc, Camarillo, CA.

Caspase-3 belongs to Interleukin-1ß Converting Enzyme (ICE) family of cysteine proteases and is present in cells as an inactive 32 kDa proenzyme. It is activated specifically during apoptosis and is therefore one of the most important markers for apoptotic activity. ApoTarget Caspase-3 protease assay is based on the recognition of the DEVD (Asp-Glue-Val-Asp) amino acid sequence linked to fluorometric label AFC (7-amino-4-trifluoromethyl coumarin) by Caspase-3. Free AFC has a fluorometric emission maximum at 505 nm but when AFC is linked in the DEVD peptide, this emission is quenched by FRET (fluorescence resonance energy transfer). Fluorescent emission maximum at 505 nm is restored when AFC is cleaved off the DEVD peptide. The resulting fluorescence is quantitated and is proportional to Caspase-3 activity in the sample.

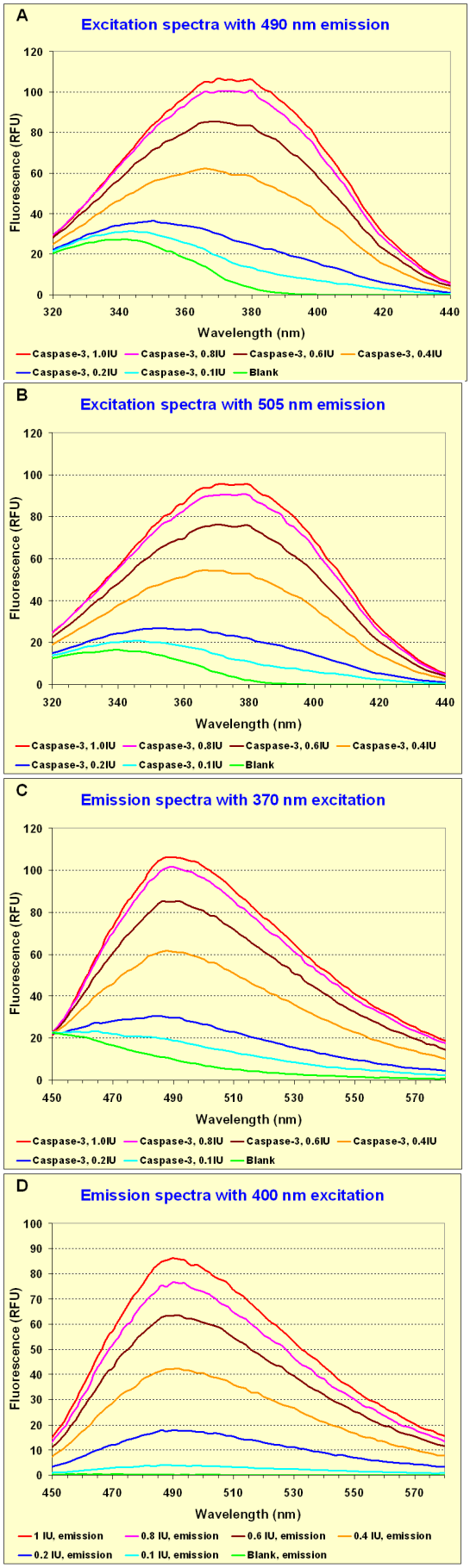


Figure 1. Excitation and emission spectra of the Caspase-3 assay with DEVD-AFC. Figures 1A and 1B show excitation spectra with two different fixed emission wavelengths and Figures 1C and 1D are showing emission spectra with fixed excitation wavelengths.

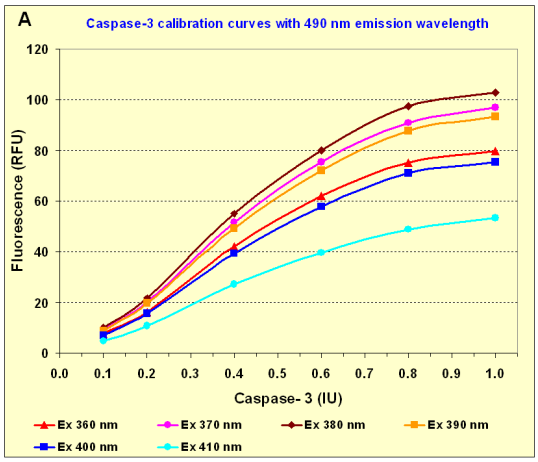
Materials and Methods

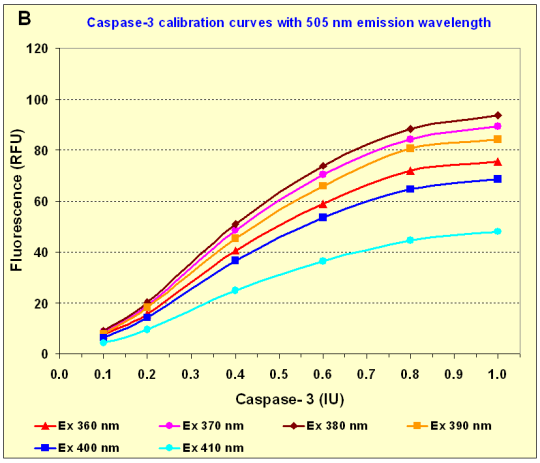
All reagents were used according to the instructions of the manufacturer with the following modifications: For Caspase-3 assay, a purified recombinant human Caspase-3 from BioSource (product number PHZ0014) was used as a sample. A dilution series from 0.1 to 1.0 IU of Caspase-3 in PBS was prepared and 50 µl of the dilution was transferred into the wells of black 96-well microtiter plate (Thermo Scientific Microtiter®). Then 50 µl of the fluorometric Caspase-3 substrate DEVD-AFC was added. Both emission and excitation spectra were measured after two hour incubation. Excitation spectra were measured between 320 - 440 nm using fixed emissions at 490 nm and 505 nm. Emission spectra were measured between 400 - 580 nm using fixed excitations at 370 nm and 400 nm. All these four spectra were measured from all Caspase-3 dilutions and from all time points.

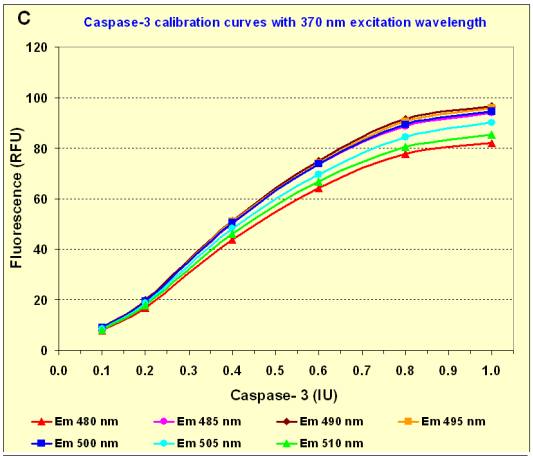
Results and discussion

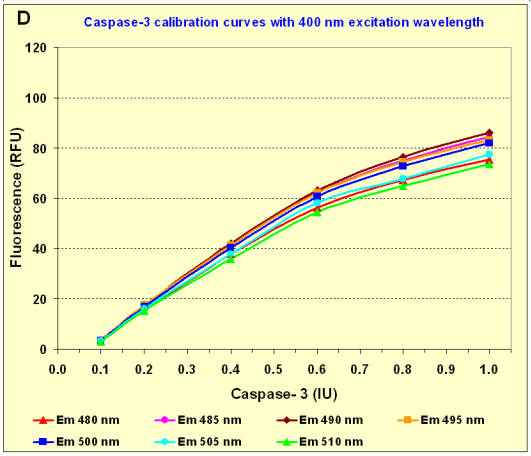
The four measured spectra of the Caspase-3 protease reaction with DEVD-AFC are shown in Figure 1. The most important findings from these figures are that both the excitation and emission maxima are clearly different from the wavelengths normally recommended for this fluorometric label. AFC is commonly measured with 400 nm excitation and 505 nm emission, when the maximum values are somewhat lower, 370 for the excitation (Figures 1A and 1B) and 490 for the emission (Figures 1C and 1D). Another important finding from Figure 1 is that the signal level obtained with the 490 nm emission is about 10% higher that the signal level from 505 nm. Even a stronger difference is noticed from the figures of the emission spectra (figures 1C and 1D). In this case, the difference is almost 25% and 370 nm excitation gives clearly higher fluorescence.

Calibration curves of Caspase-3 reaction were calculated from the spectral data for both fixed excitation and emission wavelengths. Resulting calibration curves are shown in Figure 2. It is clearly seen from the figures that the selection of excitation and emission wavelengths have a strong effect on the generated calibration curve. With this Caspase-3 assay, the selection of excitation wavelength is more important than selection of emission wavelength because there are much stronger changes in the dose response when the excitation wavelength is changed than when the emission wavelength is changed. Based on the Figures 2A and 2B, the excitation wavelength producing the strongest dose response is 380 nm with both tested emission wavelengths, therefore being optimal selection for the assay. The optimal emission wavelength can be selected based on Figures 2C and 2D and the best choice would be 490 nm.









Conclusions

Fluorometric assays have traditionally been performed with filter instruments and therefore commonly recommended excitation and emission wavelengths may be based on the filter availability in addition to the spectral behaviour of the label. Rather strong differences in the assay performance can be obtained by using fluorescence spectral scanning for optimization of the excitation and emission wavelengths even with the commercial fluorometric kits.

With the fluorometric Caspase-3 assay kit from BioSource Inc, a better dose response in the Caspase-3 assay was achieved when the Caspase-3 activity was measured using 370 nm excitation and 490 nm emission wavelengths instead of the 400 nm excitation and 505 nm emission wavelengths that are commonly used for the measurement of AFC.

References

Lazebnik Y., et al. (1994) Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. Nature 371(6495):346-347.

Casciola-Rosen L.A., et al. (1996) Apopain/CPP32 cleaves proteins that are essential for cellular repair: a fundamental principle of apoptotic death. J. Exp. Med. 183(5):1957-1964.

Anel A., et al. (1997) Inhibition of CPP32-like proteases prevents granzyme B- and Fas-, but not granzyme A-based cytotoxicity exerted by CTL clones. J. Immunol. 158(5):1999-2006.

Stennicke H.R., et al. (1997) Biochemical characteristics of Caspases-3, -6, -7 and -8. J. Biol. Chem. 272(41):25719-25723, 1997.

Talanian R.V., et al. (1997) Substrate specificities of caspase family proteases. J. Biol. Chem. 272(15):9677-9682.

Jaeschke H., et al. (1998) Activation of Caspase-3 (CPP32) like proteases is essential for TNF-α induced hepatic parenchymal cell apoptosis and neutrophil-mediated necrosis in a murine endotoxin shock model. J. Immunol. 160(7):3480-3486.

Wang, J., L. Zhen, M.G. Klug, D. Wood, X Wu, and J. Mizrahi (2000) Involvement of caspase 3- and 8-like proteases in ceramide-induced apoptosis of cardiomyocytes. Journal of Cardiac Failure 6(3):243-249.

Reffey, S.B, J.U. Wurthner, W.T. Parks, A.B. Roberts, and C.S. Duckett (2001) X-linked inhibitor of apoptosis protein functions as a cofactor in transforming growth factor-ß signaling. J. Biol. Chem. 276(28):26542-26549.

Caplen, N.J., J.P. Taylor, V.S. Statham, F. Tanaka, A. Fire, and R.A. Morgan (2002) Rescue of polyglutamine mediated cytotoxicity by double-stranded RNA-mediated RNA interference. Human Mol. Gen. 11(2):175-184.

Figure 2. Caspase-3 calibration curves from different spectral scannings. Figures 2A and 2B present calibration curves with different excitation wavelengths with two emission wavelength and figures 2C and 2D present the calibration curves with different emission wavelengths with two excitation wavelengths.

North America: USA/Canada +1 866 984 3766
Europe: Austria +43 1 801 40 0, Belgium +32 2 482 30 30, Finland +358 9 329 100, France +33 2 2803 2000, Germany national toll free 08001-536 376, Germany international +49 6184 90 6940, Italy +39 02 95059 1, Netherlands +31 76 571 4440,Russia/CIS +7 095 225 11 15, Spain/Portugal +34 93 223 3154, Switzerland +41 44 454 12 12, UK/Ireland +44 870 609 9203**Asia:** China +86 21 6865 4588 or +86 10 5850 3588, India +91 22 5542 9494, Japan +81 45 453 9220, Other Asian countries +852 2885 4613**Countries not listed:** +49 6184 90 6940 or +33 2 2803 2000

www.thermo.com

Lit no: AN0304MIB