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The effect of the storage method of Nile red solution on the fluorescence intensity in the detection of intracellular lipids

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Introduction

Nile red is a fluorescent dye that is used to detect and measure intracellular lipids. This method can detect very low amounts of intracellular lipids with high accuracy. However, the intensity of Nile red fluorescence changes over time, and this makes it difficult to use it for analyses that are performed in a time interval. Hence, it is very important to understand the changes of Nile red florescent intensity a frozen state and its effect on lipid detection results. In the present study, the effect of storage time as well as the number of freezing-thawing cycles on the florescent intensity of frozen Nile red is investigated and the best protocol to use Nile red for lipid detection with confidence is proposed.

Materials & Methods

Nile red (catalog No: 19123) and solvents Methanol and DMSO were purchased from Merck. A stock solution of Nile red was prepared by dissolving 1 mg of Nile red in 10 mL of DMSO. The stock solution was stored at -20 ℃. For the study of the effect of the number of melting and freezing times, the stock solution was melted then it was frozen for later use. For the study of the effect of storage time, after 15, 45, 90 and 120 days at -20 ℃ fluorescence intensity of Nile red stock solution was measured.

For the neutral lipids analysis, olive oil was used as the model oil. Initially, 75 µL of Nile red stock solution was added to 425 µL of methanol (solution A). Then, to stain olive oil with different

concentrations of Nile red, concentrations of 0, 60 and 120 µg/mL of olive oil in chloroform were prepared. Then 100 µL of each concentration of oil samples were mixed with 5, 10, 20 and 50 µL of solution A and finally 3 mL of methanol was added to all samples. Therefore, the final concentration of olive oil and Nile red was between 0, 2 and 4 µg/mL and 0.05 to 0.3 µg/mL in methanol, respectively. The samples were placed in the dark for 25 min at room temperature. The final concentrations of olive oil and Nile red ranged from 0 and 4 µg/mL and 0.05 to 0.3 µg/mL, respectively. In the first set of experiments, the fresh Nile red stock solution was used. In the second set of experiments, Nile red stock solution at -20 ℃ for 15 days was used. Fluorescence intensity was measured using a PerkinElmer (LS-45) fluorescence spectrometer. According to the pre-scan, the excitation and emission wavelengths of 470 nm and 515 nm were selected, respectively.

Results & discussion

Effect of the number of melting and freezing cycles and storage time

In this study, the fluorescence intensity of Nile red solution has been investigated according to the two factors of time and the number of melting and freezing cycles. The results showed that Nile red fluorescence intensity was highest in freshly prepared stock solution. By storing Nile red at -20 °C, the fluorescence intensity decreased. However, if the solution remains frozen until use, the decrease in fluorescence intensity will be less than 10%. The results show that in this condition, storage time could be prolonged to 120 days. If the stock solution is repeatedly melted and frozen (4 times or more), the fluorescence intensity decreases by nearly 80%, which is no longer suitable for analysis.

Opposite of repeated melting and freezing of Nile red, which greatly reduces the fluorescence intensity, the storage time did not affect the fluorescence intensity. If the frozen stock solution is melted only for one use, it can be kept at -20 ℃, for a long time without its fluorescence intensity decreasing significantly.

Optimal fresh and frozen Nile red concentration

Due to the change in fluorescence intensity, the optimal concentration of Nile red for lipid staining should be determined for each analysis. If the fresh stock solution of Nile red is used, for the range of lipid concentrations (olive oil) 0 to 4 µg/mL, the concentration of 0.25 µg/mL of Nile red is suitable. At 0.25 µg/mL of Nile red, a linear relationship of $F_I=34.25\times C+668.17$ ($R^2=1$) was obtained between fluorescence intensity and lipid concentration, where F_I is fluorescence intensity and C is the lipid concentration (µg/mL). The results show that there is no linear relationship between lipid concentration and fluorescence intensity in Nile red concentrations less than 0.25 µg/mL. In a similar study, 0.25 µg/mL of fresh Nile red reagent was chosen as the appropriate concentration for 2.5 to 5 µg/mL Triolein solution. In cases where the frozen stock solution is used, the concentration of 0.05 µg/mL of Nile red is suitable and it identifies the lipid and determines its concentration with

confidence. At this concentration, a linear relationship $F_I = F_I = 10.091 \times C + 71.405$ ($R^2 = 0.998$) was obtained between the lipid concentration and the fluorescence intensity of the sample. With the increase of Nile red concentration, no significant response is obtained and interference between the fluorescence intensity of the control sample and the lipid content samples was observed. This vhinterference has been observed in other studies and it is recommended to use a low concentration of Nile red to avoid this interference.

Conclusion

The use of fresh Nile red solution for intracellular lipid analysis is not always practical and most analyses are carried out using frozen reagents. To receive the best analysis outcome, it is recommended to melt the Nile red solution at the time of use. At the frozen state, the solution could be stored at -20 °C for 120 days without a significant loss of florescent intensity. Multiple freezing-thawing cycles decrease the Nile red florescent intensity significantly.

Keywords*:* *Fluorescent, Nile red dye, olive oil*

Declaration of conflict of interest: The authors declare that they have no conflicts of interest.

Statement on ethics:

We certify that the submission is original work and is not under review at any other publication.