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# Cellular detection of a mitochondria targeted brominated vinyl triphenylamine optical probe (TP-Br) by X-ray fluorescence microscopy

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**Abstract:** Triphenylamine (TP) derivatives such as two-branch cationic vinylbenzimidazolium triphenylamine **TP-2Bzim** are promising turn-on fluorescent probes suitable for two-photon imaging, labelling mitochondria in live cells. Here, we designed two **TP-2Bzim** derivatives as bimodal probes suitable for X-ray fluorescence imaging. The conjugation of the TP core with a rhenium tricarbonyl moiety in the **TP-RePyta** probe altered the localisation in live cells from mitochondria to lysosomes. The introduction of bromine on the TP core generated the **TP-Br** probe retaining good photophysical properties and mitochondria labelling in live cells. The influence of calcium channels in the uptake of **TP-Br** was studied. Synchrotron Radiation X-ray Fluorescence (SXRF) imaging of bromine enabled the detection of **TP-Br** and suggested a negligible presence of the probe in an unbound state in the incubated cells, a crucial point in the development of these probes. This study paves the way towards the development of TP probes as specific organelle stainers suitable for SXRF imaging.

## Introduction

The use of fluorescent probes in fluorescence microscopy has been instrumental in non-invasive imaging studies of the subcellular localization of a compound of interest or of biological processes for example.<sup>[1]</sup> The development of fluorescent probes that selectively localize in a specific cell organelle and meet minimal criteria such as high quantum yield, high stability and low toxicity is still the focus of intense research efforts.<sup>[2]</sup> Probes suitable for two-photon fluorescence spectroscopy are particularly desirable as they imply longer excitation wavelength and thus increased penetration depth and reduced cell damage.<sup>[3]</sup> In this context, triphenylamine derivatives are a promising class of

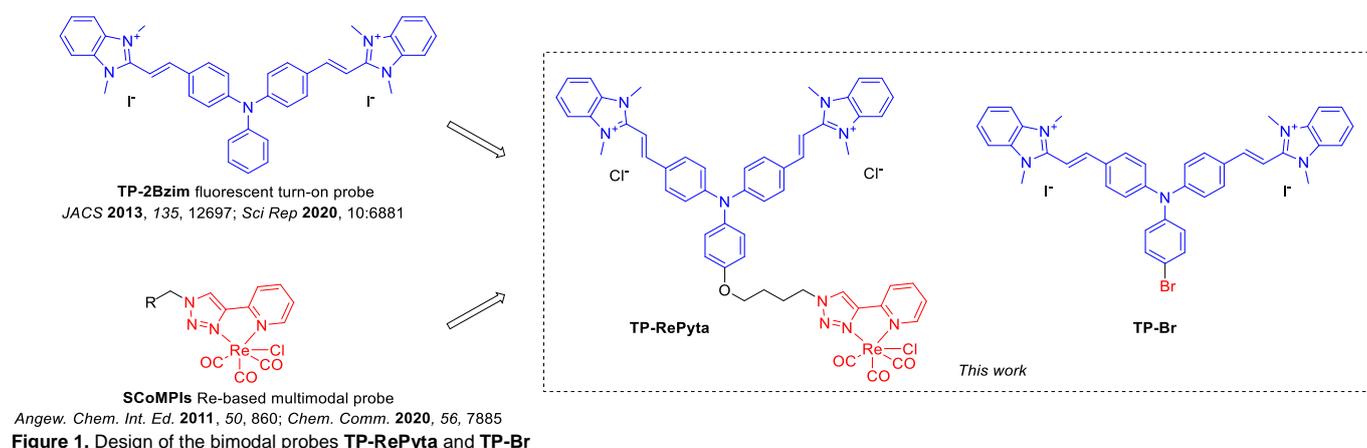
fluorescent probes.<sup>[4]</sup> They were initially designed for two-photon absorption due to their octupolar organization and they display high chemical- and photo-stability as well as good water solubility and cellular uptake. They bind to the DNA minor groove and their binding leads to a dramatic enhancement of the fluorescence signal.<sup>[4]</sup> The optimized **TP-Bzim** series, bearing two or three vinyl branches terminated by a N-methyl benzimidazolium moiety, exhibit a remarkable improvement of both their DNA affinity and fluorescence quantum yield (e.g. for the two-branch derivative (TP-2Bzim:  $K_a = 10^7 \text{ M}^{-1}$ ,  $\Phi_F = 0.54$ ), resulting in a large fluorescence emission turn-on ratio of up to 140. Nuclear DNA can be imaged in fixed cells at submicromolar concentration ([**TP-2Bzim**] = 100 nM) and ultrabright foci of centromeric AT-rich chromatin can be visualized.<sup>[4b]</sup> This outstanding combination of optical and biological properties makes **TP-2Bzim** a bioprobe surpassing the best DNA stainers. The fate and behaviour of these dyes are dramatically different in living cells.<sup>[5]</sup> The **TP-2Bzim** probe localizes in the mitochondria of living cells. This is not surprising as **TP-2Bzim** belongs to a class of delocalized cations, a specific feature found in a number of already identified small mitochondria-targeting compounds.<sup>[6]</sup>

The development of small individual multimodal probes combining more than one imaging technique has become important and it can answer crucial needs and questions.<sup>[7]</sup> First, as in the case of **TP-2Bzim**, fluorescence properties, namely quantum yield or spectrum, can be impacted by environmental factors such as binding status, molecular crowding, polarity or pH. Therefore, the apparent subcellular light distribution may not reflect accurately the spatial distribution of the probe.<sup>[8]</sup> Correlative imaging is an attractive strategy to gain new insights into the cellular distribution of **TP-2Bzim** and detect its putative presence in a non-emissive off state in the cell. Moreover,

selecting the appropriate additional modality would enable the development of specific organelle stainers for colocalization studies of non-fluorescent compounds of interest. In this study, we focused on the development of bimodal TP probes amenable for X-ray fluorescence (XRF) imaging. Scanning Synchrotron Radiation X-ray Fluorescence (SXRF) imaging is a powerful technique that combines a high detection sensitivity, a high elemental specificity ( $Z > 14$ ) and a high spatial resolution.<sup>[9]</sup> SXRF imaging has been used for mapping and quantification in biological samples owing to the depth of penetration and resolution of 100-300 nm, and with recent advances less than 40 nm.<sup>[10]</sup> The applications of SXRF microscopy has been in the areas of mapping metals/metalloids,<sup>[11]</sup> metal-based drugs in cells<sup>[12]</sup> or spheroids<sup>[13]</sup> and nanoparticle uptake in cells or animals.<sup>[14]</sup> There have been recent studies on labelling proteins with rhenium<sup>[15]</sup> and detecting manganese superoxide dismutase mimics<sup>[16]</sup> using X-ray fluorescence microscopy. Since the sample preparation of X-ray based imaging methods is compatible with fluorescence staining, correlative imaging is an area that is being explored.<sup>[17]</sup> First reports of multimodal imaging using X-ray fluorescence microscopy involved its use in combination with infrared imaging to detect an organometallic drug in worms.<sup>[18]</sup> Correlative imaging studies with synchrotron X-ray fluorescence and fluorescence imaging have focused on labelling the organelles with a conventional marker and correlating it with X-ray fluorescence information obtained<sup>[17c]</sup> or with a rhenium complex bearing both modalities.<sup>[19]</sup> We recently described rhenium carbonyl complexes conjugated to mitochondria-

targeting triphenyl phosphonium cations as antibody-free mitochondria trackers for SXRF imaging.<sup>[20]</sup>

In the present work, we designed two bimodal probes suitable for fluorescence (two-photon excitation) and SXRF imaging based on the cationic vinyl triphenylamine **TP-2Bzim** (Figure 1) for mitochondria targeting. We aim at developing low molecular weight organelle-specific probes suitable for SXRF imaging while gaining new insights into the cellular distribution of **TP-2Bzim**. The first design, **TP-RePyta**, relies on the conjugation of **TP-2Bzim** with the Re(pyta) single core multimodal probe for sub-cellular imaging (SCoMPI).<sup>[21]</sup> Rhenium complexes of general formula  $[\text{Re}(\text{N}^{\wedge}\text{N})(\text{CO})_3\text{X}]^{n+}$ , with ( $\text{N}^{\wedge}\text{N}$ ) a bidentate ligand with low lying  $\pi^*$  orbitals and X an axial ligand, display interesting features such as high stability, cell penetration abilities, luminescence emission with large Stokes shift, that have stimulated a strong interest in bio-imaging.<sup>[22]</sup> We have described fluorescence and IR correlative imaging studies using SCoMPIs<sup>[23]</sup> and their imaging by SXRF, as mentioned above.<sup>[15],[16b],[20]</sup> In an alternative strategy involving a minor modification of the vinyltriphenylamine core, a bromine atom that allows exploiting the capabilities of X-ray fluorescence<sup>[24]</sup> was introduced on the third branch of the triphenylamine core leading to the **TP-Br** probe. Live cell confocal fluorescence imaging was used to verify the localization at the mitochondria. Subsequent sample preparation by chemical fixing and air drying was optimized to preserve the mitochondrial labelling for **TP-Br**. The uptake in two cancerous and one non-cancerous cell lines was studied using light microscopy and X-ray fluorescence mapping.



**Figure 1.** Design of the bimodal probes **TP-RePyta** and **TP-Br**

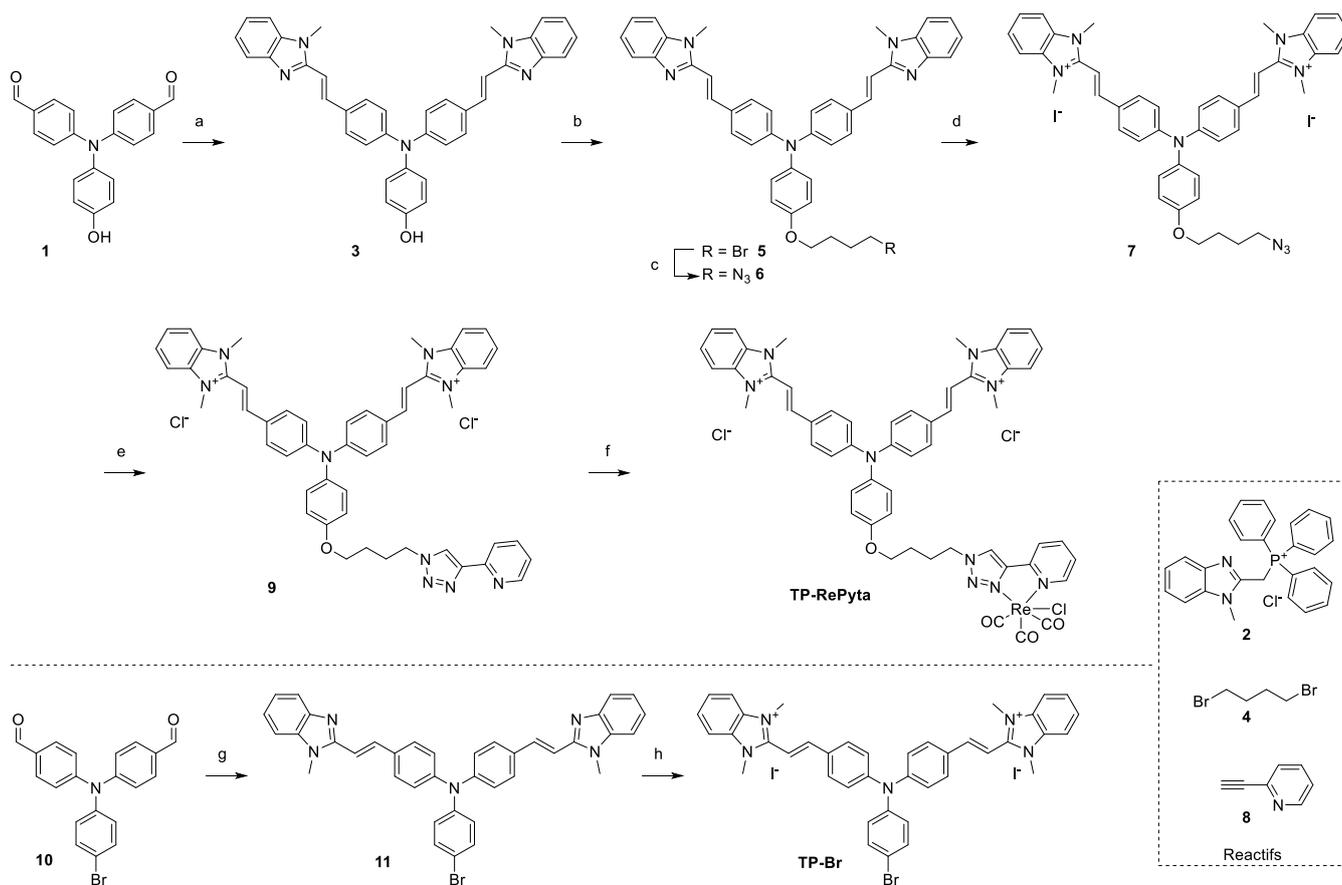
## Results and Discussion

### Design and synthesis

The synthesis of **Pyta-TP-2Bzim** started with dialdehyde **1**, already described in a previous article (Scheme 1).<sup>[25]</sup> A Wittig reaction with the phosphonium **2** on dialdehyde **1** led to the formation of the bis vinylic compound **3** in an excellent yield. Two successive nucleophilic substitutions of dibromobutane **4** respectively with phenol **3** and then with sodium azide afforded compound **6** in 66% yield. This compound was methylated using

iodomethane to give benzimidazolium salt **7**. A copper catalyzed azide alkyne cycloaddition (CuAAC) between compound **7** and ethynyl pyridine **8** followed by counter ion exchange (iodide to chloride) on resin generated the pyridine-triazole (pyta) ligand **9**. The rhenium tricarbonyl complex **TP-RePyta** was finally obtained using rhenium pentacarbonyl chloride in classical coordination conditions.<sup>[20],[26]</sup>

**TP-Br** was prepared using a two-step procedure optimized for **TP-2Bzim** (Figure 1A).<sup>[4b]</sup> A Wittig reaction between the diformyl triphenylamine **10** and the phosphonium **2** resulted in the neutral intermediate **11** with a good yield. Subsequent methylation produced the **TP-Br** probe with 95% yield.



**Scheme 1.** Synthesis of **TP-RePyta**. Reaction conditions: a) Compound **2**, DBU, THF-EtOH, r.t., 15 h, 70%; b) Compound **4**, Cs<sub>2</sub>CO<sub>3</sub>, DMF, r.t., 15h, 80%; c) NaN<sub>3</sub>, DMF, 70 °C, 16 h, 83%; d) MeI, DMF, 40°C, 15 h, 53%; e) Compound **8**, CuSO<sub>4</sub>, tris((1-benzyl-4-triazolyl)methyl)amine (TBTA), sodium ascorbate, DMF-MeOH, 40 °C, 15 h, then IRA402 Cl; 81%; f) Re(CO)<sub>5</sub>Cl, DMF, 70 °C, 15 h, 62%; Synthesis of **TP-Br**. Reaction conditions: g) Compound **2**, DBU, THF-EtOH, r.t., 15 h, 90%; h) MeI, DMF, 40 °C, 15 h, 95%.

### Photophysical properties

The absorbance and fluorescence properties of the **TP-RePyta**, **TP-Br**, and **TP-2Bzim** probes were compared in cacodylate buffer and glycerol. Spectra are shown in Fig.S2† while the comparisons with **TP-2Bzim** are provided in Table 1. **TP-Br** and **TP-RePyta** exhibit a large lowest energy absorption band with maxima at 430 and 440 nm respectively in buffer, shifted to 450 and 460 nm respectively in glycerol. Both probes are poorly fluorescent in buffer upon excitation at 450 nm. However, they are strongly fluorescent in glycerol. This behaviour means that these probes are responsive to the constraints of the environment, glycerol being a viscous solvent commonly used to restore fluorescence through restriction of molecular motions. The weak luminescence of the rhenium pyta complex (quantum yield < 1%)<sup>[26]</sup> is not observable; the emitted light (between 450 and 650 nm under excitation at 330 nm) could be reabsorbed by the triphenylamine moiety. The fluorescence quantum yields of **TP-RePyta**, **TP-Br** and **TP-2Bzim** in glycerol are respectively 7%, 29% and 35% and less than 0.005% in buffer for all of these probes (Table 1). The presence of bromine, a heavy atom, did not quench the fluorescence significantly whilst the presence of the rhenium complex impacted the fluorescence quantum yield. However, both probes retained the on/off behaviour of the cationic vinyltriphenylamine derivatives.

**Table 1.** Photophysical properties of TP derivatives in buffer and glycerol

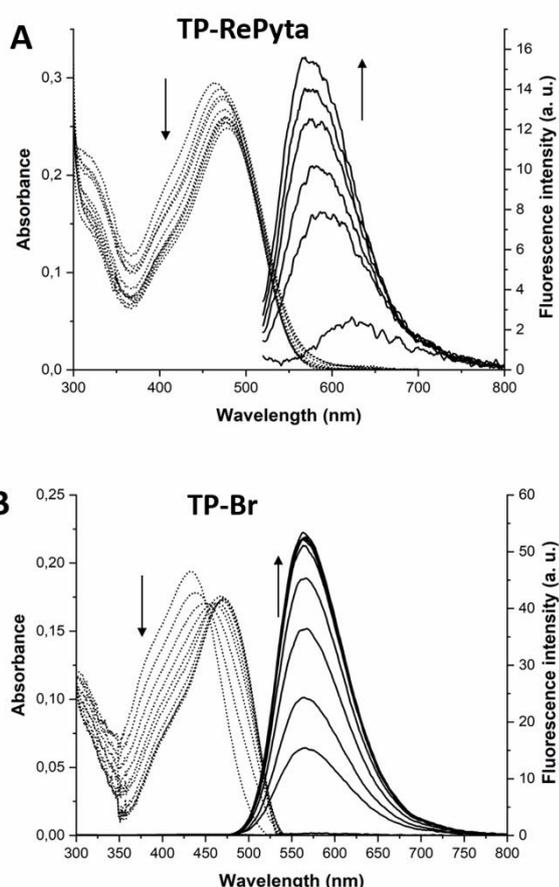
Dyes	Solvent	$\lambda_{\text{abs}}/\lambda_{\text{em}}$	$\epsilon$ (M <sup>-1</sup> .cm <sup>-1</sup> )	$\Phi_F$
<b>TP-2Bzim</b>	Buffer	432/620	42,500	<0.005
	Glycerol	448/602	40,400	0.35
<b>TP-Re-Pyta</b>	Buffer	441/619	39,000	<0.005
	Glycerol	458/598	41,000	0.07
<b>TP-Br</b>	Buffer	432/595	41,730	<0.005
	Glycerol	447/577	43,290	0.29

### DNA binding

As **TP-2Bzim** is a strong DNA binder with submicromolar dissociation constant, we first studied the impact of the modification of the new derivatives on the affinity towards DNA. To do so, the photophysical properties of **TP-RePyta** and **TP-Br** were evaluated in vitro in presence of a model oligonucleotide drevAT (figure 2). As a minor-groove binder, **TP-2Bzim** has a

strong affinity towards this palindromic sequence which is known to form an AT-rich B-DNA. The absorption decreases of TP-RePyta and TP-Br upon addition of the oligonucleotide, which demonstrated their interaction with DNA. An isosbestic point in the case of TP-Br indicates the presence of two species in equilibrium whereas none is observed for TP-RePyta.

Both compounds retain the turn-on properties of TP-2Bzim upon addition of DNA. However, the fluorescence enhancement is much lower in the case of TP-RePyta ( $F/F_0=6$ ) compared to TP-2Bzim (Table S1†). The behaviour of TP-Br is similar to the parent compound as the fluorescence enhancement is quite the same ( $F/F_0=110$ ). The affinity constants determined by titration curves fitting confirmed that TP-RePyta and TP-Br are strong DNA binders ( $K_A=2.2 \times 10^6 \text{ M}^{-1}$  and  $K_A=6.9 \times 10^6 \text{ M}^{-1}$  respectively) with good to excellent fluorescent turn-on properties (see FigS3† & Table S1†).

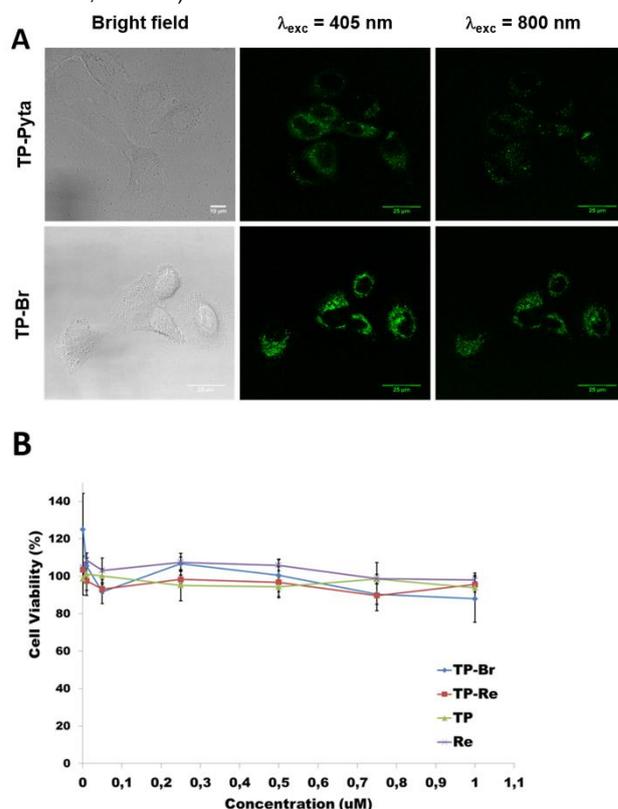


**Figure 2.** Absorption (dotted line) and fluorescence (plain line) spectra of A) TP-RePyta ( $\lambda_{\text{exc}}=510 \text{ nm}$ ) and B) TP-Br ( $\lambda_{\text{exc}}=460 \text{ nm}$ ) at  $10 \mu\text{M}$  and  $0.5 \mu\text{M}$ , respectively upon addition of DrewAT (sequence: 5'-CGCG-AAATTT-TCGCG-3') in sodium cacodylate buffer  $10 \text{ mM}$  at  $\text{pH } 7.2$ ,  $\text{NaCl } 400 \text{ mM}$

### In vitro toxicity of TP-RePyta and TP-Br probe

First, the biocompatibility of TP-RePyta and TP-Br was evaluated by MTT assay in A549 cells (Figure 3). The probes show no significant effects on cell viability (more than 80% of viable A549 cells at  $1 \mu\text{M}$  after 72 h) and can be used for live-cell imaging. The ability of these two probes for live-cell imaging was evaluated using confocal microscopy. After 2 h of incubation in A549 cells at  $0.5 \mu\text{M}$ , images were acquired after one or two-photon excitation. These two dyes are localized in the cytoplasm as shown by the

brightfield images. TP-Br exhibits higher brightness and contrast than TP-RePyta under one or two photon excitation microscopies. This was expected as TP-RePyta is less fluorescent than TP-Br under immobilization in glycerol (quantum yield of respectively 7 and 29%, Table 1).



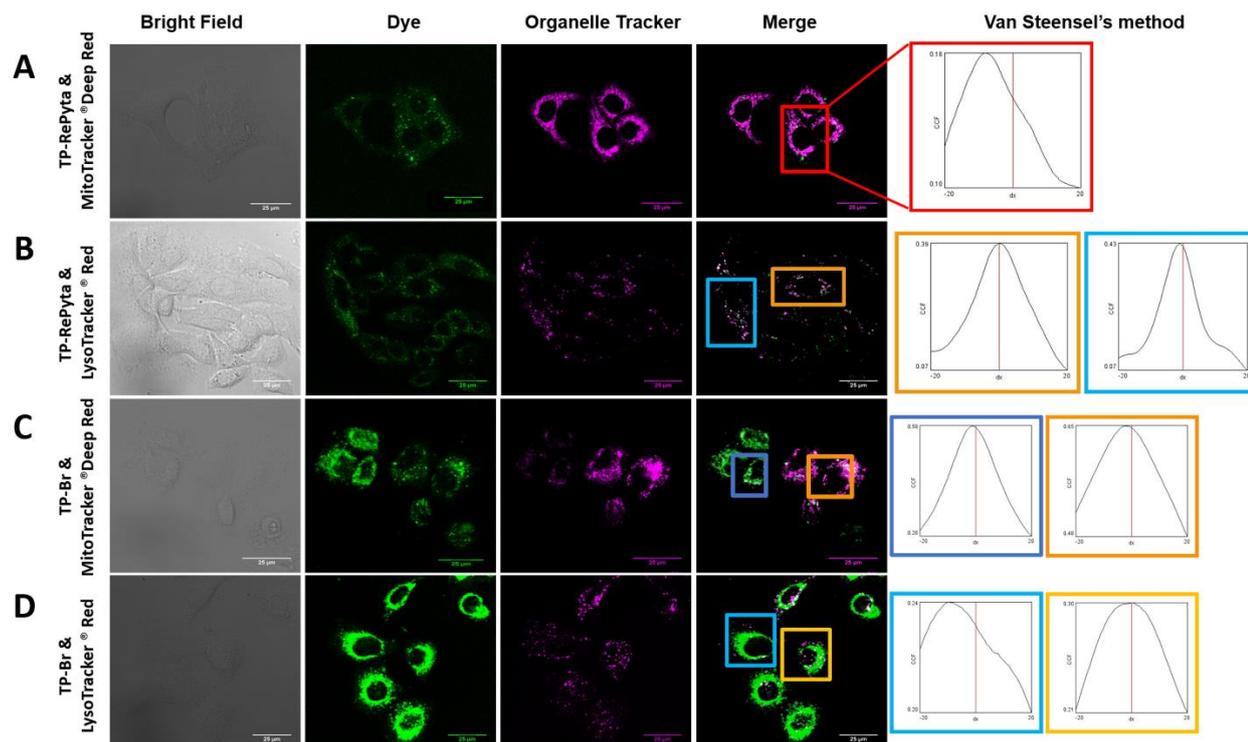
**Figure 3.** A) Live cell imaging of TP-RePyta and TP-Br ( $0.5 \mu\text{M}$ ) after incubation for 1 h in A549 under one or two-photon excitation (Emission slits  $500\text{--}700 \text{ nm}$ ); B) MTT based cell viability assay of TP-RePyta (blue), TP-Br (purple), TP-2Bzim (red) and its parent compounds RePyta (green) and TP-2Bzim (concentration range:  $0.01\text{--}1 \mu\text{M}$ , incubation time 72 h in PBS) on A549 cells.

### Mitochondrial localization and internalization mechanism of TP-RePyta and TP-Br probe in cells

We previously demonstrated that the parent compound TP-2Bzim is mainly localized at the mitochondria using colocalization studies with different organelle trackers.<sup>[5]</sup> In the present study, colocalization experiments were performed for both dyes using Mitotracker Deep Red (MTDR, Figure 4). Excitation at  $633 \text{ nm}$  led to the near infrared fluorescence of MTDR ( $650\text{--}800 \text{ nm}$ , colored in magenta) while excitation at  $405 \text{ nm}$  led to a yellow fluorescence ( $500\text{--}650 \text{ nm}$ , colored in green) for both compounds typical of triphenylamine probes (Figure 4A and C). Of note, RePyta carbonyl complexes can be imaged in cells upon excitation at  $405 \text{ nm}$  leading to an emission centered around  $550 \text{ nm}$ <sup>[8a]</sup>. However, considering the typical quantum yield of such complexes ( $<1\%$ ) and the conditions of incubation used here ( $0.5 \mu\text{M}$  for 1 h), the signal of TP-RePyta is proposed to arise mostly from the TP moiety. Spatial colocalization was analyzed using the Van Steensel's method.<sup>[27]</sup> This method applied to TP-Br showed a Gaussian maximum centered on the zero position of dx (red line) suggesting a complete overlay of TP-Br labelling with the mitochondrial stain. At the opposite, the Van Steensel method showed a shift between the Gaussian maximum and the zero

position of  $dx$  for **TP-RePyta**. This indicates that **TP-RePyta** is only poorly colocalized with MitoTracker Deep Red. The conjugation of **TP-2Bzim** with the Re-Pyta moiety seems detrimental to the mitochondrial targeting. This is quite unexpected as recent work from Bertrand et al.<sup>[20]</sup> demonstrated that the conjugation of a Re-Pyta probe with triphenyl phosphonium (TPP) cations retained the mitochondrial targeting of these TPPs. We then conducted colocalization experiments with both probes and LysoTracker Red. Excitation at 561 nm led to the red fluorescence of LysoTracker Red (600–750 nm, colored in magenta) indicating that LysoTracker Red was compatible with both probes (Figure 4B and D). These experiments demonstrated

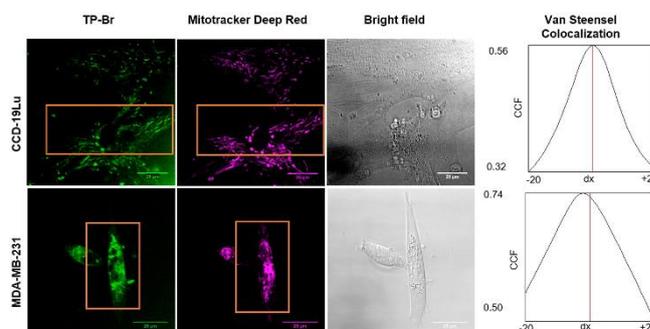
that **TP-RePyta** was mainly localized in the lysosomes with a clear alignment of the Gaussian maximum centered on the zero position of  $dx$  (Figure 4B) whereas **TP-Br** was poorly colocalized with LysoTracker Red. To conclude, **TP-Br** retains the mitochondrial labelling of its parent compound **TP-2Bzim**, whereas **TP-RePyta** mainly localizes in the lysosomes. The mitochondria targeting of **TP-Br** is not surprising since the addition of a Br represents a very slight modification of the parent compound that unambiguously accumulates in mitochondria of living cells. For that reason, along with its better photophysical properties, we decided to pursue the study on **TP-Br** alone.



**Figure 4.** Co-localization experiments with **TP-RePyta** (A) & (B) or **TP-Br** (C) & (D) (first incubation: 0.5  $\mu$ M, 1h;  $\lambda_{exc}$  = 405 nm, Emission slits 500-650 nm) and MitoTracker® Deep Red (second incubation: 100 nM, 45 min;  $\lambda_{exc}$  = 633 nm, Emission slits 650-800 nm) or LysoTracker® Red (second incubation: 100 nM, 45 min;  $\lambda_{exc}$  = 561 nm, Emission slits 600-750 nm) in A549 cells.

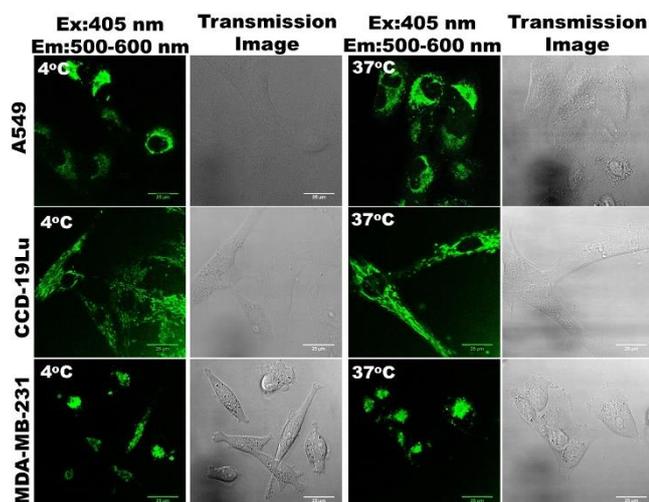
The incubation time and concentration (data not shown) were varied to assess the optimal condition for mitochondrial labelling in other cell lines MDA-MB-231 (cancerous cells) and CCD-19Lu (fibroblasts). It was observed that 30 to 60 min incubation at 0.5  $\mu$ M, depending on the cell type, was sufficient to observe labelling in cells. The morphology of the labelling matched that observed for mitochondria (Figure 5). Colocalization experiments using MitoTracker Deep Red confirmed a total mitochondrial labelling in CCD-19Lu and a partial mitochondrial labelling in MDA-MB-231 cell line (Figure 5). The MitoTracker Deep Red is known to label the intermembrane space of the mitochondria<sup>[28]</sup> and the complete overlay of **TP-Br** with the MitoTracker label suggests an intermembrane localization in CCD-19Lu cells. The partial overlay observed in MDA-MB-231 cells could be due to the localization of the probe in the matrix and the intermembrane space. As MitoTracker Green, which is known to label both the matrix and the intermembrane space,<sup>[29]</sup> has an excitation/emission which overlaps with the excitation/emission of the **TP-Br** probe, they are not a compatible pair for colocalization and hence we could not test this hypothesis. However, a partial overlay of MitoTracker

Green was indeed observed when colocalized with MitoTracker Deep Red stain in these two cell lines (Fig.S7†). Further, we also confirmed mitochondrial labelling and uptake of **TP-Br** probe in cervical cancer cells, namely HeLa (Fig.S8†).



**Figure 5.** Co-localization experiments with **TP-Br** (first incubation: 0.5  $\mu$ M, 1h;  $\lambda_{exc}$  = 405 nm, Emission slits 500-650 nm) and MitoTracker® Deep Red (second incubation: 100 nM, 45 min;  $\lambda_{exc}$  = 633 nm, Emission slits 650-800 nm) in CCD-19Lu and MDA-MB-231 cells.

The rapid uptake of the **TP-Br** in a short incubation time of 30 min led us to hypothesize diffusion to be the predominant mechanism of internalization. To verify this hypothesis, the internalization upon incubation at a reduced temperature of 4 °C, which inhibits energy mediated uptake,<sup>[30]</sup> was studied. Sodium azide-based inhibition can alter the mitochondrial oxidative phosphorylation which could hinder uptake of the probe,<sup>[30]</sup> hence this method was not chosen to inhibit energy mediated uptake. No significant effect on the internalization of the probe was observed at reduced temperature (Fig.6, control cells Fig.S9†) suggesting passive or energy independent facilitated diffusion to be the predominant mechanism of uptake and the slight reduction in the uptake speed could be assigned to reduced membrane fluidity at low temperatures.<sup>[30]</sup> At reduced temperature, the probe showed partial colocalization with MitoTracker Deep Red probe for the two cell types (Fig.S10†). CCD-19Lu cells which showed complete colocalization of **TP-Br** probe with MitoTracker Deep Red label at 37°C showed only partial colocalization at 4°C. Though the probes did not show a drastic decline in internalization at low temperature, some regions might be of low intensity to correlate with MitoTracker labelling. This could be the reason for partial overlay.



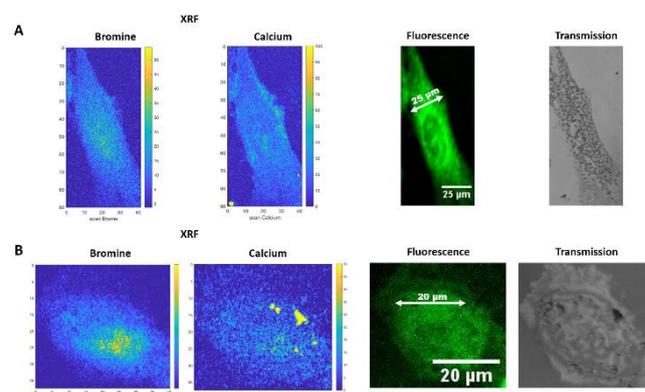
**Figure 6.** Internalization of **TP-Br** (0.5 μM) in A549, CCD-19Lu and MDA-MB-231 live cells when incubated for 60 min at 4 °C to slow down energy mediated uptake along with 30 min incubation of **TP-Br** (0.5 μM) at 37 °C. Scale bar: 25 μm

### X-ray fluorescence microscopy of **TP-Br** probe in cells

The samples for X-ray fluorescence microscopy were prepared using silicon nitride windows as described in materials and methods. We have chosen a chemical fixation/air drying method due to the ease and speed of sample preparation and focus not being endogenous ions that can diffuse during this treatment.<sup>[15],[31]</sup> As conventional fixing in 4% paraformaldehyde (PFA) caused the relocalization of the parent **TP-2Bzim** probe from the mitochondria to the nucleus in MCF7 cells,<sup>[4b]</sup> in this work, the fixing method was optimized for each cell type. HeLa cells were fixed in 2% PFA for 8 min (Fig. S11†, Control Cells Fig. S12†) which helped preserve the mitochondrial labelling with minimal nuclear relocalization. In the case of CCD-19Lu fibroblast cells, 4% PFA (Fig.S11†, control cells Fig.S12†) did not alter much the mitochondrial labelling while 2% PFA was not sufficient to preserve it (Fig. S13†). This difference could be due to the larger size of the fibroblast cells. Due to these results, 4% PFA

was the preferred fixing method for CCD-19Lu cells. To further verify that the probes retained the mitochondrial staining, cells were co-stained with MitoTracker Deep Red before fixing and air-drying the cells. These cells were imaged by confocal imaging and showed similar labelling as **TP-Br** (Fig. S14†). Since the cells were air dried, the cytoplasm collapsed on the nucleus causing apparent fluorescence in the nuclear region, which made colocalization analysis difficult between the probes.

X-ray fluorescence imaging of CCD-19Lu cells and HeLa cells (Fig. 7 and corresponding integrated spectra showing bromine peak Fig.S15† and Fig.S16†) treated with **TP-Br** indicated significant presence of bromine compared to non-treated control cells (Fig.S17†). Although it is very hard to perform a correlation study between X-ray fluorescence and optical images due to sample preparation method and use of different instruments, X-ray fluorescence imaging allowed the unequivocal detection of the **TP-Br** optical probe in these cell lines with a distribution qualitatively consistent with that observed by fluorescence. Interestingly, no dark regions in the fluorescence images were noticed with a positive X-ray fluorescence bromine map suggesting there was negligible non-fluorescent unbound probes present in CCD-19Lu and HeLa cells.



**Figure 7.** X-ray fluorescence maps of bromine and calcium normalized to 1 s/pixel measurement time (Ex energy: 17.15 keV) with color map (intensity) (left), fluorescence images ( $\lambda_{exc} = 405$  nm, Emission slits 500-600 nm) (middle) with the corresponding transmission images (right) of (A) air dried 4% PFA fixed CCD-19Lu cells (300 nm pixel size) and (B) 2% PFA fixed HeLa cells (300 nm pixel size) after incubation with **TP-Br** (0.5 μM) for 30 min. The double-headed arrows indicate the distance across distinct cell features.

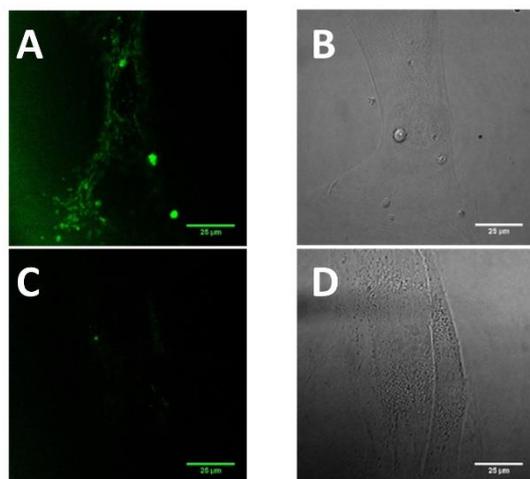
### Calcium Channel blocking and internalization

To explore further the internalization mechanism of the probe, we studied the effect of blocking calcium channels. A L and T type calcium channel blocker, Verapamil,<sup>[32]</sup> was used to inhibit uptake of the **TP-Br** probe in CCD-19Lu cells and to assess the effect of membrane potential change on its internalization. The activity of Verapamil was verified by using MitoTracker CMXRos, a mitochondrial membrane potential sensitive probe and fluo-4AM, a calcium indicator (Fig. S18†). A decrease in membrane potential and increase in calcium signal was observed in CCD-19Lu cells, as Verapamil blocked efflux pumps along with influx pumps, reducing calcium transients<sup>[33]</sup> or intracellular calcium increase induced by membrane potential decrease from internal stores or external media.<sup>[34]</sup>

CCD-19Lu cells showed significant reduction in uptake of **TP-Br** probe in the presence of Verapamil as compared to control cells not treated with Verapamil (Fig.8).

Verapamil blocking on MDA-MB-231 cell line showed a weak reduction in mitochondrial membrane potential compared to control cells without Verapamil and a heterogeneous calcium distribution with increase in some cells as was also observed in control cells. No effect on the uptake of **TP-Br** was observed compared to control cells (Fig.S19†). Human fibroblasts are reported to have only L type calcium channels.<sup>[35]</sup> This in turn suggests a strong influence of L type calcium channels in CCD-19Lu cells on the uptake of **TP-Br**. MDA-MB-231 cancerous cells express other calcium channels such as Orai3 or TRP which could be involved in the internalization which are not blocked by Verapamil.<sup>[36]</sup>

Upon treatment with Verapamil, the signal intensity of MitoTracker CMXRos, a mitochondrial membrane potential sensitive probe, was significantly reduced in CCD-19Lu cells whereas it was poorly affected in MDA-MB231 cells. This is in line with the impact on the uptake of the **TP-Br** probe in these two cell lines.



**Figure 8.** Internalization of the **TP-Br** (0.5  $\mu$ M, 30 min) in the absence (A & B) and presence (C & D) of Verapamil (VP, 20  $\mu$ M) calcium channel inhibitor in CCD-19Lu cells. A & C: confocal microscopy ( $\lambda_{exc}$  = 405 nm, emission slits: 500-600 nm), B & D: transmission images. Scale bar: 25  $\mu$ m

## Conclusion

We designed two new bimodal vinyl triphenylamine probes **TP-RePyta** and **TP-Br** for optical and X-ray fluorescence imaging. These probes mostly retain the photophysical properties of the parent compound **TP-2Bzim** with fluorescence enhancement upon DNA binding. The probes are not toxic and can be imaged in cells in single and two-photon absorption excitation. While the conjugation with a rhenium pyta tricarbonyl [Re(pyta)(CO)<sub>3</sub>X] complex modified the localisation of the probe to the lysosomes in living A549 cells, the brominated probe **TP-Br** retained a subcellular localization consistent with mitochondria staining in live cells of the three cell lines used (CCD-19Lu, HeLa, MDA-MB231). **TP-Br** is suitable for X-ray fluorescence detection and imaging in cells. SXRF imaging in CCD-19Lu and HeLa cells did not reveal significant positive bromine signals in places

where no fluorescence was detected, suggesting a negligible presence of non-fluorescent unbound probes. This is a crucial information for the development of these turn-on fluorescent probes, that is revealed thanks to the X-ray fluorescence imaging modality introduced by the bromine atom. The **TP-Br** probe holds potential as a multimodal light and X-ray fluorescence imaging probe owing to diffusion mediated uptake and low toxicity in 2D cell culture. Future perspective of this study would be to extend X-ray fluorescence and fluorescence microscopy of the probe in 3D spheroids. Moreover, emerging upgrade of synchrotron sources, including the planned upgrade of Synchrotron Soleil, will provide significant flux increase (e.g. two order of magnitudes at Nanoscopium<sup>[37]</sup>) resulting in one order of magnitude increased sensitivity. These probes, in the future, can be developed as organelle targeted X-ray fluorescence contrast agents along with fluorescence imaging capabilities.

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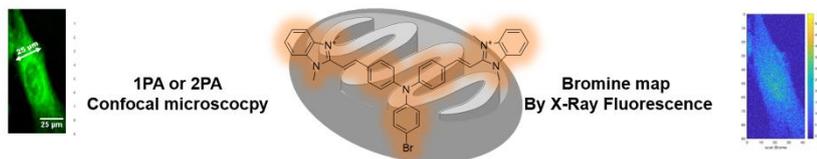
**Keywords:** organic chemistry • fluorescence microscopy • X-Ray fluorescence • bimodal probes • mitochondria

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## Entry for the Table of Contents



We designed and synthesized a new bimodal vinyltriphenylamine probe. **TP-Br** is an off-on fluorescent probe that targets mitochondria thanks to its lipophilic cationic structure. The probe was imaged by two modalities in cells: by one-photon or two-photon excitation fluorescence microscopy and by Scanning Synchrotron Radiation X-ray Fluorescence.