Fluorophores from fungi

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Introduction

Fluorescence has many advantages over traditional colour and radioactive labels, and is playing an increasingly important role in the most powerful analytical techniques.

For example, fluorescence is at the heart of many nucleic acid based diagnostics (e.g. DNA microarray, real time-PCR, fluorescence *in situ* hybridisation, etc), immunofluorescence assays, defined substrate technologies and differential display proteomics and is gradually replacing or complementing other techniques based on colour or radiolabels. There are several reasons for this:

- Fluorescent techniques are $\geq 10^6$ x more sensitive than those based on colour and suffer minimal interference.
- Fluorescence signals are proportional to the concentration of the substance being investigated. Fluorescence-based measurements are readily quantifiable over many orders of magnitude compared with two orders of magnitude for colour-based assays.
- Fluorescence is non-invasive and changes in fluorescence intensity can be measured remotely and very rapidly (in picoseconds if necessary). This allows the study of dynamic processes, in real-time, such as rapid physiological changes inside a cell.
- Fluorescence techniques are generally simple and safe compared to similarly sensitive radioactive methods, which create disposal problems and require specialised facilities and training.

Fluorescence is detected and quantified using a variety of instrumentation such as fluorescence microscopes, scanners, plate readers, fluorometers as well as flow cytometers. Despite the widespread use of fluorescence techniques, only a limited number of fluorochrome families are known (Table 1). Thus there is a need for new colours of fluorescent labels that can be used to complement existing fluorophores in multiplex assays (measuring more than one analyte simultaneously). Natural products from microorganisms, such as fungi, represent a relatively unexplored resource for the discovery of new fluorophores.

Fluorescence

Fluorescence is a fundamental physical property of some molecules with rigid conjugated systems that allow them to be excited by light of one particular wavelength and then emit light at a different wavelength. Typically, fluorochromes emit light at a longer wavelength than the excitation source.

The difference between the excitation and emission frequencies is known as the

Table 1. Commonly used fluorescent probes for protein conjugation (adapted from http://pingu.salk.edu/flow/fluo.html).

Probe	Excitation	Emission	Stokes' Shift	MW
Beljian Red	394, 510	605	95, 211	410
Hydroxycoumarin	325	386	61	331
Aminocoumarin	350	445	95	330
Methoxycoumarin	360	410	50	317
Cascade Blue	375; 400	423	23, 48	596
R-Phycoerythrin (PE)	480; 565	578	13, 98	240,000
Fluorescein (FITC)	495	518	23	389
Oregon Green	493	519	26	368
BODIPY range of dyes	500-647	503-660	All short	292-813
TRITC	554	573	19	444
X-Rhodamine	570	576	6	548
PerCP	490	675	185	35,000
Texas Red	595	615	20	625
Cy3	552	565	15	767
Cy5	650	670	20	792
Cy7	743	767	24	818
Allophycocyanin (APC)	650	660	10	104,000

Stokes' shift. The Stokes' shift is proportional to the change in dipole moment between the ground and excited state of the fluorophores[1]. Long Stokes' shift fluorochromes have particular utility because they suffer from fewer problems associated with self-quenching and because they can be used in combination with short Stokes' shift fluorochromes in multiplex assays.

The use of fluorescence in research applications is diverse and ranges from studies in pharmacology and immunology to proteomics and genomics. It would be difficult to find a modern life science or medical research laboratory that does not routinely use fluorescent dyes and tags. However, there is a continuing need for new fluorophores with specific chemical and physical properties.

Fluorescence in microbiology

Differential staining of microorganisms is almost as old as microbiology. These techniques have been developed for an increasing number of microorganisms and have perhaps reached their most sophisticated in the range of chromatogenic agars which enable identification of a wide range of different species on a single medium[2].

Combined chromogenic and fluorogenic substrates can be used for the detection of coliforms and faecal coliforms respectively in water using products such as Colilert (IDEXX). Fluorogenic substrates can also be used as the basis of bioassays to measure diverse functions such as membrane potential, membrane integrity or specific enzyme activity[3].

The use of fluorescence in microbiology is really just an extension of existing culture and microscopic staining techniques. The advantage of fluorescence is that it offers additional multiplex options, greater sensitivity, lower backgrounds and greater dynamic range. These advantages really come into their own when analysing individual microbial cells, particularly using automated systems such as flow cytometry [4]. The high throughput possible with flow cytometers (>10^4 cells per second) enables statistically valid samples to be rapidly analysed.

There are a variety of fluorescent stains that can be used in conjunction with flow cytometry to measure a variety of different parameters. For example, microbial cells can be stained using immunofluorescent techniques, fluorescence *in situ* hybridisation or a variety of physiological stains.

Sources of fluorescent molecules

Fluorochromes used in biological detection systems originate from both chemical and biological sources. Fluorescein, which was originally isolated from coal tar in the 1800s is the most widely used fluorochrome and has led to a range of synthetic analogues such as Texas Red, Oregon Green and Rhodamine.

The fluorescein family are short Stokes' shift fluorophores (Table 1). For multiplex applications there is a need for fluorescent molecules that have spectral characteristics that are complementary to fluorescein. Of particular value are fluorescent molecules that are excited by the same light source as fluorescein but which emit a different colour (longer Stokes' shift).

There are a number of biological sources of fluorescent pigments. The photosynthetic pigments of plants and algae (e.g. Phycoerythrin, PerCP) are used as fluorescent labels in the biological sciences. Phycoerythrin and PerCP are long Stokes' shift fluorochromes that can be excited using blue light (Table 1) and would be suitable as dual labels. However, being proteins severely limits their applications as they cannot, for example, be used as intracellular probes.

Although not used as a fluorescent label, green fluorescent protein from the jellyfish *Aequorea victoria* has become very important as a genetically encoded marker to follow physiology of cells or patterns of gene expression in living organisms and biosensors[5]. Recently a new family of GFP-like proteins have been identified in Australian reef building corals[6].

Fungi as a source of fluorescent molecules

The use of fungi as a source of dyes dates back to antiquity and is experiencing a renaissance as 'ecologically friendly'[7]. The Chinese have used the red rice fungus *Monascus purpureus* as a food and textile dye since ancient times and, in Europe, cudbear was a popular red dye produced from lichens *(Roccella and Ochrolechia)* until they were replaced by cheaper aniline dyes derived from coal tar.

Some fungi are known to produce fluorochromes: for example, the aflatoxins of *Aspergillus flavus* are fluorescent and toxin production can readily be assessed by the fluorescent ring seen under UV-light illumination[8]. Similarly, the mycotoxin citrinin is detected by fluorescence after acidification with 1M hydrochloric acid[9]. There are a few other scattered reports of natural fluorescent products from fungi including mushrooms[10] but, to the best of our knowledge, fungi have never been investigated specifically as a source of fluorchromes.

In 1996, we were developing flow cytometry as a technique for high throughput screening of improved yeast strains for industry. Classical mating experiments with industrial strains of yeast are difficult to follow because they mate with a low frequency and it is undesirable to introduce selectable markers (such as antibiotic resistance) into yeast that will ultimately be used for foods and beverages.

Dan Deere came up with the idea that one parental strain could be stained with a green fluorescent tracking dye and one with a red tracking dye. By using fluorescence activated cell sorting, the rare hybrids that were stained both red and green as a consequence of plasmogamy (fusion) could be isolated. Whilst fluorescein was a suitable green stain, we were unable to find a suitable red stain despite spending a fortune on commercial products.

In a classic case of the sometimes random and serendipitous nature of scientific discovery, Philip Bell and Jian Shen

decided to spot some previously untested fluorophores onto old yeast colonies destined for the autoclave due to fungal contamination. The fluorophores tested were useless but, to their great surprise, colonies of yeast growing near a contaminating fungus (later identified as *Epicoccum nigrum)* were seen to fluoresce bright red. We extracted enough of this red pigment (Beljian Red) to demonstrate its utility for flow cytometry (using the blue laser) and for the yeast mating experiments[11].

Phil worked on the fungus in his garage for two years to identify how to increase yields of the dye. Although production of the fluorochrome was a hit and miss affair, eventually sufficient quantity was produced to allow Peter Karuso and Hayley Brown to start work on the extract. In four months they had developed an isolation method (Figure 1) and determined that the active principle was a small molecule (MW 410) of polyketide origin. With the assistance of Martin Slade, we were able to isolate a small quantity of the pure natural product and determined its structure, principally by NMR spectroscopy.

Beljian Red has some unique and useful spectral characteristics: probably of most importance is its long Stokes' shift (Table 1). It fluoresces red when illuminated with blue (Ar) or green (He-Ne) laser. This feature made it ideal for the yeast mating experiments (Figure 2) as it is the perfect partner for the green (fluorescein based) dyes that we were using. This long Stokes' shift also means the dye is useful for other multicolour fluorescence applications.

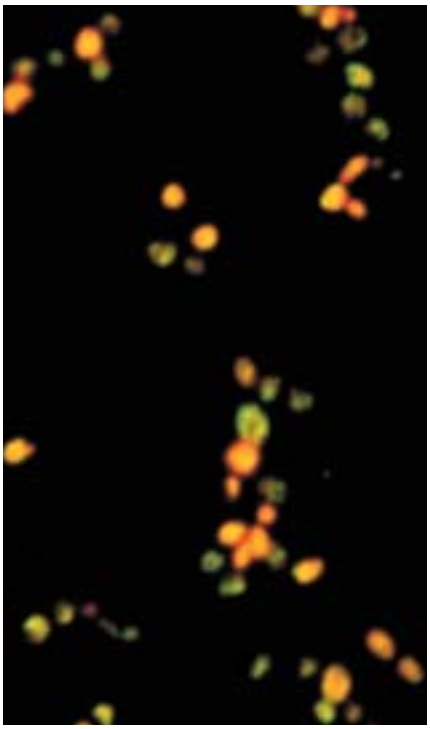
The combination of small size, lack of charge and long Stokes' shift is potentially a very useful set of characteristics for intracellular staining and many other biotechnological applications. Beljian Red can also be excitable by the new generation of cheap solid-state violet lasers (395 nm) that have been developed for DVDs.

Figure 1. Bioassay directed separation of Beljian Red.
(A): multicoloured bands eluted from a Sephadex LH-20 Column were tested
(B): for their ability to stain yeast cells fluorescently (λ_{ex} 365 nm).

Conclusion

Fungi are extremely diverse in both phylogeny and physiology and yet have only been poorly investigated as a source of novel fluorescent compounds. The accidental discovery of Belijan Red, a relatively simple but potentially very useful molecule produced by a common fungus, demonstrates well the rich treasure trove of natural products that are awaiting discovery from the fungi.

Figure 2. Yeast mating experiments (λ_{ex} 488 nm) showing two strains of brewer's yeast and their hybrids (green and red) resulting from plasmogamy.



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