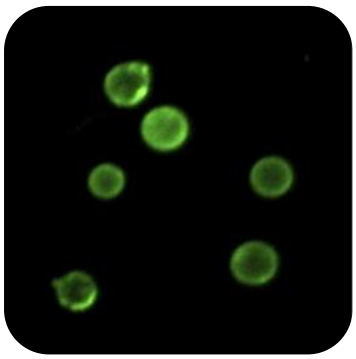
AnnexinV-FITC Staining as Early Marker of Apoptosis

Introduction



In cell line development and upscale bioreactor cultivation it is important to monitor the viability of the culture to determine the growth rate of cells and to check the quality of the culture conditions. In this assay SynenTec demonstrates a very sensitive method to get an overview of the cell status.

The cell membrane consists of different types of phospholipids. In living cells, phospholipid phosphatidylserine (PS) is located on the inside of the cell membrane (Fig. 1 a). If cellular stress like UV-irradiation, oxidative stress or DNA damage leads into the intrinsic apoptosis pathway, one of the first steps is that PS translocates from the internal to the external leaflet of the cell membrane (Fig. 1 b). The phospholipid asymmetry of the cell membrane gets lost. In the standard way of apoptosis, PS on the outside of the membrane is a signal for macrophages to digest this specific cell [1].

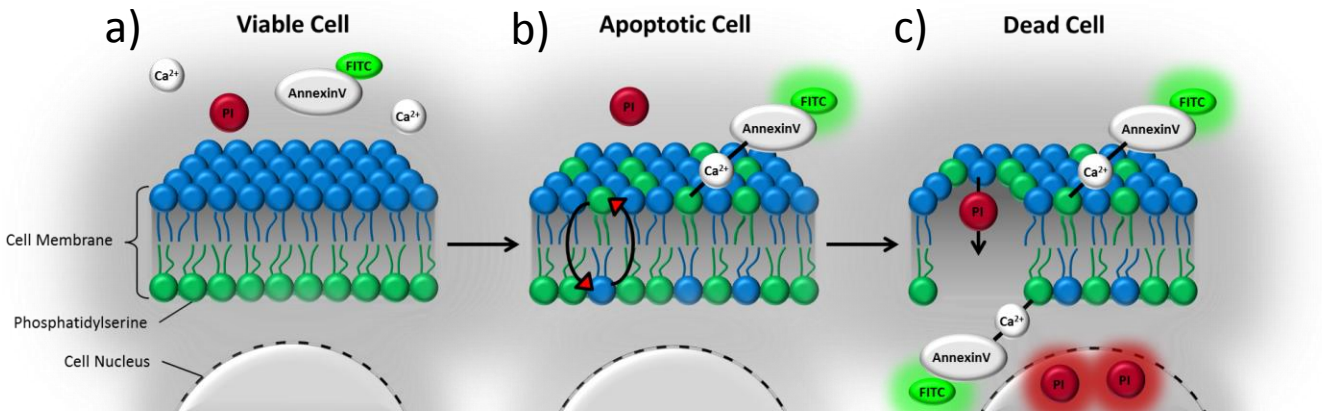


Fig. 1 a-c: Simplified scheme of the difference between viable, apoptotic and dead cells
a) Shown is a viable cell, phosphatidylserine is only in the inner leaflet of the bilayer, AnnexinV-FITC cannot bind, PI stays outside of the cell. **b)** Shown is a apoptotic cell, PS has moved to the outside of the membrane, AnnexinV binds, PI stays outside. **c)** Shown is a dead cell, the membrane is permeable, PI can get into the cell and stains the cell nucleus, AnnexinV-FITC binds to the inner aud outer leaflet.

AnnexinV is a protein which has a high affinity to bind phosphatidylserine in the presence of calcium ions (Fig. 1 b)[1]. If AnnexinV is added to the cell culture it binds to all cells which have accessible PS' and it binds to all apoptotic cells when PS turned to the outside and to dead cells. When a cell dies due to necrosis the cell membrane becomes permeable and AnnexinV can get into the cell and also binds to the PS in the inner leaflet of the bilayer (Fig. 1 c).

The fact that AnnexinV is a very early indicator of apoptosis allows an earlier detection of problems within the cell culture e.g. in fermenters than the analysis of apoptosis by DNA-based assays. With AnnexinV it is still possible to retrieve the culture e.g. by modification of the pH-value, the amount of nutrients, the stirring rate and/or the temperature. This possibility saves a lot of money and time.

To distinguish between viable, apoptotic and dead cells we stained with an AnnexinV-FITC-conjugate, Propidium iodide (PI) and Hoechst 33342 simultaneously. The Hoechst staining is to detect all cells in the blue channel, it stains all nuclei. Propidium iodide marks only the dead cells in the red channel and AnnexinV-FITC stains all apoptotic and dead cells in the green channel. Therefore, we have three different types of cells: The viable with a blue nucleus (Fig. 2 a), the apoptotic ones with a blue nucleus and a green membrane (Fig. 2 b) and the dead cells with a blue and red nucleus and a green membrane (Fig. 2 c). Due to this difference the NyONE cell imager with its associated image analysis software differentiates highly sensitive and very precisely between live, dead and apoptotic cells.

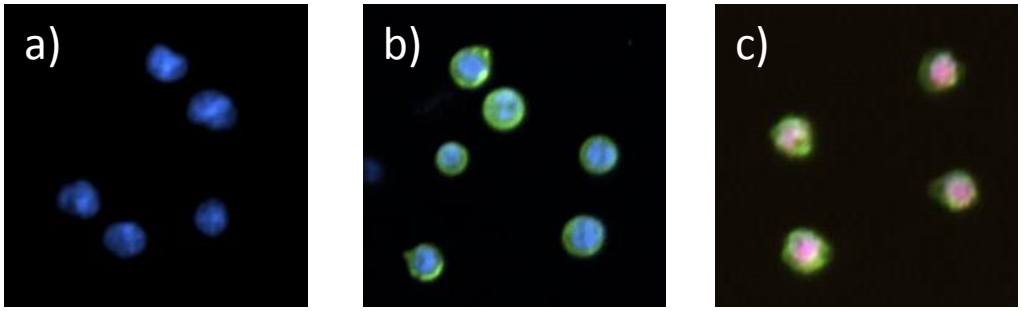


Fig. 2: These images show the difference between viable, apoptotic and dead cells
a) Shown are viable cells, the nuclei, stained with Hoechst, AnnexinV and PI, could not bind. **b)** Shown are apoptotic cells with a green AnnexinV-FITC stained cell membrane and a blue Hoechst stained cell nucleus. **c)** Dead cells with PI (red) and Hoechst (blue) in the cell nucleus and a green membrane with AnnexinV-FITC.

In this assay we treated the cell samples with different concentrations of staurosporine to obtain various numbers of apoptotic cells. Staurosporine is a protein kinase inhibitor which induces the intrinsic apoptosis pathway.
To have a control with 100 % dead cells we used a high concentration of hydrogen peroxide (H_2O_2).

Materials

Cells/Reagents

- Molt-4 cells (human T cell leukemia)
- RPMI medium with 10 % FBS and 1 % Penicillin/Streptomycin
- Phosphate Buffered Saline (w/o Ca^{2+} & w/o Mg^{2+} (PBS^{--}))
- Staurosporine, 1 mM stock solution (e.g. Santa Cruz Biotechnology #sc-3510)
- AnnexinV-FITC, 71.4 mg/mL stock solution (e.g. Sigma-Aldrich #A9210, from human placenta)
- AnnexinV 10x binding buffer (e.g. SouthernBiotech #10045-01)
- Hoechst 33342, 8.9 mM stock-solution in H_2O_{dest} (e.g. Santa Cruz Biotechnology #sc-391054)
- Propidium iodide, 4 mM stock solution in H_2O_{dest} (e.g. Santa Cruz Biotechnology #sc-3541)

Equipment

- Microplate with transparent bottom (e.g. Greiner Bio-One µclear 96-well plate (cat.# 655090); black plates are optimal for fluorescence imaging
- Centrifuge tubes
- Swing out plate centrifuge
- Cell incubator (37 °C, 5 % CO_2)
- Pipettes and tips
- NyONE microplate imaging microscope

Procedure

1. Dilute the cell sample in serum free medium: 2850 µL medium + 150 µL cell suspension (The dilution factor depends on the expected cell concentration in your culture. It is possible to measure a final cell concentration up to $2.5 \cdot 10^5$ cells/mL.)
2. Allocate the cell suspension to 8 microcentrifuge tubes: 300 µL per tube
3. Dilute the staurosporine 1:10: 45 µL serum free medium + 5 µL staurosporine stock solution
4. Add staurosporine dilution to 7 tubes:
   1) + 0 µL (≙ 0 µM staurosporine)
   2) + 0.15 µL (≙ 0.05 µM)
   3) + 0.3 µL (≙ 0.1 µM)
   4) + 0.45 µL (≙ 0.15 µM)
   5) + 0.6 µL (≙ 0.2 µM)
   6) + 0.75 µL (≙ 0.25 µM)
   7) + 0.9 µL (≙ 0.3 µM)
5. Incubate 1 h at 37 °C
6. Pipet 3.33 µL H_2O_2 into the 8th tube (final concentration of 100 µM)
7. Incubate all tubes again for 1 h
8. Centrifuge 5 min at 300 x g
9. Prepare staining solution:
   4000 µL PBS^-
   + 1.308 µL Hoechst (≙ 2.88 µM)
   + 10 µL PI (≙ 10 µM)
10. Aspirate the supernatant from the cells
11. Resuspend the cells in 300 µL staining solution
12. Incubate 15 min at 37 °C
13. Prepare the AnnexinV-FITC solution while incubating:
    300 µL binding buffer + 12.26 µL AnnexinV-FITC stock solution
14. Add 34,87 µL Annexin solution to each tube (≙ 0.375 µg/mL) and mix well
15. Incubate 15 min at room temperature
16. Pipet 100 µL per well (3 wells per concentration) into a microplate
17. Centrifuge 1 min at 30 x g to ensure that all cells are at the bottom of the plate
18. Remove the plate carefully from the centrifuge and place it into the sample holder of the NyONE imager
19. Measure the cells with the Virtual Cytoplasm 2F image analysis operator

Results

In our performed experiments we could show that the NyONE and its analysis software are very well suited for the detection of the cell culture health. By using AnnexinV, a very early marker of apoptosis, it can be recognized at an early stage, whether the cell culture is in good condition. This applies both to small cell culture units as well as to bioreactors.

The Virtual Cytoplasm 2F-operator of SynenTecs' analysis software was used. As shown in Fig. 4 each culture includes dead and apoptotic cells under normal conditions. Here, 6.2 % of the cells were stained with AnnexinV-FITC which means that 6.2 % of the cells are apoptotic or dead. 4.8% of the cells were stained with PI which conforms to the dead cells. The apoptosis rate of the culture amounts to 1.4 %.

Combining the apoptosis-inducing staurosporine in a concentration of 0.15 µM with an incubation time of 2 h, a distinct induction of apoptosis can be achieved. Here, the apoptosis rate of the sample amounts to 36.8 %, 80.8 % are stained with AnnexinV and 44 % with PI .

If only PI or Trypan blue would be used at this point to determine the viability of the culture as in standard practice, a significantly lower value of 44 % stained cells, instead of 80.8 %, would have been detected.

The advantage of using AnxV is that you can detect the degradation of the culture health even before the cells finally die. This enables an early intervention in the cell culture e.g. by adding nutrients, by modification of the pH value or by adjusting the stirring rate or the

temperature.
As a 100 % dead control we used H_2O_2 at a concentration of 100 µM. When the cells are incubated with this concentration they are all lethal by default, as see in the measurement below (Fig. 4). This again shows that the additional use of PI is very important to distinguish apoptotic from necrotic cells. Here in most instances the AnxV staining takes place because the membrane integrity gets lost in primary and secondary necrotic cells and thus the AnxV can also bind to the intracellular PS.

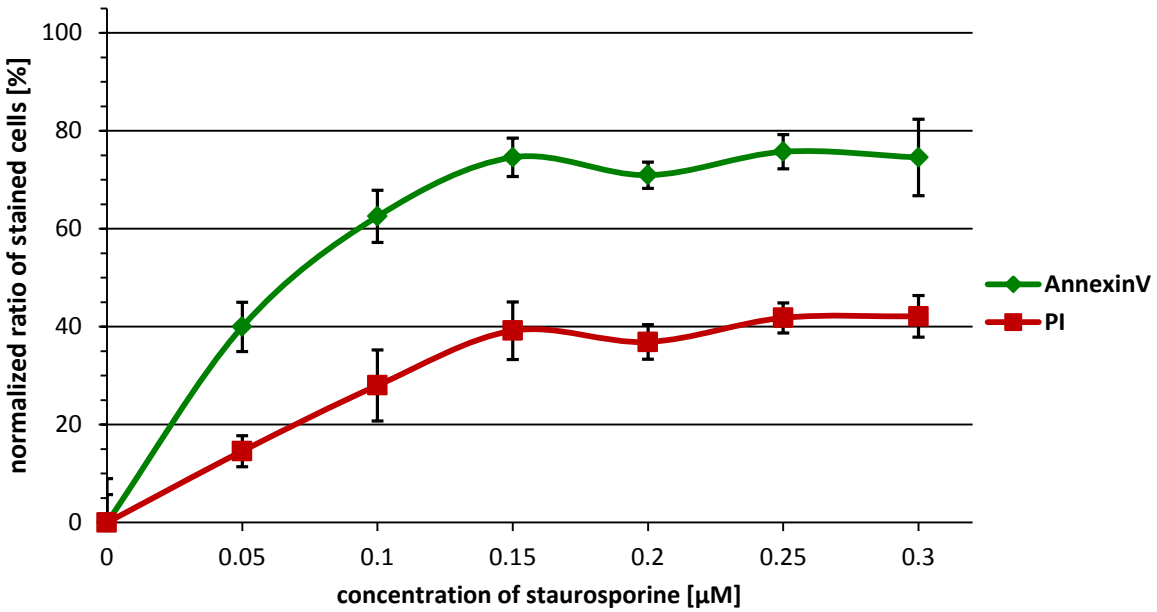


Fig. 3: AnnexinV-FITC and PI stained Molt-4 cells depending on different staurosporine treatments
Represented is the increase of the apoptosis rate depending on the used staurosporine concentration. As it can be seen in both curve progressions the ratio of the AnnexinV stained cells (green) rises faster than the PI stained cells (red) at a low staurosporine concentration. Therefore the detection of the apoptosis is more sensitive than the detection of the necrosis or later apoptosis.

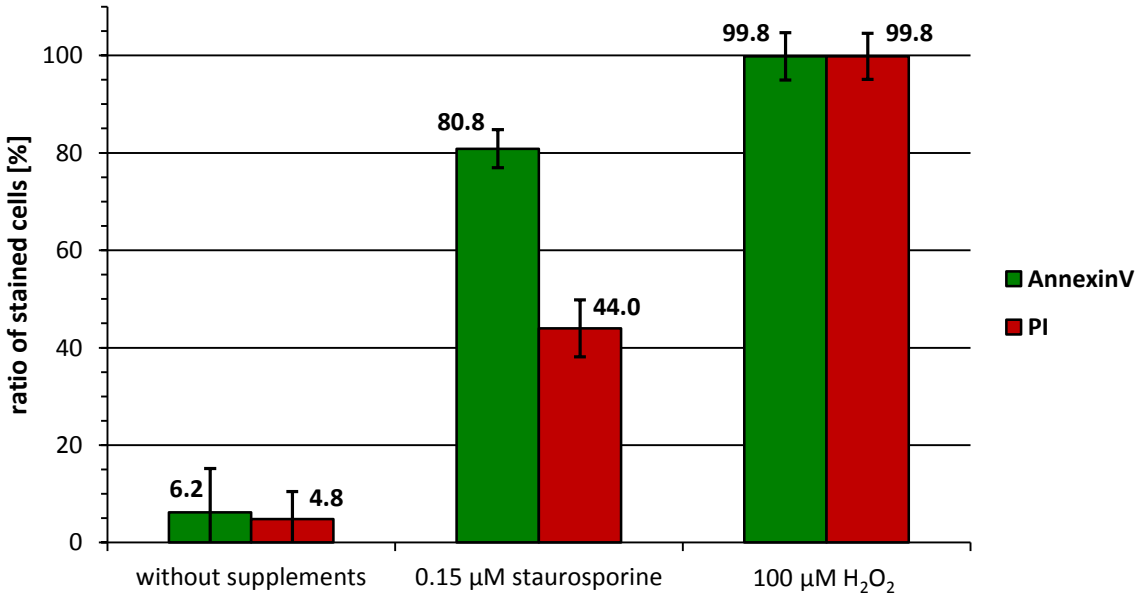


Fig. 4: AnnexinV-FITC and PI staining of different treated Molt-4 cells
The first bars show a AnnexinV-FITC (green) and PI (red) staining of a untreated Molt-4 culture. For the second bars the cells were treated with 0.15 µM staurosporine for 2 h to reach a high apoptosis rate. The third pair shows the ratio of all apoptotic and necrotic cells after a treatment with 100 µM H_2O_2 for 1 h as a 100% dead control. The apoptosis rate is the result of the difference between the green and the red bar.

In Fig. 3 the biggest distance between the AnnexinV and the PI stained cells respectively the apoptosis rate is at a treatment with 0.15 µM staurosporine for 2 h, therefore this concentration was chosen for the creation of Fig. 4.

The figures 5 to 7 show the change from less to many apoptotic and dead cells. The images were taken with our NyONE imaging system and the Virtual Cytoplasm 2F image processing operator.
Our image analysis software can very easily differentiate between viable, apoptotic and dead cells (Fig. 5-7 e). All green circles mark the viable cells, which means that fluorescence was only detected in the first fluorescence channel. This is the Hoechst channel with UV excitation and blue emission. All yellow circles mark the apoptotic cells. They were detected in the first and the second channel. The second one is the AnnexinV-FITC channel with blue excitation and green emission. The red circles mark the dead cells. There was fluorescence in the first, the second and the third channel. The third channel is for the Propidium iodide with blue excitation and red emission.

It is obvious that the AnnexinV in the dead cells is much brighter than the AnnexinV in apoptotic cells. The reason for this is that the AnnexinV in dead cells can bind to the inner and the outer PS and not only to the outer PS like in apoptotic cells (Fig. 6 b).
And it is also notable that all nuclei with two stainings (Hoechst and PI) are a little bit darker in the first channel than nuclei with only Hoechst inside. An artefact pending on the counterstaining.

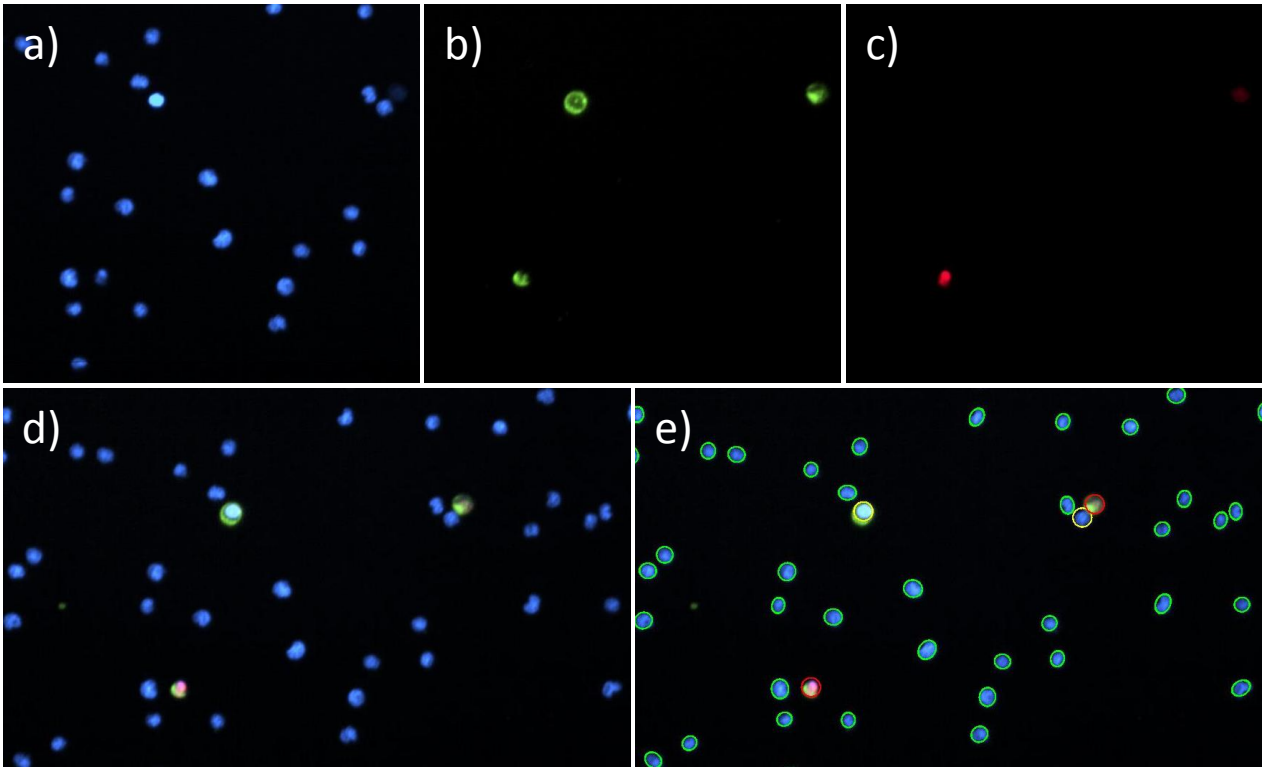


Fig. 5: These images show the stained Molt-4 cell sample without supplements
a) Hoechst staining; total cell count. **b)** Apoptotic and dead cells stained with AnnexinV-FITC. **c)** Dead cells stained with PI. **d)** Overlay image with Hoechst, AnnexinV and PI stainings. **e)** Overlay image with image processing results, green for viable cells, yellow for apoptotic and red for dead cells.

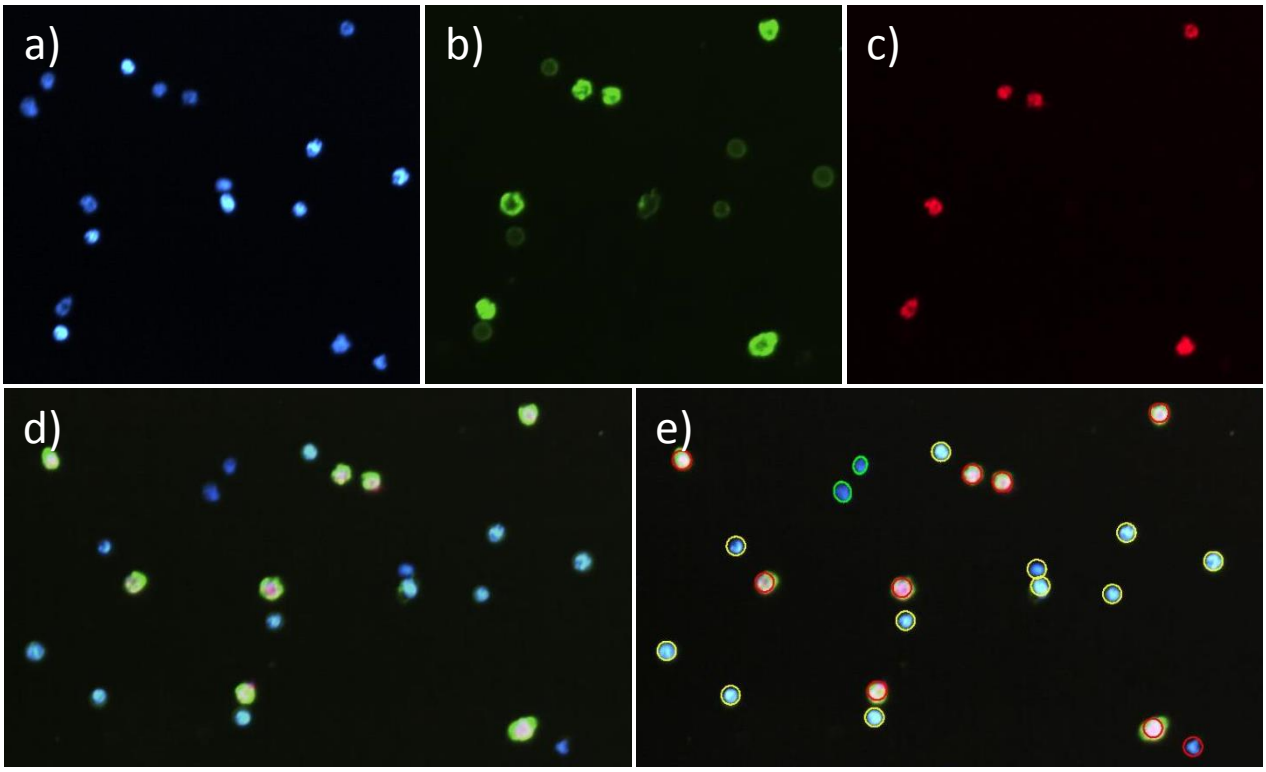


Fig. 6: These images show the stained Molt-4 cell sample treated with 0.15 µM staurosporine for 2 h
a) Hoechst staining; total cell count. **b)** Apoptotic and dead cells, stained with AnnexinV-FITC. **c)** Dead cells stained with PI. **d)** Overlay image with Hoechst, AnnexinV and PI stainings. **e)** Overlay image with image processing results, green for viable cells, yellow for apoptotic and red for dead cells.

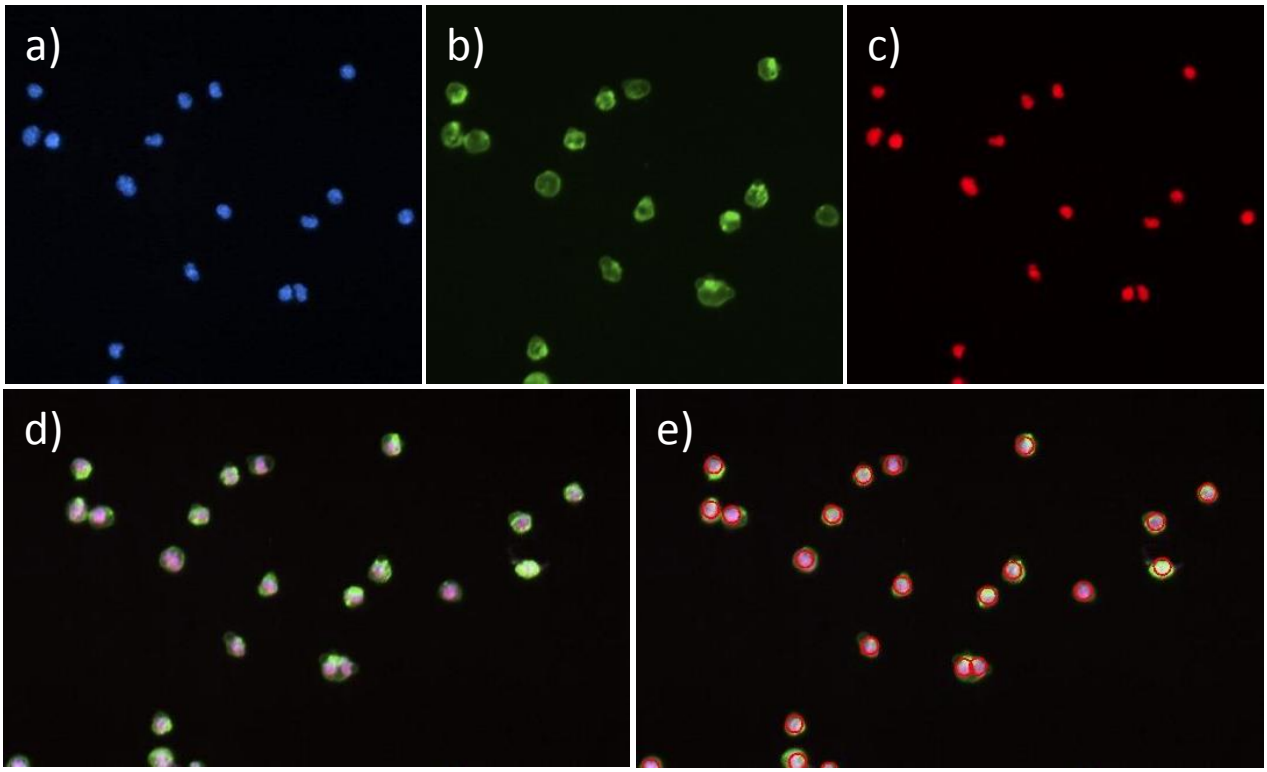


Fig. 7: These images show the stained Molt-4 cell sample treated with 100 µM H_2O_2 for 1 h
a) Hoechst staining; total cell count. **b)** Apoptotic and dead cells stained with AnnexinV-FITC. **c)** Dead cells stained with PI. **d)** Overlay image with Hoechst, AnnexinV and PI stainings. **e)** Overlay image with image processing results, red cirlces for dead cells.

Conclusions

1. The usage of AnnexinV is substantially more sensitive and earlier indicates a bad culture condition than just a PI staining!

2. The NyONE cell imager and its Virtual Cytoplasm 2F-operator can distinguish very easily and well between viable, apoptotic and necrotic cells!

3. This method is suitable for both the determination of apoptosis in toxin testing and the monitoring of batch cultures in cell line development and protein and antibody production!

4. When using AnnexinV, Hoechst for total cell count and PI for dead cell discrimination should always be used too!

References

[1] Demchenko, A. P., The change of cellular membranes on apoptosis: Fluorescence detection, Experimental Oncology 34, 263–268, September 2012

[2] Godard, T., Deslandes, E., Lebailly, P., Vigreux, C., Sichel, F., Poul, J.M., Gauduchon, P. (1999) Early detection of staurosporine-induced apoptosis by comet and annexin V assays. Histochem Cell Biol., 112, 155-161.

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