8. Publications and Patents
The earthworm, *Eudrilus eugeniae*, has a prodigious ability to regenerate lost segments. The skin of the worm has an outermost epidermal layer followed by a thick circular muscle layer and an innermost thin longitudinal cell layer. During the process of regeneration, the circular muscle layer decreased in thickness, and longitudinal cell layer increased. The histological analysis of the regenerated worm shows that the longitudinal cell layer forms the regeneration blastema. BrdU-labeling retention assay confirmed that the circular muscle and longitudinal cell layers have BrdU-positive cells, which migrate from the adjacent segments to the regeneration blastema. In addition, it was noted that the cells of the earthworm, *E. eugeniae*, have the property of autofluorescence. Autofluorescence was found in the cytoplasm, but not in the nucleus. It has been also found that the major source for autofluorescence is riboflavin. Further, it was also demonstrated that supplementation with riboflavin increases the rate of regeneration, while regeneration was hampered by reduced levels of riboflavin. The importance of riboflavin in regeneration was also confirmed by rescue assay. In addition, it was also identified that BrdU-positive cells are highly fluorescent compared to the surrounding cells.

**Introduction**

The segmented worm *Eudrilus eugeniae* belongs to the family Eudrilidae. The mature worm has about 80–100 segments [1]. The vital organs of *E. eugeniae* are located in the first 13 segments and consist of a mouth, simple brain, 2 pairs of heart, testis, seminal vesicle, rudimentary ovary, oviduct, and accessory glands of the ovary. A thick cylindrical collar-like structure, called the clitellum, which plays an important role in reproduction, is present in segments 13–18 [2]. The posterior part has some vital organs such as a pair of prostate glands, intestine, and the anus. All the internal organs are in the coelomic fluid, which also has different types of cells called coelomic cells. They have the property of autofluorescence [3]. The source of the fluorescence is mainly riboflavin. Autofluorescence has been reported in the body setae [4] too. The fluorescence activity of the other types of cells in the worm has not yet been studied.

In animals, autofluorescence has been reported in the scorpion [5], spider [6], butterflies [7], jellyfish [8], frog [9], and many sea animals [10]. Chemical substances such as coumarin [5] or the modified amino acid dityrosine [11] have been reported as sources of autofluorescence in living systems. In humans, autofluorescence has been reported in the cytoplasm of the human hair follicle stem cell [12]. The fluorophore responsible for autofluorescence in the stem cell is not yet known.

Many animals have been used to study regeneration and stem cell biology. The salamander has been shown to regenerate its limb, tail, upper and lower jaws, ocular tissues such as the lens and retina, the intestine, and small sections of the heart [13–16]. In axolotl, the transplantation of blastema from the amputated limb to the anterior chamber of the eye or a tunnel bored in the connective tissue of the dorsal fin leads to normal regeneration [17,18]. It was also reported that cells retain the memory of their tissue origins during limb regeneration in axolotl [19]. Zebrafish has the capacity for regeneration and renews the myocardium vigorously and restricts scar formation [20,21]. Axon regeneration in *Caenorhabditis elegans* reveals the regeneration of several neuron types, including motor and sensory neurons, upon injury [22]. The process of regeneration, from the planaria to amphibians, starts with the formation of regeneration blastema, which is a proliferative mass of undifferentiated progenitor cells from which new differentiated cells arise [23,24]. The earthworm has a prodigious regeneration capacity, and studies on regeneration have been reported in earthworms such as *Eisenia andrei*, *Eisenia fetida*, *Lumbricus rubellus* [25–29], *Ptychodera flava* [30], and *Enchytraeus japonensis* [31]. In *E. japonensis*, regeneration is controlled by the nervous...
system [32]. In addition, it has been reported that in most of the oligochaete worms, a common morphological feature of the neoblast is the presence of undifferentiated cell types having a high nucleo-cytoplasmic ratio, a large nucleus with a large nucleolus and a basophilic cytoplasm [33–37]. Even though the earthworm is easy, economical to rear, and a convenient model system, the process of regeneration has not been worked out in detail.

In the present study, it has been found that the longitudinal cell layer in the skin forms the regenerative blastema. The BrdU-labeling retention assay revealed that BrdU-positive cells are present in the boundary of the circular muscle layer close to the epidermis and in the longitudinal cell layer. The BrdU-positive cells migrated into the blastema during regeneration. Interestingly, it was found that these cells have a strong fluorescent property compared with the surrounding cells, and the major source of fluorescence is riboflavin. Riboflavin is important for normal regeneration of the earthworm.

Materials and Methods

Culture and maintenance of earthworm

The earthworm, *E. eugeniae*, was maintained in a tub containing soil, cow dung, and leaf litter at an ambient temperature [38].

Autofluorescence of coelomic fluid cells

To observe the autofluorescence of coelomic fluid cells, coelomic fluid was collected from the mature worm. It was smeared on a clean glass slide. The smear was fixed with 4% paraformaldehyde, briefly washed with 1×PBS, and examined under a Nikon Ti-S inverted fluorescence microscope.

Histology

To study autofluorescence in the worm and the patterns of tissue formation during regeneration, histology was performed. The worm tissues were fixed with 10% formalin for 24 h and allowed to dehydrate (by gradient isopropyl alcohol from 60% to 100%), followed by clearing of the isopropyl alcohol with xylene, and then embedded in paraffin wax (Purchased from HiMedia Laboratories Private Limited). The block was sliced into 6-μm-thick sections using a microtome (Besto), and the sections were mounted on slides and viewed under a Nikon Ti-S inverted fluorescent microscope for autofluorescence, after which the slides were stained with a hematoxylin–eosin combination. Then, the staining pattern was documented. Solvents used for histology were purchased from Merck Specialities Private Limited, India.

Sample preparation for thin-layer chromatography

Earthworm tissues were homogenized with sterile distilled water. The homogenates were centrifuged at 5,000 rpm for 10 min (Eppendorf Mini Spin Centrifuge), and the supernatant was resolved by thin-layer chromatography.

Thin-layer chromatography

The slurry was prepared by mixing silica gel and water in the ratio 3:2. It was applied on the glass plate at a thickness of about 0.25 mm and the plate was allowed to dry at room temperature for 15–30 min. Then, the plate was kept in a hot-air oven at 100°C–120°C for 2 h [39]. Samples were loaded on the plate, and resolved with a solvent mixture [butanol (7):chloroform (4):acetic acid (5):ammonia (1):water (1)] and visualized under UV light. The thin layer chromatography (TLC) plate was further stained with ninhydrin. The spot visualized under UV was scraped off using a surgical blade and the scraped material was transferred to a microfuge tube. For eluting the fluorophore and riboflavin from the silica gel, distilled water was added and mixed well. The tube was then centrifuged at 10,000 rpm for 10 min, and the supernatant was used for thin-layer chromatography and spectrofluorometric analysis. Solvents used for the TLC were purchased from Merck Specialities Private Limited, India.

Estimation of riboflavin

The known concentration of riboflavin was resolved using thin-layer chromatography and visualized under UV light. By using imaging software NIS-Elements BR 3.1, the grayscale intensity was measured. From the data, a standard graph was generated and the concentration of riboflavin in the different samples of worm was determined.

A solution of riboflavin in distilled water was scanned using a spectrophotometer and the absorbance maxima were determined. A standard graph was then plotted by taking different concentrations of riboflavin. Using the standard graph, unknown concentrations of riboflavin in worm samples were calculated.

Spectrofluorometric analyses

Spectrofluorometric analyses were performed using the lysate of earthworm. Earthworm tissues were homogenized with sterile distilled water, and the lysate obtained was centrifuged at 5,000 rpm for 10 min (Eppendorf Mini Spin Centrifuge). The supernatant was collected, and it was sub-

BrdU labeling retention assay

The BrdU (purchased from Sisco Research Laboratories Private Limited) stock solution was prepared at a concentration of 10 mg/mL in 1×PBS and each worm was injected with 10 μL of BrdU from the 20th segment to the 24th segments. The worm injected with BrdU was maintained for 30 days for chasing. On the 30th day the worm was amputated at the 11th segment and allowed to regenerate the lost part. The regeneration blastema appeared on the 4th day, and it was formalin-fixed, paraffin-embedded, sectioned, and stained with anti-BrdU antibody.

Immunohistochemistry

To visualize BrdU incorporation, paraffin-embedded earthworm tissue sections (6μm) were de-paraffinized with xylene and hydrated. Endogenous peroxidase was inhibited by incubation of the sections for 30 min with freshly prepared 10% H2O2 and 10% methanol in 1×PBS. The sections were
then treated with 0.1% trypsin in 0.1% CaCl$_2$ at 37°C for 10 min. DNA was denatured by incubating the section with 2N HCL at 37°C for 45 min. Nonspecific staining was blocked by treatment with 2% BSA for 1 h at ambient temperature. The sections were then incubated overnight at 4°C with mouse monoclonal anti-BrdU antibody (Clone BU-33; Sigma) at a dilution of 1:200 in BSA. After incubation with primary antibody, tissue sections were washed and incubated for 1 h with goat anti-mouse IgG conjugated with horseradish peroxidase (Lot No: 062100; GeNei) at a dilution of 1:500. Staining was developed with Diaminobenzidine (DAB Kit; GeNei) substrate and the sections were counterstained with Ehrlich hematoxylin. The prepared slides were mounted with DPX and observed under a Nikon Ti-S microscope.

### Statistical analysis

Statistical analyses such as standard deviation, $P$ value for BrdU-positive cells versus a positive control, and regeneration growth kinetics were performed using Microsoft Office Excel 2007.

### Results

The earthworm, *E. eugeniae*, is shown in Fig. 1A. The clitellum of the worm begins at the 13th segment and ends at the 18th [2]. The segments upstream (from the 1st to the 13th) and downstream (from the 18th to the last) of the clitellum are called the anterior and posterior parts, respectively.

![Regeneration studies in the earthworm, Eudrilus eugeniae.](image)

**FIG. 1.** Regeneration studies in the earthworm, *Eudrilus eugeniae*. (A) The earthworm, *E. eugeniae*, has head, clitellum (from 13th to 20th segment), intestine, and tail. (B) Histological analysis of a control worm from 11th to 13th segments. (C) Transverse section of 13th segment of normal worm shows outermost epidermal layer followed by circular muscle layer, and the innermost layer of the skin was a longitudinal cell layer. The next lining is the intestinal layer. (D) The worms were amputated at 11th segment, and the wound was healed after 24 h. (E) The amputated worm starts regeneration and forms the regeneration blastema at 48 h. (F) Histology analysis of 48 h regenerated earthworm. The longitudinal cell layers (third layer) cover the wounded area and proliferate to form the regeneration blastema as marked by the asterisk. (G) Magnified image of the regenerated site. The longitudinal cell layer proliferates and forms the regeneration blastema, as marked by the asterisk. (H) Regeneration blastema at 7th day after amputation. (I) The figure shows the histological analysis of 7th day regenerated blastema. In the regeneration blastema, septum was formed as marked by the arrow. B, blastema; E, epidermal layer; C, circular muscle layer; L, longitudinal cell layer; SV, seminal vesicle; BV, blood vessel; IL, intestinal lumen.
To study the regeneration process, 6 worms were taken and amputated at the junction of the 10th and 11th segments. The first 10 segments (1–10) contain the important organs as described earlier. The amputated worms were maintained in a rearing bed, and it took 24 h for the wound to heal at the posterior part of the worms (Fig. 1D). The regeneration blastema was formed at the 48th hour in all the worms (Fig. 1E).

The normal anterior segments (segments 1–10) and the regenerating blastema were subjected to histology analysis (Fig. 1B, C) and (Fig. 1F, G). The pattern (Fig. 1B, C) shows that the worms have an outermost epidermal layer followed by a circular muscle layer. The innermost layer in the skin is longitudinal cells. These layers are shown clearly in the longitudinal (Fig. 1B) and transverse section (Fig. 1C) of the worms, indicating that all 3 layers (epidermal, circular, and longitudinal layers) are not even in the body of the earthworm. They are thicker in the dorsal side and their thickness gradually decreases toward the ventral side, as shown in Fig. 1C.

During regeneration, there was no change in the epidermal layer. In contrast, the thickness of the circular muscle

**FIG. 2.** BrdU labeling retention assay. (A) Intense brown color BrdU-positive cells were observed in the regenerated blastema; the boxes indicate the clumps of BrdU-positive cells. The line separates regeneraded blastema and the adjacent segment. The image was taken with the 4× objective. Asterisk (*) indicates the fold artifact. (B–E) Magnified image of BrdU-positive cells of panel (A). The image was taken with the 40× objective. (F) Intense brown color BrdU-positive cells marked by boxes were observed in the nearby segment (12th) of regenerated blastema. The image was taken with the 20× objective. (G) The worms injected with BrdU were processed after 34 days with anti-BrdU antibody for non-regenerating control purpose. The BrdU-positive cells in brown color were shown in the box. (H) The bar diagram shows the BrdU-positive cells in control and regenerating worms with standard deviation. *P value (0.0003). E, epidermal layer; C, circular muscle layer; L, longitudinal cell layer. Color images available online at www.liebertonline.com/scd
layer reduced from 170±3 to 98±2 μm at the mid-part of the dorsal side. In contrast, the longitudinal cell layer, which was 100±2 μm thick in normal worms, increased in thickness to 188±3 μm during regeneration. In addition, the section of the worm at 48 h of regeneration shows that the regeneration blastema is formed by the longitudinal cells (Fig. 1F, G).

In the 7th day blastema shown in Fig. 1H, the formation of septum (Fig. 1I) (indicated by the arrow) was observed. Based upon the data it is concluded that the regeneration blastema is formed by longitudinal cells, and that these cells divide further to produce different types of cells to regenerate the lost segments.

BrdU-labeling retention assay was performed in order to locate the stem cells. Six worms were injected with BrdU (100 μg/g of worm) into the ceolomic fluid from the 20th segment to the 24th. The BrdU-injected worms were maintained carefully for 30 days. On the 30th day, the worms were amputated at the junction of the 10th and 11th segments. The regenerated blastema of the 4th day was subjected to sectioning, followed by immunohistochemistry with the anti-BrdU antibody and a brief staining with hematoxylin. The data (Fig. 2A) show the presence of a cluster of BrdU-positive cells in the blastema. These cells are shown in the boxes of Fig. 2A. The regions containing the BrdU-positive cells were documented with the 40× objective (Fig. 2B–E). The data clearly show that the BrdU-positive and surrounding cells are stained dark brown and blue (by hematoxylin), respectively. The data show that the mass of slow-proliferating

**FIG. 3.** Autofluorescence of BrdU-positive cells. (A) BrdU-positive cells at the regeneration blastema, marked by arrow, stained with BCIP/NBT system. (B–D) Autofluorescence of BrdU-positive cells under red, blue, and green filters, respectively. (E, F) BrdU-positive cells were also documented in adjacent segments of regeneration blastema, marked by arrow, stained with diaminobenzidine system (E) and under fluorescent mode with red filter (F). E, epidermal layer; C, circular muscle layer; L, longitudinal cell layer.
BrdU-positive cells is surrounded by fast-proliferating cells that are BrdU-negative. To study the presence of BrdU-positive cells in the upstream segments of the blastema, segments 11–13 were sectioned and processed for immunohistochemistry with the anti-BrdU antibody. The data (Fig. 2F) clearly show that BrdU-positive cells are present in the circular muscle layer, being particularly abundant in the circular muscle layer at the base of the epidermis. At the same time, a few BrdU-positive cells are also found in the longitudinal layer shown and marked by boxes in Fig. 2F. To study BrdU-positive cells in the normal worm, 3 worms were injected with BrdU (100 μg/g of worm) into the coelomic fluid from the 20th segment to the 24th. These worms were maintained carefully for 34 days, after which they were processed for immunohistochemistry using the anti-BrdU antibody. The data from this study are shown in Fig. 2G, which clearly reveals the presence of a higher population of BrdU-positive cells in the regenerating worm (Fig. 2F) than in the normal worm (Fig. 2G). To verify these results, the experiments were repeated thrice and cells in the different layers of the skin and BrdU-positive cells were counted in each mid-part section of the 11th and 12th segments of regenerating and normal worms (Supplementary Table S1; Supplementary Data are available online at www.liebertonline.com/scd). The counts of BrdU-positive cells were 258 ± 13.92 and 129 ± 5.97 per segment in the regenerating and normal worm, respectively, as shown in Fig. 2F. Hence, it was concluded that the number of BrdU-positive cells increases in the adjacent segment of the blastema during regeneration. In agreement with the data of Fig. 1F and G, the data shown in the Supplementary Table S1 also show that the number of circular-muscle cells decreased (from 333.84 ± 11.58 cells to 202.66 ± 19.73 cells) and that of longitudinal cells increased (from 178.84 ± 7.25 cells to 291.33 ± 11.53 cells) in the regenerating worm compared with the normal worm.

As a negative control, earthworm tissue sections were processed with control anti-mouse serum and data from this experiment are shown in Supplementary Fig. S1. The data clearly show the absence of any reaction involving DAB, confirming that the earlier data with the BrdU antibody (Figs. 2 and 3E) are, in fact, signals corresponding to the reaction of DAB with the anti-BrdU antibody.

It has been reported that stem cells of the human hair follicle have a capacity for autofluorescence [12]. This property of stem cells has been documented by different groups [40–42]. In addition, cells that were differentiated from stem cells were not autofluorescent [43]. To check for the presence of autofluorescence in stem cells of the earthworm, two 6-μm sections were made longitudinally, and, first, it was examined by fluorescence microscopy (data shown in Fig. 3B–D, F). Then, the sections were immunostained with anti-BrdU antibody. One of the sections was developed with BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) and the other with DAB. The results are presented in Fig. 3A and E, respectively. The data show almost all cells in the section fluoresced. Interestingly, BrdU-positive cells are highly autofluorescent compared with their surrounding cells. As a negative control, a thin section of rat kidney tissue was examined under the fluorescence microscope. No cells with the property of autofluorescence could be observed (data shown in Supplementary Fig. S2). The BrdU-positive cells fluoresced 2.3-fold more than the surrounding cells. To understand the statistics of the strong fluorescence of BrdU-positive cells, fluorescence cells were counted in a total of 100 BrdU-positive cells randomly selected from the regenerated blastema, circular muscle layer and longitudinal cell layer of the downstream segments of regenerated blastema. It was found that 79 ± 6 BrdU-positive cells were more fluorescent than the surrounding cells. The counting was performed thrice with 3 different mid-part sections.

It has been reported that the major source of autofluorescence in the earthworm *E. fetida* is riboflavin [44,45]. To find the source of autofluorescence in the earthworm *E. eugeniae*, the tissues of the worm were homogenized and centrifuged, and the supernatant was subjected to thin-layer chromatography. The TLC data clearly showed yellowish-green spots that were visible to the naked eye. Under UV light, the spots fluoresced brightly with a yellowish-green color. The fluorescent compound in the worm and riboflavin had the following common characteristics: (1) the color of the compound, (2) fluorescence under UV, and (3) Same Rf values in the TLC. To verify the data further, riboflavin (10 µg), 10 µL of the earthworm extracts, and a mixture of the supernatant of earthworm extract and riboflavin (5 µg) were resolved by TLC. Figure 4A shows only one spot from the mixture with an Rf value identical to that of riboflavin, confirming that riboflavin mixed with the supernatant has the same Rf value as the other samples chromatographed. From the data, it was concluded that the major source of autofluorescence of the worm tissues is due to riboflavin.

Spectrofluorometry was performed to further confirm the identity of the fluorescent material and also explore the existence of any other fluorescent material in earthworm tissue. The excitation and emission spectra of riboflavin, FAD, FMN, and nicotinamide adenine dinucleotide hydride (NADH) was obtained for comparison. These spectra are shown in Fig. 4C. The excitation and emission maxima of riboflavin, FAD, FMN, and NADH are 386 & 448, 450 & 522, 445 & 523, and 340 & 466 nm, respectively. The data obtained were compared with the excitation and emission maxima of the above-mentioned

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**FIG. 4.** Thin layer chromatography and spectrofluorometry analysis of riboflavin, FMN, FAD, and earthworm extracts. (A) Lane 1: riboflavin (10 µg); lane 2: riboflavin (5 µg) mixed with an earthworm extract; lane 3: earthworm extract alone. (B) Lane 1: normal earthworm extracts; lane 2: antibiotic-injected worm extract, which shows that the presence of riboflavin in the tissue was reduced to 1/7-fold. (C) Excitation and emission spectrum of riboflavin, FMN, and FAD. (D) Excitation and emission spectrum of the normal worm extracts of circular muscle layer and longitudinal cell layer. (E) Emission spectrum of riboflavin, eluted from TLC. (F) Emission spectrum of earthworm lysate of circular muscle layer and longitudinal cell layer, eluted from TLC. (G) Excitation and emission spectrum of the regenerated worm extracts of circular muscle layer and longitudinal cell layer. (H) Excitation and emission spectrum of the regenerated worm blastema. The *dotted* and *solid* lines represent spectra of excitation and emission respectively; asterisk (*) indicates the 2 unknown fluorophore at 587 and 635 nm. FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; NADH, nicotinamide adenine dinucleotide hydride; TLC, thin layer chromatography; PL, photoluminescence.
vitamins and their cofactors, as shown in the Supplementary Table S2. This confirmed that the experimental data are within the range of the reported excitation and emission maxima of the above vitamins and their cofactors. Further, the lysate of the circular and longitudinal muscle layers of the worm was subjected to spectrofluorometric analysis, and data shown in Fig. 4D show that in addition to the 520–525 nm peaks, which are the emission maxima of riboflavin, FAD [51], and FMN [52], there is a peak at 497 nm. To investigate the source of the emission maximum at 497 nm, the lysate of the circular and longitudinal muscle layers (80 \( \mu \)g of protein sample) and pure riboflavin was subjected to thin layer chromatography. Under UV illumination, both riboflavin and the fluorophore of the worm lysate showed identical \( R_f \) values (Supplementary Fig. S3A). The spots corresponding to the fluorophore were scraped from the plate, and the substance (fluorophore) eluted as described in Materials and Methods. The eluates (from lysate and pure riboflavin) obtained were again resolved by TLC, and the fluorophore eluted from the scraped material as above. The eluates from the silica gel were then subjected to spectrofluorometric analysis (Fig. 4E, F for pure riboflavin and lysate, respectively).

Comparison of the spectrofluorometric data of the eluted fluorophore from the lysate (Fig. 4F) with that from direct spectrophotometric analysis of the lysate (Fig. 4D) showed no great difference in the peaks at 497 and 522 nm. The data (Fig. 4E, F) clearly illustrate that both the eluted fluorophores from TLC materials (the lysate and the riboflavin) have an emission maximum at 497 nm. The experiment was repeated carefully 4 times and consistent data were obtained. The results confirm that during TLC, riboflavin undergoes modification. Hence, the emission maximum of eluted riboflavin shifted from 522 to 497 nm. Similarly, it has been reported by Chapman and Reid [48] that riboflavin can exist in various forms in the cell as follows: mostly, it is in the oxidized form in the cell. It is converted to the semiquinone by addition of a proton and an electron. The addition of one more electron to the semiquinone produces hydroquinone. Then, C4a-peroxyflavin was produced by oxidation of the hydroquinone by addition of 2 oxygen molecules to the carbon at C4. Finally, C4a-hydroperoxyflavin results by the addition of a proton to C4a-peroxyflavin. The report suggests that riboflavin exists in different forms in biological systems. In addition, as already known, carrier proteins bind with riboflavin for transport, and FMN & FAD as coenzymes bind with different proteins of the Krebs cycle [53], amino acid synthesis [53], and electron transport chain [53]. Hence, the reason for the 497 nm emission maximum of fluorescence of worm lysate could be protein interaction with riboflavin, FAD, or FMN. To confirm this, the presence of protein in the purified fluorophore of worm lysate was tested by the Bradford’s method. The data showed the absence of protein in the eluted fraction of the fluorophore (data not shown). To confirm the data further, TLC technique was used as follows: The protein sample (80 \( \mu \)g) of circular muscle layer and longitudinal cell layer was loaded onto the TLC plate, and the protein was detected in purple at both the lysate loading spots. The path of the fluorophore on the TLC plate showed a purple color, and a trace of the color was also seen in at the fluorophore Supplementary Fig. S3A and B. The eluted fluorophore from 80 \( \mu \)g of protein lysate of circular and longitudinal tissue were resolved by TLC again and visualized under UV light (Supplementary Fig. S3C). The plate was then stained with ninhydrin (Supplementary Fig. S3D). The data show the absence of protein (Supplementary Fig. S3D) in the loading spot, and also in the running path of the fluorophore. The data suggest that the protein present in the eluate was effectively removed by the TLC separation and elution protocols. The absence of proteins in the eluted fluorophore could be due to the complete denaturation and precipitation of proteins by the mobile phase of TLC [butanol (7):chloroform (4):acetate acid (5):ammonia (1):water (1)], and the further centrifugation at 10,000 rpm for 10 min removed the trace amount of the protein contaminants from the fluorophore.

Taken together, the data shown in Supplementary Fig. S3 and the Bradford protein assay reveal that the fluorophore of worm lysate eluted from the TLC plate has no protein. Hence, it is clear that the peak for the 497 nm emission maximum is not due to the protein complex of riboflavin, FAD, or FMN. Thus, it is concluded that the fluorophore (from worm lysate) responsible for the 497 nm emission maximum is nothing, but a derivate of riboflavin. Further studies are necessary to understand the nature of the riboflavin derivatives present in the tissues of the worm. The data also suggest that the tissues of the circular and longitudinal muscle have significant amounts of NADH (emision maximum at 466 nm).

To extend the work further, lysate was prepared from the circular and longitudinal muscles of the regenerating worm, and the regenerative blastema, on the 4th day. They were subjected to spectrofluorometric analysis, and the data for these are shown in Fig. 4G and H, which clearly show that both the samples predominantly contain derivatives of riboflavin (497 nm emission maxima), along with lesser amounts of riboflavin, FAD, and FMN. In addition to the above, a small amount of NADH is also present in both the samples. There was no significant difference between the normal and regenerating worm with respect to the fluorophores from circular and longitudinal cells. Interestingly, in the regenerative blastema, there were 2 unknown fluorophores: one with an emission maximum at 587 nm and another at 635 nm (Fig. 4H). Further studies are needed to characterize these unknown fluorophores.

To extend the study further, the coelomocytes of E. eugeniae were observed under the fluorescence microscope. It was found that coelomocytes have the capacity to fluoresce (Fig. 5A–D). The fluorescent property of the coelomocytes had been reported in many species of the earthworm. Cholewa et al. reported that only 11%–35% of coelomocytes in Allolobophora chlorotica, Dendrodrilus rubidus, E. fetida, and Octolasion sp. have the ability to fluoresce [3]. In contrast, it was found that all coelomic cells in E. eugeniae have the fluorescent property (Fig. 5A).

The data given in Fig. 5E, G, and H show that all cells do not fluoresce with the same intensity. Cells at the boundary of the epidermis and circular muscle layer fluoresce more strongly. In addition, a few cells in the longitudinal cell layer also exhibit a higher degree of fluorescence. The cells fluorescing at higher intensity were counted in the section of the 11th and 12th segments. It was found that 112 ± 8 cells per segment had a 2.3-fold higher fluorescence than the others. Careful observation revealed that the fluorescence is from the cytoplasm, not from the nucleus (Fig. 5G, H). The data fit with the report that riboflavin is localized in the cytoplasm rather than in the
To determine the amount of riboflavin in the worm, riboflavin in the tissues was estimated by TLC and spectrophotometry. It was found that the worm has abundant riboflavin, at a concentration of 477.5 ± 15 µg/g. It would be of interest to understand the biological significance of riboflavin enrichment in the earthworm tissues.

To demonstrate the link between riboflavin enrichment in the worm’s tissues and its regenerative capacity, 3 sets of 6 worms each was injected with a specified quantity of riboflavin once a day for 3 consecutive days. First of the sets was injected with 1.4 µg riboflavin/g of body mass, while the second set received 2.8 µg riboflavin/g of body mass. For control, the third set of the worms were injected sterile water at the same frequency. On the 3rd day, all 3 sets of worms were amputated at the 10th segment, thus blocking the intake of food as the mouth was removed. The kinetics of regeneration in the riboflavin-injected worms was compared with the control set of worms. The data (Fig. 6D–F) show that an increase in the dosage of riboflavin accelerates the regeneration process. On the 3rd day after amputation, 1 ± 1 mm blastemas were observed with the lower-dose injection of riboflavin, while the blastema size increased to 2 ± 1 mm in worms given the higher-dose injection of riboflavin; in control worms, the blastema size was 1 ± 1 mm, similar to that of worms given the lower dose of riboflavin. Similarly, on the 5th day, blastema size was 2 ± 1, 4 ± 1, and 2 ± 1 mm in the lower-dose injected, higher-dose injected, and control worms, respectively. Likewise, on the 7th day, 4 ± 1, 8 ± 1, and 4 ± 1 mm blastema size was observed, respectively. These data clearly show that riboflavin promotes the regeneration process.

It is known that the riboflavin can be synthesized by microbes [55–57] and plants [58–60]. To understand the source of riboflavin production, a set of 6 worms was injected once a day for 3 consecutive days with a mixture of antibiotics at the following concentrations: penicillin, 10 U; amphotericin B, 0.025 µg; streptomycin, 0.010 mg. The control set of worms received an injection of 10 µL of sterile water at the same frequency. Penicillin and streptomycin have a broad-spectrum action against both gram-positive and gram-negative organisms, while amphotericin B is an anti-fungal agent. It was observed in worms given the higher-dose injection of antibiotics. The control worms had a blastema size of 1 ± 1 mm, while the blastema size increased to 2 ± 1 mm in worms given the lower-dose injection of riboflavin. Similarly, on the 5th day, blastema size was 2 ± 1, 4 ± 1, and 2 ± 1 mm in worms given the lower-dose injection, higher-dose injected, and control worms, respectively. Likewise, on the 7th day, 4 ± 1, 8 ± 1, and 4 ± 1 mm blastema size was observed, respectively. These data confirm that riboflavin promotes the regeneration process.

nucleus [54]. Figure 5F shows thin sections of the above segments briefly stained with hematoxylin. In addition, it was also observed that solvents such as formaldehyde, isopropanol, and xylene used in histochemistry of the samples do not interfere with the fluorescent property of the cells.
FIG. 6. Regeneration kinetics upon injection of antibiotics, riboflavin (vitamin B2), and antibiotics + riboflavin on earthworm. (A) The control worm was injected with sterile water and amputated at 10th segment. After the 2nd day wound healing, on day 3, the regeneration blastema was observed. (B, C) The observed growth of regeneration blastema on 5th day and 7th day, respectively. (D) The worms were injected with 2.8 µg/g riboflavin once a day for 3 days. After 3rd day the worm was amputated at 10th segment. The wound was healed at 2nd day. On 3rd day, the regeneration blastema was observed. (E, F) The growth of regeneration blastema on 5th and 7th day, respectively. (G) The worms were injected with antibiotics (penicillin, 10 U; amphotericin B, 0.025 µg; streptomycin, 0.010 mg) once a day for 3 days. After 3rd day the worm was amputated at 10th segment. The wound was healed at 2nd day. On 3rd day, no regeneration blastema was observed. (H, I) The growth of regeneration blastema on 5th day and 7th day. (J) The worms were injected with antibiotics (penicillin, 10 U; amphotericin B, 0.025 µg; streptomycin, 0.010 mg) and 2.8 µg/g riboflavin with a time interval of 12 h once a day for 3 days. After 3rd day the worm was amputated at 10th segment. The wound was healed at 2nd day. On the 3rd day regeneration blastema was observed. (K, L) The growth of blastema on 5th and 7th days, respectively. (M) The regeneration growth kinetics upon injection of sterile water, antibiotics, riboflavin, and antibiotics + riboflavin on earthworm was shown in a bar diagram with standard error. *P value < 0.024.
the 3rd day. On the 5th day, 2 ± 1, 1 ± 1, and 2 ± 1 mm blastemal size was observed in the lower-dose, higher-dose antibiotics, and sterile water-injected worms, respectively. Likewise, on the 7th day, 3 ± 1, 2 ± 1, and 4 ± 1 mm blastemal size was observed in the lower-dose, higher-dose, and zero-dose antibiotics-injected worms, respectively. Taken together, the results showed that antibiotic injection reduces the concentration of riboflavin in the worm, and that antibiotic administration hampers the kinetics of blastema formation. The reduction of blastemal growth may also be due to the side effects of the antibiotics.

To confirm the results obtained on suppression of blastemal growth by antibiotics, a rescue assay was performed. A group of 6 worms was first injected with antibiotics (penicillin, 10 U; amphotericin B, 0.025 μg; streptomycin, 0.010 mg), followed by an injection with riboflavin (2.8 μg riboflavin/g body mass) after 12 h. The control set of worms received 10 μl of sterile water injection at the same frequency. This injection pattern was repeated for the next 2 days. On the 3rd day, all the sets of worms were amputated from segments 1–5, thus blocking the intake of food as the mouth was removed. Regeneration was observed on the 3rd day after amputation. The regeneration rates of the control worm and those injected with antibiotics and riboflavin are shown in Fig. 6A–C and J–L. The results clearly show that upon injection of antibiotics followed by riboflavin, the regenerative capacity of the worms, hampered by the injection of antibiotics, was revived by the subsequent injection of riboflavin. On the 3rd, 5th, and 7th day after amputation, 2 ± 1 & 1 ± 1 mm, 4 ± 1 & 2 ± 1 mm, and 6 ± 1 & 4 ± 1 mm blastemas were observed in worms injected with antibiotics followed by riboflavin, and control worms, respectively. Taken together, riboflavin is observed to reverse the inhibitory effect of antibiotics on blastemal growth. For example (Fig. 6M), the riboflavin-injected worm generated 2 ± 1 mm of blastema on the 3rd day, while injection of antibiotics alone did not result in blastemal growth till the 3rd day. Similarly, the rescue effect of riboflavin was observed on the 5th day. In contrast, the inhibition of blastemal growth was not completely rescued even on the 7th day in worms injected with antibiotics alone. The difference in the size of the blastemas on the 7th day between riboflavin-injected worms and those injected with antibiotics followed by riboflavin is 1 mm. This difference might be due to the adverse effect of antibiotics. It is known that antibiotic injection causes such adverse effects [61]. Hence, the rescue assay data clearly confirm that riboflavin is not only the major source of auto-fluorescence but also the key factor for regeneration in the earthworm, *E. eugeniae*.

**Discussion**

Regeneration is the process of restoration of lost body parts. It was reported that during the regeneration process, a regeneration blastema was formed with a proliferative mass of undifferentiated progenitor cells, from which newly differentiated cells arise [23,24]. The wound of an amputated site was healed quickly at 24 h in the earthworm, *E. eugeniae*. The regeneration blastema formed at 48 h after amputation is soft and transparent. During regeneration, the reduction of cell density in the circular muscle layer and the increase of the longitudinal cell layer suggest that the circular muscle cells may be differentiated into longitudinal cells or may undergo apoptosis. The amputation at segments 10–11 removed not only vital organs such as the heart and the simple brain, but also the mouth. The removal of the mouth blocks the intake of nutrients. In this critical situation, apoptosis leading to loss of circular muscle cells may not occur. Hence, the removal of the circular muscle layer by apoptosis may cause additional energy loss, and differentiation of the circular muscle cells into the longitudinal cell layer could save the energy for the rest of the regeneration process. Hence, there is a greater possibility for differentiation of the circular muscle layer into the longitudinal cell layer.

The longitudinal cells further divide and form the blastema. The complete regeneration of the anterior part of the worm took 24 days. Hence, the experimental data suggest that cells of the blastema, which is formed by longitudinal cells, have the ability to differentiate into many different types of cells to regenerate the organs present in segments 1–10. The histological data of the 7th day regeneration blastema clearly show the formation of septa, but organs were not yet developed in the segments. The data suggest that the internal organs are developed after the formation of segments.

The presence of BrdU in the blastemal tissue (Fig. 2A), which is produced newly, confirms the migration of BrdU-positive cells from the adjacent segments to the blastema. Besides, the predominant niche of BrdU-positive cells in the 11th and 12th segments (upstream of the blastema) in the junction of the circular muscle layer and epidermis (Fig. 2F) further supports the stem cells to migrate from the 2 layers of skin to the blastema. The presence of more BrdU-positive cells in the 11th and 12th segments during regeneration (Fig. 2F, H) than in the control worm (Fig. 2G, H) might be due to either the migration of stem cells from the downstream segments to amputation site or renewal of stem cell.

In accordance with the previous reports [44,45], the data of TLC and spectrofluorometry (Fig. 4) show that the major source of fluorescence is riboflavin, and its derivatives. It has been reported that riboflavin is an electron carrier in the Krebs cycle [53], electron transport chain [53], amino acid synthesis [53], lipid biosynthesis [53], and DNA repair [62]. In this study, it is proved that riboflavin is essential for regeneration too (Fig. 6J–L). In general, gut microbes produce ethanol, riboflavin, other vitamins, and secondary metabolites. In the scenario, Rawls et al. [63] reported that in a microbe-free (sterile) environment, the development of zebrafish was affected, leading to the formation of developmentally abnormal young ones. However, they did not find the substances whose absence was responsible for the developmental defects. The abnormal development of zebrafish in a sterile environment may be due to the riboflavin deficiency as the regeneration of organs from the blastema is comparable with the normal process of embryogenesis.

The data on strong fluorescence from BrdU-positive cells (Fig. 2) confirm that stem cells have the capacity to accumulate more riboflavin than the surrounding cells. The fluorescence of stem cells has been reported in rat and mice [40–42]. Interestingly, Wun et al. reported autofluorescence of human skin stem cell. Hence, it is concluded that the fluorescent property of stem cells is conserved from worms to humans. The studies on riboflavin accumulation in the cell can shed light on stem cell and regeneration biology. The earthworm, *E. eugeniae*, is an ideal model to study the mechanism of riboflavin accumulation in stem cells, and the role of riboflavin in stem cell biology and regeneration.
Acknowledgments

This work was financially supported by Department of Biotechnology, New Delhi. We sincerely thank Dr. H. Nellaiah, Department of Biotechnology, Kalasalingam University, for critical reading of the article.

Author Disclosure Statement

The authors declare no potential conflicts of interest.

References


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E-mail: sudhakarmsu@yahoo.com

Received for publication August 25, 2011
Accepted after revision December 8, 2011
Prepublished on Liebert Instant Online December 9, 2011
To

Ramesh Chand Meena I.A.S.,
Member Secretary,
Tamilnadu State Council For Science and Technology,
Directorate of Technical Education Campus,
Chennai 600 025.

Respected Madam,

Sub: Forwarding the patent application of Dr. S. Sudhakar - Reg

Herewith I am forwarding the patent application of Dr. S. Sudhakar, Head,
Department of Biotechnology, Manonmaniam Sundaranar University, Alwarkuruchi,
entitled "Identification, Characterization and Structural Elucidation of new anti
tuberculosis drug” for filing the patent. Kindly do the needful.

Thank you.

Yours faithfully,

[Signature]

Dr. K. VELURAJA
Professor and Head
Department of Physics
Manonmaniam Sundaranar University
Tirunelveli - 627 012.
Tamilnadu, India.
# FORM 1

THE PATENTS ACT 1970
(39 OF 1970)
&
The Patents Rules, 2003

APPLICATION FOR GRANT OF PATENT
(See section 7,54 & 135 and rule 20 (1))

---

**1. APPLICANT (S)**

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<tr>
<th>Name</th>
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</tr>
</thead>
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<tr>
<td>MANONMANIAM SUNDARANAR UNIVERSITY</td>
<td>India</td>
<td>Abishekapatli, Tirunelveli - 627 012. India.</td>
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**2. INVENTOR (S)**

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<tr>
<th>Name</th>
<th>Nationality</th>
<th>Address</th>
</tr>
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<tr>
<td>Sivasubramaniam Sudhakar*</td>
<td>Indian</td>
<td>Associate professor and Head</td>
</tr>
<tr>
<td>Yesudhason Beryl Vedha*</td>
<td>Indian</td>
<td>Research Scholar</td>
</tr>
<tr>
<td>Selvan Christyraj Johnson Retnaraj Samuel*</td>
<td>Indian</td>
<td>Research Scholar</td>
</tr>
</tbody>
</table>
| Selvan Christyraj Jackson Durairaj* | Indian | *Department of Biotechnology,  
Manonmaniam Sundaranar University,  
Alwarkurichi- 627412. Tamilnadu. |
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<tr>
<th>Name</th>
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<td>Muthalah Muthuraj</td>
<td>Indian</td>
<td>Bacteriologist,</td>
</tr>
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<td></td>
<td></td>
<td>Department of Microbiology,</td>
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<tr>
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<tr>
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<td>Professor and Head</td>
</tr>
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<td>Indian</td>
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3. **TITLE OF THE INVENTION**

Identification, Characterization and Structural Elucidation of new anti tuberculosis drug
4. ADDRESS FOR CORRESPONDENCE OF APPLICANT / AUTHORIZED PATENT AGENT IN INDIA

MANONMANIAM SUNDARANAR UNIVERSITY
Abishekappatti,
Tirunelveli – 627 012. India.

Telephone No.: 0462- 2338632
Fax No. 0462- 2334363
E-mail : theregistrarmsu@sancharnet.in

5. PRIORITY PARTICULARS OF THE APPLICATION (S) FILED IN CONVENTION COUNTRY

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7. PARTICULARS FOR FILING DIVISIONAL APPLICATION

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8. PARTICULARS FOR FILING PATENT OF ADDITION

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9. DECLARATIONS:

(i) Declaration by the Inventor(s)

I/We, the above named inventor(s) are the true & first inventor(s) for this invention and declare that the applicant(s) herein is/are my/our assignee or legal representative.

(a) Date:

(b) Signature(s)

(c) Name(s):

Sivasubramaniun Sudhakar
Yesudhason Beryl Vedha
Selvan Christyraj Johnson Retnaraj Samuel
Selvan Christyraj Jackson Durairaj
Muthaiar Muthuraj
Kasinadar Veluraja
Jeyasigamani Fermin Angelo Selvin
Devadasan Velmurugan

(ii) Declaration by the applicant(s) in the convention country

I/We, the applicant(s) in the convention country declare that the applicant(s) herein is/are my/our assignee or legal representative

(a) Date: 27/11/10

(b) Signature(s)

(c) Name(s) of the signatory: Dr. C. Sudhakaran, Annamalai University, Tamilnadu, India

(iii) Declaration by the applicant(s):

I/We, the applicant(s) hereby declare(s) that:

- We are in possession of the above-mentioned invention.
- The provisional/complete specification relating to the invention is filed with this application.
• The invention as disclosed in the specification uses the biological material from India and the necessary permission from the competent authority shall be submitted by me/us before the grant of patent to me/us.

• There is no lawful ground of objection to the grant of the Patent to me/us.

• I am/We are the assignee or legal representative of true & first inventors.

• The application or each of the applications, particulars of which are given in Para – 5 was first application in convention country/countries in respect of my/our invention.

• I/We claim the priority from the above mentioned application(s) filed in convention country/countries and state that no application for protection in respect of the invention had been made in a convention country before that date by me/us or by any person from which I/We derive the title.

• My/our application in India is based on international application under Patent Cooperation Treaty (PCT) as mentioned in Para – 6.

• The application is divided out of my/our application particulars of which are given in Para – 7 and pray that this application may be treated as deemed to have been filed on ___________ under sec. 16 of the Act.

• The said invention is an improvement in or modification of the invention particulars of which are given in Para – 8

10. FOLLOWING ARE THE ATTACHMENTS WITH THE APPLICATION:

(a) Complete specification.

(b) Complete specification (in conformation with the international application) / as amended before the International Preliminary Examination Authority (IPEA), as applicable (2 copies).

   No. of pages: 32   No. of claims: 3

(c) Drawings (in conformation with the international application) / as amended before the International Preliminary Examination Authority (IPEA), as applicable (2 copies). No. of Sheets- 12

(d) Priority documents

(e) Translation of priority document / specification/ International Search Report
(f) Statement and undertaking on Form 3

(g) Power of Authority

(h) Declaration of inventorship on Form 5

(i) Sequence listing in electronic form (floppy disc)

(j) ........................................

Fee Rs. ..................................... in Cash/Cheque/Bank Draft bearing no. ........................................

Date .......................... On ............................... Bank.

I/We hereby declare that to the best of my/our knowledge, information and belief the fact and matters stated herein are correct and I/We request that a patent may be granted to me/us for the said invention.

Dated this ...... day of ......... 2011

To,

The Controller of Patent


Signature: 

ASSOCIATE PROFESSOR AND HEAD
Dept. Of Biotechnology
Manonmaniam Sundaranar University
Alwarthirunagari-627412 Tamilnadu, India
F O R M 2

THE PATENTS ACT, 1970

(39 of 1970)

COMPLETE SPECIFICATION

(See section 10 and rule 13)

Identification, Characterization and Structural

Elucidation of new Anti Tuberculosis Drug

MANONMANIAM SUNDARANAR UNIVERSITY

Abishekappati, Tirunelveli - 627 012. India.
FORM 3

THE PATENT ACT, 1970

(39 OF 1970)

&

The Patents Rules, 2003

STATEMENT AND UNDERTAKING UNDER SECTION 8

(See section 8, rule 12)

1. Namely of the applicant(s) I/We Manonmaniam Sundaranar University hereby declare:

2. Name, address and nationality of the joint applicant:

   (i) that I/We who have made any application for the same/substantially the same invention outside India.

   Or

   (ii) that I/We who have made this application No.

                    Dated

alone/jointly, made for the same/substantially same invention, application(s) for patent in the other countries, the particulars of which are given below:

<table>
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<th>Application No.</th>
<th>Status of the application</th>
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3. Name and address of the assignee (iii) that the rights in the application(s) has/have been assigned to self and that I/We
undertake that up to the date of grant of the patent, by the Controller. I/We would keep him informed in writing the details regarding corresponding applications for patents filed outside India within three months from the date of filing of such application.

Dated this 9th day of August 2011

4. To be signed by the applicant or his authorized registered patent agent.

Signature

ASSOCIATE PROFESSOR AND HDR
Dept. Of Biotechnology
Manonmaniam Sundaranar University
Aluva, Cochin-682 012, Kerala

To,

The Controller of Patent

5. Name of the natural person who has signed.

Dr. S. EMMAUEL JOSHUA JEBASINGH, Ph.D.
Assistant Professor
Department of Biotechnology
Manonmaniam Sundaranar University
Annamalai University - 608 002
FORM 5
THE PATENT ACT, 1970
(39 OF 1970)
&
The Patents Rules, 2003
DECLARATION AS TO INVENTORSHIP
[See section 10(6) and rule 13(6)]

1. NAME OF APPLICANT(S)

hereby declare that the true and first inventor(s) of the invention disclosed in the complete specification filed in pursuance of my / our application numbered ........................................ dated ........................................ is/are ........................................

2. INVENTOR(S)

a. Name : 1. Dr. Sivasubramaniam Sudhakar
           2. Yesudhason Beryl Vedha
           3. Selvan Christyraj Johnson Retnaraj Samuel
           4. Selvan Christyraj Jackson Durairaj
           5. Dr. Muthaiah Muthuraj
           6. Dr. Kasinadar Veluraja
           7. Jeyasigamani Fermin Angelo Selvin
           8. Dr. Devadasan Velmurugan

b. Nationality : Indian
c. Address:

1. Associate Professor and Head
   Department of Biotechnology,
   Manonmaniam Sundaranar University,
   Alwarkurichi- 627 412. Tamilnadu.

2. Research scholar,
   Department of Biotechnology,
   Manonmaniam Sundaranar University,
   Alwarkurichi- 627 412. Tamilnadu.

3. Research Scholar.
   Department of Biotechnology,
   Manonmaniam Sundaranar University,
   Alwarkurichi- 627 412. Tamilnadu.

4. Research scholar,
   Department of Biotechnology,
   Manonmaniam Sundaranar University,
   Alwarkurichi- 627 412. Tamilnadu.

5. Bacteriologist,
   Department of Microbiology,
   Government Hospital for chest diseases,
   Gorimedu -605006. Puducherry.

6. Professor and Head,
   Department of Physics,
   Manonmaniam Sundaranar University,
   Abishakapatti-627 012. Tamilnadu.

7. Research scholar,
   Department of Physics,
   Manonmaniam Sundaranar University,
   Abishakapatti-627 012. Tamilnadu.

8. Professor and Head,
   Department of Crystallography and Biophysics,
   University of Madras, Guindy Campus,
   Chennai - 600 025. Tamilnadu.
Dated this:

Signature :- 1. [Signature]

2. [Signature]

3. [Signature]

4. [Signature]

5. [Signature]

6. [Signature]

7. [Signature]

8. [Signature]

Name of the signatory :- 1. Dr. Sivasubramaniam Sudhakar

2. Yesudhason Beryl Vedha

3. Selvan Christyraj Johnson Retnaraj Samuel

4. Selvan Christyraj Jackson Durairaj

5. Dr. Muthaiy Muthuraj

6. Dr. Kasinadar Veluraja

7. Jeyasigamani Fermin Angelo Selvin

8. Dr. Devadasan Velmurugan
3. DECLARATION TO BE GIVEN WHEN THE APPLICATION IN INDIA IS FILED BY THE
APPLICANT (S) IN THE CONVENTION COUNTRY:-

We the applicant(s) in the convention country hereby declare that our right to apply for a patent in
India is by way of assignment from the true and first inventor(s).

Dated this 2.9.14

Signature: [Signature]

Name of the signatory: ASSOCIATE PROFESSOR AND
Dept. Of Biotechnology
Dr. S. Balakrishnan
Manonmaniam Sundaranar Univ.
N. WARKURCHI 627 412, Tamilnadu

4. STATEMENT (to be signed by the additional inventor(s) not mentioned in the application form)

If/We assent to the invention referred to in the above declaration, being included in the complete
specification filled in pursuance of the stated application.

Dated this 2.9.14

Signature of the additional inventor (s): [Signature]

Name: Dr. S. Balakrishnan
ASSOCIATE PROFESSOR AND
Dept. Of Biotechnology
Manonmaniam Sundaranar Univ.
N. WARKURCHI 627 412, Tamilnadu

To,
The Controller of Patent
The Patent Office, at Chennai
FORM 18

THE PATENTS ACT 1970
(39 of 970)
&
THE PATENTS RULES, 2003

REQUEST/EXPRESS REQUEST FOR
EXAMINATION OF APPLICATION FOR PATENT
[See section 11B and rules 20 (4) (ii), 24B (1) (i)]

FOR OFFICE USE ONLY

RQ. No:
Filing Date:
Amount of Fee Paid:
CBR No:
Signature:

1. APPLICANT(S)/OTHER INTERESTED PERSON

Name : Manonmaniam Sundaranar University
Nationality : Indian
Address :

2. STATEMENT IN CASE OF REQUEST FOR EXAMINATION MADE BY THE APPLICANT(S)

I/ we hereby request that my/our application for patent no. _______________ filed on _______________ for the invention titled "IDENTIFICATION, CHARACTERIZATION AND STRUCTURAL IDENTIFICATION OF NEW ANTI-TUBERCULOSIS DRUG" shall be examined under section 12 and 13 of the Act.

or

I / We hereby make an express request that my / our application for patent no. _______________ filed on _______________ based on Patent Cooperation Treaty (PCT) application no. _______________ dated _______________ made in country _______________ shall be examined under section 12 and 13 of the Act, immediately without waiting for the expiry of 31 months as specified in rule 20 (4)(ii).
3. STATEMENT IN CASE OF REQUEST FOR EXAMINATION MADE BY ANY OTHER INTERESTED PERSON

I/we the interested person request for the examination of the application no. ________________

Dated ____________ filed by the applicant MANONMANIAM SUNDARARAJ UNIVERSITY

Titled "IDENTIFICATION, CHARACTERIZATION AND STRUCTURAL ELUCIDATION OF NEW ANTI-TUBERCULOSIS DRUG" __________________ under sections 12 and 13 of the Act.

As an evidence of my/our interest in the application for patent following documents are submitted:-

(a) Complete Specification, No. of Pages ____, No. of Claims ___

(b) Drawings, No. of Sheets ___

© Other Documents (Forms 1, 3, 5, 18 and 26)

4. ADDRESS FOR SERVICE:

Dr. S. Sudhakar, Associate Professor And Head
Department of Biotechnology, Manonmaniam Sundaranar University, Alvakalurichi-Tirunelveli (Dt)- 627 412.

Mobile No. 9940998936

Email- Sudhakarmsu @ yahoo.com.

Dated this

Signature __________________

Name of the signatory

To,

The Controller of Patents

The Patent Office at Chennai

E. Sethuraman

21st __________

[Signature]
FORM 26
THE PATENTS ACT, 1970
(39 of 1970)
&
The Patents Rules, 2003
FORM FOR AUTHORISATION OF A PATENT AGENT/OR ANY 
PERSON IN A MATTER OR PROCEEDING UNDER THE ACT
[See sections 127 and 132; and rule 135]

1. Insert name, address and nationality. I/We\(^1\) hereby authorize\(^2\) to act on my/out behalf in connection with\(^3\) and request that all notices, requisitions and communication relating thereto may be sent to such person at the above address unless otherwise specified.

2. Insert the name, address and nationality of the person(s) to be authorized.

3. State the particular matter or proceeding for which the authorisation is made. I/We hereby revoke all previous authorizations, if any made, in respect of same matter or proceeding. I/We hereby assent to the action already taken by the said person in the above matter.
4. To be signed by the person(s) making this authorization.

Dated this ______day of ________ 20__

Signature

(__________________________)

Dr. S. Sudhanar
ASSOCIATE PROFESSOR AND
Dept. Of Biotechnology
Manonmaniam Sundaranar Unive
ALWARKURICHI-627412 Tamilnadu.

5. Name of the natural person who has signed along with designation and official seal, if any.

Dr. S. Emmanuel Joshua Jebasingh, Ph.D.
Assistant Professor,
Department of Biotechnology,
Manonmaniam Sundaranar University,
Alwarkurichi - 627 412.

To

The Controller of Patents,
The Patent Office, at Chennai

To be stamped under the Indian Stamp Act, 1899 (2 of 1899).