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Review

How to use Nile Red, a selective fluorescent stain for microalgal neutral lipids

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ABSTRACT

The use of Nile Red for rapid monitoring of the neutral lipid content in microalgae has gained interest over the last decade, since neutral lipids are feedstock for renewable transportation fuel. In this review, we discuss the main considerations needed to make an NR protocol reliable for staining neutral lipids in microalgae. Cell wall permeability must be enhanced by using stain carriers: DMSO (5% v/v to 25% v/v), glycerol (0.1 to 0.125 mg/mL), or EDTA (3.0 to 3.8 mg/mL). Temperatures between 30 and 40 °C facilitate the diffusion of NR through the cell wall without incurring excess quenching. Good NR-lipid interaction requires using a low NR/cell ratio; the NR concentration must be between 0.25 μg/mL and 2.0 μg/mL, and the cell concentration $>5 \times 10^4$ cells/mL. In order to have the maximum and stable NR fluorescence, it is necessary to scan the excitation/emission wavelengths for up to a 40-min of incubation time. We outline a five-step method to customize the Nile Red protocol to a specific strain: 1) Evaluate the strain's suitability by checking for the presence of neutral lipid, 2) Select of the best excitation/emission wavelength, 3) Optimization of incubation time, stain carrier, dye concentration, and temperature, 4) Prepare single-strain algal cultures with different lipid contents to calibrate NR fluorescence with neutral-lipid content, and 5) Correlate NR fluorescence intensity to neutral lipid content for the same strain. Once the protocol is customized, the NR method allows for rapid and reliable monitoring of neutral lipid content of a microalgae strain.

Contents

1. Introduction

The transportation sector contributes ~14% of greenhouse gas (GHG) emissions worldwide (The Intergovernmental Panel on Climate Change (IPCC), 2014), and almost 95% of transportation energy comes from fuels derived from petroleum, such as diesel and gasoline. Today,

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the transportation sector has no competitive alternatives to fossil fuels (Alemán-Nava et al., 2014; Mata et al., 2010). One possible alternative source for transportation fuel is lipid obtained from microalgae, as these lipids are suitable as renewable feedstock for biodiesel, jet fuel, ethanol, and butanol (Mata et al., 2010). Microalgae are photosynthetic microorganisms able to accumulate significant amounts of neutral lipids (20–50% of dry weight) mainly in the form of tricacylglyceride (TAG), which are stored in cytosolic lipid bodies (Day et al., 1999; Hu et al., 2008). Because microalgae use sunlight as their source of energy and CO_2 as their source of carbon (Singh and Gu, 2010), the feedstock that they produce can be renewable and carbon-neutral. It is possible to produce larger amounts of biomass per unit area from microalgae than from any kind of terrestrial plant, and microalgae cultivation does not compete with food production (Chisti, 2007; Cuellar-Bermudez et al., 2014; Rittmann, 2008).

A rapid measuring technique for the lipid content of microalgae would be of high value for monitoring growth when operating cultivation facilities (Chen et al., 2011). Today, the methods for quantification of lipids require solvent extraction and gravimetric assay, both of which are time and labor intensive. Several new techniques have been proposed to allow in situ measurement of lipid content: e.g., spectrophotometry, dielectric spectroscopy, and dielectrophoresis (Cooksey et al., 1987; Izard and Limberger, 2003). The simplest and most cost-effective method that measures lipid content is fluorescence spectroscopy, in which a dye binds to lipid droplets and emits a fluorescence signal that can be correlated to the lipid content.

Nile Red (NR, 9-diethylamino-5Hbenzo[α]phenoxazine-5-one), one of the most used fluorescent dyes, is lipophilic, i.e., able to bind to intracellular neutral-lipids (Greenspan and Fowler, 1985) with a linear correlation between NR fluorescence and the neutral lipid content (Cooksey et al., 1987). NR is a red phenoxazone dye that is present in small amounts in commercial preparations of Nile Blue (Conn, 1953). Studying the composition of Nile Blue and other phenoxazine dyes, Thorpe (1907) discovered that they contained traces of oxidized products called phenoxazones that have yellow or red colour. Phenoxazines could be converted to phenoxazones by boiling in dilute acid (illustrated in Fig. 1). Smith et al. then discovered that Nile Blue bound to polar lipids, but the traces of NR bound to neutral-lipids (Smith, 1911). From then, NR started to be used for determining neutral lipids in different kind of cells. As shown in Fig. 2, interest in NR to measure neutral lipids has increased rapidly since 2000.

This article critically reviews the use of NR for quantifying neutral-lipids in different microalgae strains. Based on our review, we propose a strategy to apply the NR method for reliable and rapid quantification of neutral lipids in different microalgae strains.

2. Staining dyes

In 1907, it was discovered that phenoxazine dyes (Nile Blue A, methylene-blue, and Mendola's Blue) could stain lipids: blue or red depending on the nature of the lipids and the dye (Thorpe, 1907). Further studies led to other lipophilic dyes that can be used with microorganisms: e.g., Sudan Black B (Evans et al., 1985) was used to measure lipids in yeast colonies, 1,8-naphthoylene-1′-2′-benzimidazole and its methoxy-substituted derivative (Pomoshchnikova et al., 1981) were

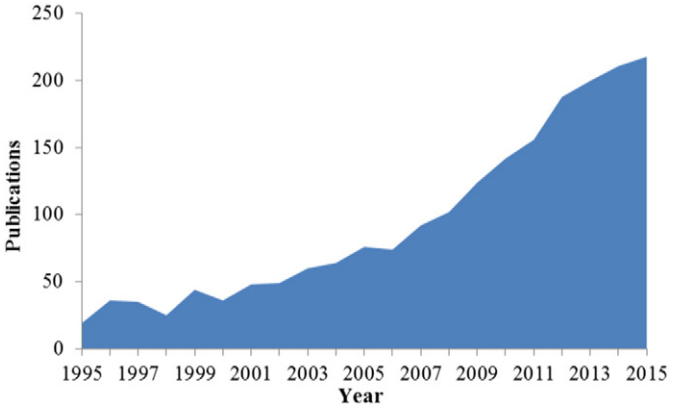

Fig. 2. Number of publications from 1995 to 2015 concerning the use of NR. Source: Scopus. Keyword: Nile Red. Last updated May 30th 2016.

used in yeast and mycelial fungi, and luminor 490PT (Pomoshchinikova et al., 1983) was used for fungal mycelia. Staining dyes also allow visualization of cells with lipids under fluorescence microscopy (Rumin et al., 2015). A major drawback of these early dyes was they had to be synthetized by the users. Furthermore, the staining time was several hours, and a washing step was needed after staining to remove excess dye (Cooksey et al., 1987).

Recently, BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4adiaza-s-indacene) (Haugland, 1992) has been used for staining neutral lipids in vesicles in human hepatocytes (De Gottardi et al., 2007), mammalian cells (Gocze and Freeman, 1994; Listenberger and Brown, 2007), and algal cells (Cooper et al., 2010). While BODIPY is widely useful, its disadvantages are background fluorescence in the medium and dye-dye interactions, both of which increase the variability of fluorescence values during neutral-lipid measurement (Rumin et al., 2015). Recently, NR also was adapted for staining different kind of cells: mammalian cells (Genicot et al., 2005), bacteria (Izard and Limberger, 2003), yeast (Evans et al., 1985; Kimura et al., 2004), zooplankton (Kamisaka et al., 1999), and microalgae (Elsey et al., 2007; Eltgroth et al., 2005). The NR protocol involves correlating fluorescence intensity to neutral lipid content through a calibration curve. To do the calibration curve, the intracellular neutral lipid content must be determined by another technique, such as chromatography (Alonzo and Mayzaud, 1999; Kimura et al., 2004). The accuracy of the calibration can be undermined by a number of factors affecting neutral lipid staining with NR: cell concentration, temperature, and dye concentration (Siegler et al., 2012).

3. Applications of Nile Red in microalgae

NR has proven to be suitable for monitoring the content of neutral-lipids during microalgae cultivation; this is of value because neutral lipids are feedstock for biodiesel production (Cuellar-Bermudez et al., 2014) (Damiani et al., 2010; Hu et al., 2008; Krohn et al., 2011; Popovich et al., 2011). Table 1 summarizes the strains and conditions reviewed in this section.

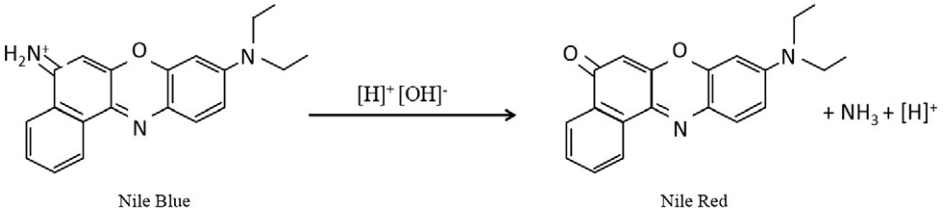

Fig. 1. Synthesis of Nile Red from Nile Blue (Frick et al., 2014).