Excited state proton transfer of some substituted naphthols in liposomes

N Pappayee & A K Mishra*
Department of Chemistry, Indian Institute of Technology, Madras.
Chennai 600 036, India
*Tel +91 (044) 4458251. Fax +91 (044) 2352545
e-mail: mishra@acer.iitm.ac.in

Received 14 December 1999; revised 19 May 2000

Fluorescence probes based on excited state proton transfer (ESPT) have been recently developed for microheterogeneous media. The ESPT behaviour of a series of substituted naphthols (4-chloro-1-naphthol, 5-amino-1-naphthol, 4-methoxy-1-naphthol, 7-methoxy-2-naphthol, 3-amino-2-naphthol, 6-bromo-2-naphthol and 1-bromo-2-naphthol) is investigated in liposomes, using steady state and time resolved fluorescence studies. 4-Chloro-1-naphthol emerges as a successful ESPT probe even though 7-methoxy-2-naphthol and 4-methoxy-1-naphthol are also useful. The possible reason for this success is accounted on the basis of the difference in proton dissociation constants in excited state ($pK_a^*$) and ground state ($pK_a$). The failure of aminonaphthols as ESPT probe is due to the existence of multiple equilibrium between different species and the low quantum yield of these molecules in non-polar media. The increased intersystem crossing in the case of bromonaphthol excludes its possibility as an ESPT probe. Among the seven monosubstituted naphthols that have been screened, 4-chloro-1-naphthol emerges as a better choice for an ESPT probe. The neutral form fluorescence of this molecule correctly reflects the phase transition behaviour of DMPC liposome membranes. This probe also successfully senses the cholesterol-induced phase changes in lipid bilayer membranes.

The phenomenon of excited state proton transfer (ESPT) is well known from the works of Forster [1] and Weller [2]. Molecules like aromatic amine and phenols show large enhancement in acidity due to migration of charge from the electronegative atom center to the ring in the excited state compared to the ground state [3]. As a result, their ionization constant decreases by several units in the electronic excited state ($pK_a^*$) relative to that in the ground state ($pK_a$). Hence if the $pH$ of the medium is intermediate between $pK_a$ and $pK_a^*$, the molecule when excited in its neutral form readily deprotonates to produce the anion in the excited state, from which the Stokes shifted anion emission originates.

Among the known ESPT molecules, naphthols [4,6] stand out due to their extremely fast excited singlet state deprotonation rate. They are commonly called as excited state acids. 1-Naphthol in water exists in its neutral form in the ground state ($pK_a = 9.2$). Upon excitation, rapid proton transfer occurs ($pK_a^{1/2} = 0.4$) [7] and emission is observed only from the excited anion species. However, addition of a solvent like methanol affects the proton transfer equilibrium in the excited state. Depending on the solvent composition, emission from both neutral and anion forms is observable. The shift in the emission of these two forms is often appreciable which makes it a convenient parameter to study microheterogeneous media. Also, the prototropic equilibrium between these two forms is sensitive to the microenvironment and any change in the surrounding leads to a change in the ratio of anionic to neutral peak intensities. Since this phenomenon of ESPT is a strong function of the local environment in which it occurs, information regarding a microenvironment in a microheterogeneous medium can be obtained. Studies of fluorescent ESPT molecules in environment like micelles [8,9], reverse micelles [10,11], liposome [12,13], cyclodextrins [14], crown ethers [15] and colloidal solutions like proteins [16] have been amply carried out.

Recent work from our laboratory has reported the potential use of 1-naphthol as an ESPT probe for lipid bilayer membranes [15]. The partition coefficient of 1-naphthol for lipids is very high [15]. It has an excellent ability to sense the phase transition temperature in liposome and report on the membrane permeability changes. These changes can be conveniently monitored.
simply by noting the intensity changes of the neutral form fluorescence. It can be also used to sense the membrane modifications caused by addition of perturbants like cholesterol and surfactants\textsuperscript{17}.

Since 1-naphthol works remarkably well as a probe, it is worthwhile to investigate some of the substituted naphthols as ESPT probes for liposomes. In this regard, the following monosubstituted naphthols: 4-chloro-1-naphthol, 5-amino-1-naphthol, 4-methoxy-1-naphthol, 7-methoxy-2-naphthol, 3-amino-2-naphthol, 6-bromo-2-naphthol and 1-bromo-2-naphthol (Aldrich Chemicals), were taken up for study. A variation of substituents with electron donating and electron withdrawing groups in naphthol molecule affects this disposition of $pK_a$ changes and as a result emissions from either one form or both the forms are seen. Chloronaphthol exhibiting electron withdrawing inductive effect favours proton transfer at ortho and para positions to -OH group. Bromonaphthols, which also belong to the same category, have the bulky bromine atom, which affects the rate of fluorescence, enhancing the rate of intersystem crossing. The electron donating resonance effect of methoxynaphthols depending on the position of the methoxy group in the naphthol ring affects the proton transfer process. Aminonaphthols containing two acidic groups, exhibit multiple equilibrium between different forms (cation, zwitterion, anion) depending on the $pH$ of the medium. Hence examination of these various substituted naphthols as ESPT probes becomes interesting.

Liposomes made from dimyristoylphosphatidylcholine (DMPC) lipid were chosen for the study, as this membrane is well characterized\textsuperscript{18}. DMPC lipid belongs to the class of glycerophospholipids, which are zwitterionic in nature. This lipid contains saturated chains of twelve carbon atoms each. The transition of gel to liquid crystalline state at 23°C makes it convenient to monitor phase changes.

Materials and Methods

4-Chloro-1-naphthol, 5-amino-1-naphthol, 4-methoxy-1-naphthol, 7-methoxy-2-naphthol, 3-amino-2-naphthol, 6-bromo-2-naphthol and 1-bromo-2-naphthol were purchased from Aldrich Chemicals. Dimyristoylphosphatidylcholine (DMPC) was purchased from Sigma Chemicals Co and was used as such. Cholesterol was bought from S.D.Fine Chemicals. All solvents were distilled before used. Doubly distilled water was used in all cases.

Liposome preparation

DMPC lipid stock was prepared in chloroform. The solvent was removed using a rotary evaporator; residual solvent, if any, was removed by keeping the flask under vacuum for sufficient time. Multilamellar vesicles were prepared by adding an appropriate volume of phosphate-buffered saline at pH 7 to the lipid film, with vigorous stirring and warming at 45-50°C, to yield a final lipid concentration of 0.1mM. Freshly prepared liposomes were used for all experiments. DMPC-cholesterol liposomes were prepared by adding the same volume of lipid to different volumes of cholesterol stock such that the molar ratio of cholesterol varied from 0 to 50 mol% of lipid.

Labeling

Stock solutions of all the probes were prepared in phosphate buffered saline at pH 7. After adding the probe to the prepared liposome, it was equilibrated for one hour at 30°C before the start of experiments. For all the experiments, a control solution containing the same concentration of liposome, but no probe was added and used as a blank. Concentration of probe used was $2 \times 10^{-6}$ M for chloronaphthol and 7-methoxy-2-naphthol and $5 \times 10^{-6}$ M for 4-methoxy-1-naphthol, aminonaphthols and bromonaphthols.

Fluorescence measurement

Fluorescence measurements were made using a Hitachi F-4500 spectrofluorometer. The excitation wavelengths were varied according to the probe molecule (different naphthols have different excitation wavelength) and emission spectrum was recorded in the wavelength range 300-600nm with the excitation and emission slit widths having a bandpass of 5nm, except in the case of low fluorescing bromonaphthols where 5/10nm slit width was used. Fluorescence lifetime measurements were made using an Edinburg Analytical Instrument at 30°C. For all measurements a peak count of 10000 was taken. The pulse width of the hydrogen lamp used was 1.7ns. The amplitudes and the lifetimes were determined by an iterative convolution method as developed by O' Connor et al\textsuperscript{19}. This method gives fairly accurate lifetime of 0.1ns for a lamp pulse excitation width of 2ns. Lifetime measurements for all the samples were made at the saturation lipid/probe ratio.
Table 1 - Excitation and emission wavelengths for all naphthols in water.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Excitation wavelength (nm)</th>
<th>Emission wavelength in water (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Neutral</td>
</tr>
<tr>
<td>4-Chloro-1-naphthol</td>
<td>300</td>
<td>378</td>
</tr>
<tr>
<td>4-Methoxy-1-naphthol</td>
<td>315</td>
<td>410</td>
</tr>
<tr>
<td>7-Methoxy-2-naphthol</td>
<td>325</td>
<td>344</td>
</tr>
<tr>
<td>5-Amino-1-naphthol</td>
<td>310</td>
<td>410</td>
</tr>
<tr>
<td>3-Amino-2-naphthol</td>
<td>330</td>
<td>384</td>
</tr>
<tr>
<td>1-Bromo-2-naphthol</td>
<td>290</td>
<td>356</td>
</tr>
<tr>
<td>6-Bromo-2-naphthol</td>
<td>290</td>
<td>361</td>
</tr>
</tbody>
</table>

Table 2 - Ionization constant for all naphthols in the ground and excited state.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pK_a</th>
<th>pK_a^*</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Chloro-1-naphthol</td>
<td>8.75</td>
<td>1.73</td>
</tr>
<tr>
<td>4-Methoxy-1-naphthol</td>
<td>10.5</td>
<td>10.3</td>
</tr>
<tr>
<td>7-Methoxy-2-naphthol</td>
<td>9.9</td>
<td>8.4</td>
</tr>
<tr>
<td>5-Amino-1-naphthol</td>
<td>9.5</td>
<td>9.5</td>
</tr>
<tr>
<td>3-Amino-2-naphthol</td>
<td>9.3</td>
<td>9.2</td>
</tr>
<tr>
<td>1-Bromo-2-naphthol</td>
<td>7.89</td>
<td>7.4</td>
</tr>
<tr>
<td>6-Bromo-2-naphthol</td>
<td>9.23</td>
<td>8.9</td>
</tr>
</tbody>
</table>


Results and Discussion

Steady state fluorescence measurement

The excitation and emission wavelengths of all substituted naphthols examined are listed in Table 1. The pK_a and pK_a^* values for all these naphthols are given in Table 2. The pK_a and pK_a^* values for both the methoxynaphthols and the pK_a^* value for both the bromonaphthols were not available in the literature. The usual absorbometric titration for determination of pK_a and fluorimetric titration for pK_a^* were, therefore, carried out¹. The spectral characteristics of each naphthol are discussed below.

(a) 4-Chloro-1-naphthol

The emission spectrum of 4-chloro-1-naphthol in presence and absence of DMPC lipid is shown in Fig. 1. In aqueous solution, emission of neutral form (378nm) is very weak, the predominant emitting species being the anion (460nm). This is because the pK_a is 8.75 and pK_a^* is 1.73 (ref. 3). In the ground state at pH 7, the probe exists as a neutral molecule but on excitation it readily deprotonates to give only the anion emission. On addition of DMPC, the emission intensity of the neutral form increases with a decrease in the anionic form. The fact that the fluorescence intensity of the neutral form of chloronaphthol increases with progressive addition of DMPC liposome clearly shows that this molecule partitions into the membrane. The presence of an isoemissive point suggests that the neutral form fluorescence appears at the expense of the anionic form fluorescence.

(b) 4-Methoxy-1-naphthol and 7-methoxy-2-naphthol

The ground state pK_a is determined from the absorption measurements. From the plot of optical density against pH, the pK_a was estimated to be 9.9 for 7-methoxy-2-naphthol and 10.52 for 4-methoxy-1-naphthol. The excited state pK_a was determined from the fluorimetric titration, which ultimately yielded the ground state dissociation constant for both the methoxy substituted naphthols. The pK_a^* values for 7-methoxy 2-naphthol and 4-methoxy-1-naphthol are 8.4 and 10.35 respectively. Fluorimetric titration gives ground state pK_a when the rate of fluorescence emission is faster than the rate of proton transfer.

The emission spectrum of 4-methoxy-1-naphthol is shown in Fig 2. This molecule exhibits only neutral form emission (410nm) at pH 7. This is due to the fact that pK_a^* is 10.35 which furnishes the anion only beyond pH 11. The enhancement of the neutral form fluorescence intensity in the membrane could be due to the reduction of non-radiative decay rates in the restrictive environment.
7-Methoxy-2-naphthol behaves in a similar manner as chloronaphthol with neutral form showing emission at 344 nm and anion at 416 nm. Since $pK_a$ is 8.4 for this probe, a weak anion emission is seen along with a predominant neutral emission in water. As expected, there is a progressive increase in the neutral form emission with increasing lipid concentration (Fig. 3).

(c) 5-Amino-1-naphthol and 3-amino-2-naphthol

Due to the presence of two functional groups these molecules exist in nearly six different prototropic forms at different $p$Hs. They are dication, monocation, normal form, zwitterion, anion and dianion. The neutral form exists in the $p$H range 4 - 9. The ground state $pK_a$ is 9.5 for 5-amino-1-naphthol and 9.3 for 3-amino-2-naphthol. In this case also, the fluorimetric titration yielded only the ground state $pK_a$. Since $pK_a$ for both the amino naphthols is around 9.5, only neutral form emission is seen in water.

3-Amino-2-naphthol shows neutral form emission (384nm) in water (Fig. 4). There is a small decrease in fluorescence intensity in the presence of the lipid. This decrease can be rationalized on the basis of the lower fluorescence quantum yield of this molecule in non-polar media like cyclohexane (0.16) compared with the quantum yield in water (0.41) (ref. 21). The reduction of fluorescence intensity in lipid medium is a clear indication of the incorporation of this probe into the lipid.

The emission spectrum of 5-amino-1-naphthol in water and in lipid is shown in Fig. 5. Neutral form emission occurs at 410 nm. There is hardly any change in the emission intensity of this molecule with lipid addition. The fluorescence quantum yields of this molecule are fairly low in both non-polar (cyclohexane, 0.04) and aqueous media (0.02) (ref. 20). The fairly low intensity and lack of significant change in the spectrum makes this molecule unsuitable for probe purposes.

(d) 6-Bromo-2-naphthol and 1-bromo-2-naphthol

The $pK_a$ and $pK_a$ values for 6-bromo-2-naphthol are 9.23, 8.9 and for 1-bromo-2-naphthol are 7.89, 7.4 respectively. The fluorescence intensity is very low in both bromonaphthols due to the heavy atom effect of bromine, which increases the rate of intersystem crossing. The extremely weak fluorescence and its
insensitivity to the presence of lipid (Fig. 6) make it inappropriate for probing. 1-Bromo-2-naphthol fluorescence however shows the usual pattern expected from ESPT probes. There is a progressive enhancement of neutral form fluorescence (356nm) at the expense of anionic form fluorescence (417nm) with increasing lipid concentration (Fig 7). Both the forms of this molecule show almost equal intensity in water. This is because the $pK_a^*$ is 7.4 which is close to the neutral pH.

The above observations clearly suggest that the range and the magnitude of $\Delta pK_a$ ($pK_a^* - pK_a^-$) and its disposition with respect to the pH of the liposome medium is a crucial parameter in selecting an ESPT probe. 4-Chloro-1-naphthol with a large $\Delta pK_a$ (~7) in the range spanning across the neutral pH of the liposome medium shows the largest variation of neutral and anionic form fluorescence intensity. This behaviour of 4-chloro-1-naphthol is similar to that of 1-naphthol.

**Determination of partition coefficient**

The partition coefficient for these naphthols in DMPC liposomes was determined by using the fluorescence measurement techniques 22. 4-Chloro-1-naphthol, 4-methoxy-1-naphthol, 7-methoxy-2-naphthol and 1-bromo-2-naphthol showed an enhancement in neutral form intensity with increasing lipid concentration. 3-Amino-2-naphthol alone showed a decrease in fluorescence intensity. For all these five naphthols a saturation effect is seen. A representative curve for the variation of the neutral form fluorescence intensity is shown in Fig.8a for 4-chloro-1-naphthol. A double reciprocal plot of $1/F$ against $1/L$, according to Eq. 1

$$\frac{1}{F} = 55.6 \left( \frac{K_F L}{F_0} \right) + \frac{1}{F_0}$$

is linear, from the slope of which the partition coefficient is calculated (Fig.8b). In this equation $F$ and $F_0$ are the fluorescence intensities of the probe in the presence and absence of liposome suspension.

The partition coefficients for other naphthols mentioned above were calculated in a similar way. The $K_p$ values are listed in Table 3. These high partition coefficient values obtained suggest that all these probes partition well into lipid bilayer.

**Time resolved measurements**

Fluorescence lifetimes are often very sensitive indicators of microenvironment 27. For 1-naphthol in liposome membrane, the neutral form emission has
been found to have two component lifetimes. The shorter lifetime component corresponds to the presence of 1-naphthol in the membrane-water interface and longer lifetime component to 1-naphthol in interior of the membrane\(^7\). The fluorescence lifetimes of various prototropic forms for the chloro, methoxy and aminonaphthols are given in Table 4.

The lifetimes for bromonaphthols could not be obtained because of their low fluorescence intensity. A representative decay curve for the neutral form emission of 4-chloro-1-naphthol is given in Fig 9. The lifetimes for both the neutral and anionic forms in water are monoexponential. In liposome membrane, the lifetime of anionic form shows monoexponential decay. For the neutral form fluorescence, 4-chloro-1-naphthol and 4-methoxy-1-naphthol showed biexponential decays, whereas 7-methoxy-2-naphthol and both the aminonaphthols showed monoexponential decay.

The biexponential decay of 4-chloro-1-naphthol neutral form shows that this molecule is distributed in
the membrane at two different sites analogous to the
distribution of 1-naphthol. The shortest lifetime
component (0.87 ns) can be assigned to the molecule on
the surface site and the longer lifetime (3 ns) to the
molecule present in the interior site. The amplitude
ratios indicate that the molecule is present predominately in the surface site (87%). Only the
surface site molecule undergoes deprotonation in the
excited state because of its accessibility to water.
Hence anionic form fluorescence is monoexponential.

Similar biexponential decay is observed for 4-methoxy-
1-naphthol. Since the emitting form is only the neutral
species even in water, this molecule does not show an
ESPT behavior. 7-Methoxy-2-naphthol, however,
shows monoexponential emission from both the neutral
and anionic forms even at saturation partitioning. This
indicates that this molecule is present in surface site
accessible to water.

The aminonaphthols, which have only neutral form
emission, do not show any appreciable changes in
fluorescence lifetime both in water and lipid.

**Effect of temperature**

The phospholipid membranes undergo a well-defined
thermotropic phase transition in which the lipid chain
changes from an ordered gel state to a fluid liquid
crystalline state\(^{24,25}\). The temperature at which these
lipids undergo phase changes is known as chain melting
temperature (\(T_c\)). The \(trans/gauche\) transition of the
hydrocarbon chains of the lipid molecule increases
tremendously with increasing temperature and gives
rise to temperature dependent increase in membrane
permeability and fluidity. The ESPT of 1-naphthol was
shown to sense the phase transition\(^{12}\).

The ability of these substituted naphthols to sense the
phase changes in DMPC liposomes was examined.
DMPC liposome has a convenient phase transition
temperature at 23°C. To monitor these changes, a
variable temperature study was performed for the
lipid/probe ratio of 100 at the saturation point where
most of the probe gets partitioned into the membrane.
Only chloronaphthol and 7-methoxy-2-naphthol showed
variation in the intensity with increasing temperature.
The temperature profile for the change in neutral form
intensity is shown in Fig 10. There is a remarkable
increase in intensity at the phase transition temperature.
The explanation for this observation has been proposed
in a model for 1-naphthol distribution in our earlier
work\(^{15}\). This model proposes a redistribution of probe
population between the surface and interior sites due to
changes in membrane structure. Any change that
increases membrane porosity facilitates increased
population of interior sites, which results in an
enhancement of neutral form fluorescence. The
simultaneous existence of gel and liquid crystalline
phases at $T_c$ introduces large number of defects in the membrane that increases membrane porosity.

The inability of 3-amino-2-naphthol and 1-bromo-2-naphthol to sense the phase transition temperature of the membrane may be due to preference to stay in the surface site rather than to get redistributed into the interior site. 3-Amino-2-naphthol has a very low quantum yield in non-polar (0.12) medium compared to that in polar medium (0.41), which allows it to remain in the interfacial region itself.

Among all the seven naphthols screened as ESPT probes for monitoring lipid membrane phase changes, 4-chloro-1-naphthol is expected to be a better probe. A few further experiments were carried out with this molecule to ascertain its applicability as a probe.

**Quenching studies**

Fluorescence quenching studies on membrane-bound fluorophore provide valuable information regarding their distribution and microenvironment. In the present study iodide ion was used as a quencher. Iodide ion is an efficient hydrophilic quencher and it is expected that only the water accessible fraction of the neutral form in membrane would be quenched. Since chloronaphthol showed surface and interior site distribution behaviour and a positive response to phase changes of membrane, quenching studies were performed with this molecule. The quenching experiments were carried out above, below and at the phase transition temperature of the DMPC liposome.

The Stern-Volmer plot of $(F/F_0)$ vs $[Q]$ for the neutral form in DMPC liposome is shown in Fig. 11(a) which shows a leveling effect and a negative deviation from linearity at all three temperatures. Such a leveling would be obtained only if there are two populations of fluorophores, out of which one is accessible to quencher.

The deviation obtained above could be analyzed using a modified Stern-Volmer (SV) equation. The total fluorescence $F_t$ in the absence of quencher is given by

$$F_t = F_a + F_n$$

where “$a$” refers to the quencher accessible fraction and “$n$” refers to the fraction of fluorophore not accessible to quencher. Modification of the SV equation
considering quenching of only the quencher accessible component leads to the following equation given by Lehrer 27.

$$\frac{F}{\Delta F} = \frac{1}{f_s} K[Q] + \frac{1}{f_s}$$

where $f_s = \frac{F}{F_0}$ is the fraction of initial fluorescence which is accessible to quencher and $K$ is the Stern-Volmer quenching constant, $\Delta F$ is ($F_s - F$), where $F$ is the fluorescence intensity in the presence of quencher. The plot of $F/\Delta F$ Vs $1/[Q]$ is linear, the intercept giving $f_s$ (Fig.11b). From a high value of 0.81 in the gel state (15°C) the water accessible fraction decreases to a low value of 0.72 at $T_c$ and then again increases to 0.77 at the liquid crystalline state (30°C). This can be easily rationalized according to the distribution model discussed earlier 12. The probe accommodation ability in the interior site of the membrane is minimum at the compact gel state, maximum at $T_c$ because of membrane imperfections and intermediate in the liquid crystalline state.

**Effect of cholesterol**

Cholesterol is known to decrease membrane area compressibility and increase membrane cohesion 26,29. Cholesterol induces more efficient packing of the lipid bilayer membrane, as the combined area taken up by fatty acyl chains and cholesterol together is equal to that occupied by the head group. Cholesterol is also known to prevent lipid crystallization and maintain the bilayer in the liquid crystalline state. This results in an elimination of the acyl chain melting phase transition characteristics of the membrane. Since chloronaphthol showed remarkable changes among other naphthols, this probe was chosen to study the effect of cholesterol on membranes. The response of chloronaphthol to cholesterol-induced phase changes is shown in Fig. 12. The variation in neutral form intensity with temperature, which was large in the absence of cholesterol, progressively decreased with addition of cholesterol and by 50 mol% cholesterol the variations were minimum. The blurring of phase transition with cholesterol addition thus comes out very clearly with ESPT probing. This is similar to the behaviour of 1-naphthol in liposomes 12.

**Conclusions**

Out of seven naphthols tested, 4-chloro-1-naphthol emerges as the successful probe. The two-emission wavelengths of neutral and anion forms of this probe are well separated. Chloronaphthol duplicates 1-naphthol well in all respects. This success can be attributed to the large $\Delta pK_a$ value and the appropriate disposition of the $pK_a \sim pK_a^*$ range across the neutral pH of the medium. Even though 7-methoxy-2-naphthol also exhibits two forms, the spectral changes are minimum. 4-Methoxy-1-naphthol is also shown to partition into lipids but exhibits only one form. Thus among various substituted naphthols studied, 4-methoxy-1-naphthol can be used as a one state (only neutral form) probe while chloronaphthol and 7-methoxy-2-naphthol can be used as two state (both neutral and anion form) probes for lipid membranes. The high partition coefficient obtained for all the above three naphthols ensures that they can be used as membrane probes at neutral pH. Chloronaphthol and 7-methoxy-1-naphthol are able to sense the phase changes in liposome. The cholesterol-induced change was also monitored by chloronaphthol.

**Acknowledgement**

The authors thank Dr. Gopidas, Regional Research Laboratory, Trivandrum for extending facility for fluorescence lifetime measurements.

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