SUPPLEMENTAL MATERIALS AND METHODS

Staining of *S. cerevisiae* cells with VAD-FMK and DEVD-FMK caspase inhibitors can be artefactual

Presence of active caspase (early apoptotic marker) in higher eukaryotic cells is usually monitored by *in situ* staining of cells with fluorochrome-labelled inhibitors of caspases (FLICA). This kind of caspase inhibitors is composed of peptides with Asp residue at P1 position (acting as caspase pseudosubstrates) linked to e.g. fluoromethyl ketone (FMK) which makes them irreversible competitive inhibitors of active caspasas and with blocked N end by N-benzoylcarbonyl group (Z-VAD-FMK) (Ekert et al., 1999) (Fig. S1A). In FLICA, fluorochrome substitutes the N-terminal group (Fig.S1B). FMK derivatives are usually prepared as methyl esters of the aspartic acid to allow better entry of the inhibitor into living cells. The prerequisite of their proper function as specific caspase inhibitors after entering the cells is methyl ester hydrolysis by cellular esterases (Fig.S1A).

![Fig.S1](image)

Fig.S1. Examples of caspase inhibitors. A. Non-fluorescent O-methylated Z-VAD-FMK; cleavage of methyl ester is marked by arrow. In FITC-VAD-FMK, the fluorochrome fluorescein isothiocyanate (B) substitutes the N-benzoylcarbonyl group in Z-VAD-FMK.

This FLICA staining of active caspase was used by Madeo et al. (2002) also for staining of yeast metacaspase Mca1p. However, Wysocki and Kron (2004) questioned this method, saying that FITC-VAD-FMK stains nonspecifically only propidium iodide (PI) positive, i.e. permeabilised yeast cells. Some problems concerning specificity of FLICA binding and thus active caspase detection in mammalian apoptotic cells are also discussed by Pozarowski et al. (2003). Therefore, we checked applicability of FLICAs in our yeast colony model using following approaches.

A) Cells from *S.cerevisiae* colonies (10 days old) were stained with FITC-VAD-FMK, (CaspACE™ FITC-VAD-FMK *In Situ* Marker, Promega), FITC-DEVD-FMK, (CaspGLOW™ Fluorescein Active Caspase-3 Staining Kit, BioVision), Red-VAD-FMK (CaspGLOW™ Red Active Caspase Staining Kit, BioVision) for 30 min, 1 and 2 h and with FITC (Sigma) for 30 min at 30 °C in Wash Buffer (BioVision) and in different combinations as indicated in Tab.S1. In parallel, to test the FLICA binding specificity, cell samples were preincubated with non-fluorescent Z-VAD-FMK (BioVision) and stained as indicated (Tab.S1). The number of stained cells was counted under the fluorescence microscope.

VAD-FMK is a broad spectrum caspase inhibitor also mentioned as pan-caspase inhibitor. We compared staining of apoptotic yeast cells with FITC-VAD-FMK and Red-VAD-FMK. Additionally, we used also...
FITC-DEVD-FMK because Madeo et al. (2002) assessed that DEVD-AMC (AMC = 7-Amino-4-methylcoumarin) is not the substrate of yeast Mca1p.

Tab.S1: Staining of *S.cerevisiae* cells with different combinations of FLICA and FITC.

<table>
<thead>
<tr>
<th>1st treatment</th>
<th>2nd treatment</th>
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<th>FITC-VAD-FMK</th>
<th>Red-VAD-FMK</th>
<th>FITC-DEVD-FMK</th>
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<td>Z-VAD-FMK</td>
<td>G</td>
<td></td>
<td>R</td>
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</tbody>
</table>

* All cells stained with FITC-VAD-FMK or FITC-DEVD-FMK were stained also with Red-VAD-FMK (Fig.S2A)
** All stained cells were stained both with FITC and Red-VAD-FMK (Fig.S2B)

G, green fluorescence, R, red fluorescence, R/G or G/R, red and green fluorescence of the same cells. The fraction of stained cells was the same in all parallel stainings (18 %, approximately).

B) Cells stained with FITC and FITC-VAD-FMK were counterstained with propidium iodide (PI) (Fig.S2C, D). Besides cells stained both with FITC-VAD-FMK and PI (permeabilized cells), significant fraction of cells (50 % of all stained cells, approximately) was stained only by FITC-VAD-FMK (Fig.S2C). Same result was obtained for double staining with FITC and PI (Fig.S2D).

Fig.S2: Comparison of staining with FLICA and FITC and counterstaining with PI.
C) Yeast cells permeabilized either with 50 % EtOH or by 2 min boiling at 95°C were stained by FITC and FITC-VAD-FMK (Fig. S3).

**Fig.S3:** Staining of dead cells with FITC and FITC-VAD-FMK. Bar 5 µm.

**CONCLUSIONS:**
- FITC-VAD-FMK, Red-VAD-FMK and FITC-DEVD-FMK stain the same fraction of cells that are not stained by PI (cells that are not permeabilized), i.e. FLICAs stain living cells. Staining with Red-VAD-FMK cannot be inhibited by pretreatment with FITC-VAD-FMK and vice versa. Moreover, staining with any of the three FLICA cannot be inhibited by pretreatment with non-fluorescent Z-VAD-FMK. This implies that at least in our case, the FLICA inhibitors do not specifically bind to active caspase-like protease.
- FITC stains the same non-permeabilized fraction of cells as Red-VAD-FMK, i.e. staining is not dependent on peptide VAD specific binding to active caspase. **This means that FLICA staining is not suitable for caspase-like protease detection in yeast cells.**
- Permeabilized cells are effectively stained by FITC-VAD-FMK, Red-VAD-FMK as well as by FITC.

**Staining of S.cerevisiae cells with D₂R substrate appears to be specific for a detection of caspase-like or another ASPase activity.**

To avoid artefacts caused by non-specific binding of caspase inhibitor FITC-VAD-FMK in yeast cells (Wysocki and Kron, 2004; see above), we monitored intracellular caspase-like (or other protease able to cleave its substrates after Asp residue; ASPase) activity directly by use of its substrate D₂R ((Asp)₂-Rhodamine 110) (Fig.S4D), which was shown to become fluorescent only after its cleavage by caspase (Hug et al., 1999). Non-fluorescent substrate D₂R enters the intact cell and can be cleaved by direct action of activated caspases (or by other ASPases) to green fluorescent monosubstituted rhodamine 110 and free rhodamine 110 (Hug et al., 1999) (Fig.S4D). In order to check specificity of the staining, we performed several tests with following results:

- **A)** D₂R and Red-VAD-FMK stain different cells (Fig.S4A).
- **B)** D₂R and propidium iodide stain different cells (Fig.S4B).
- **C)** Yeast cells permeabilized either by 50 % ethanol or by 2 min boiling at 95°C are not stainable by D₂R but they are stainable by PI (Fig.S4C).
D) Z-VAD-FMK inhibitor does not decrease a number of D₂R positive cells. There are three possible explanations: i) Z-VAD-FMK does not enter effectively yeast cells; ii) methyl group blocking Z-VAD-FMK activity is not properly removed in yeast, or iii) Mca1p independent ASPase activity in S. cerevisiae is not sensitive to Z-VAD-FMK inhibitor. Problems with binding of this inhibitor to caspases during apoptosis detection in human cells by FLICA were reported previously (Pozarowski et al., 2003).

![Fig.S4: Staining of active ASPase in cells from yeast colonies with D₂R substrate. Consequent staining with D₂R and Red-VAD-FMK (A), D₂R and PI of non-permeabilised cells (B) and of cells permeabilised by 50% ethanol (C). Formula of D₂R (D); sites of peptide bond hydrolysis by ASPase are marked by arrows. Bar, 5 µm.](image)

Thus, D₂R substrate appears to be good alternative for detection of caspase-like activity (or, generally, ASPase activity) in living-yeast cells. It enables direct monitoring of ASPase activity and avoids non-specific background caused by fluorophore binding to yeast cells as described for FLICA inhibitors.

REFERENCES:


