Twin-layer biosensor for real-time monitoring of alkane metabolism

Twin-Layer Biosensor for Real-Time Monitoring of Alkane Metabolism

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Abstract

Intracellular metabolic sensors can be used for efficient screening and optimization of microbial cell factories. In particular, the sensors are useful in acquiring information about pathway dynamics and bottlenecks in a straightforward manner. Here, we developed a twin-layer biosensor which functions simultaneously at two levels: through transcription factor mediated sensing and enzyme-metabolite interaction, providing insights into the dynamics of alkane metabolism. In addition, the sensor can be used for monitoring either alkane degradation or biosynthesis, depending on the used cellular context. Alkanes are monitored using a fluorescent reporter green fluorescent protein placed under a native alkane-inducible promoter, whereas a bacterial luciferase producing bioluminescence signal enzymatically detects a specific metabolic intermediate in the alkane production/degradation pathway. First, we employed the sensor to investigate the native alkane degradation route in Acinetobacter baylyi ADP1. The highest fluorescence and luminescence signals were obtained for dodecane. Secondly, we constructed a non-native alkane synthesis pathway in A. baylyi ADP1, of which the functionality was confirmed with the sensor. The twin-layer approach provides convenient means to study and optimize the kinetics and performance of the heterologous pathway and will facilitate the development of an efficient cell factory.

Introduction

In synthetic biology, biological systems are harnessed for the production of valuable molecules by means of rational design and construction. The typical workflow consists of design, construction, characterization, and revision of the synthetic systems. With the advances in computational modeling as well as synthetic DNA and cloning technologies, the phenotypical characterization of the constructed organisms remains the bottleneck for the efficient development of high-performing microbial hosts (Zhang et al., 2015). This is mainly because the analysis of the metabolic end products and intermediates is laborious and time consuming. In rare exceptions, the positive changes are readily detectable as changes in the phenotype (e.g. colorful product such as lycopene), but usually the metabolites cannot be quantified in a high-throughput manner. Furthermore, for the identification of bottlenecks or imbalances in the metabolic network, the analysis of the end products alone is insufficient and should be complemented with the analysis of key metabolic intermediates.

One solution is to use a suitable biological sensor that is able to respond to changes in the concentration of a specific metabolite and report it with an easily quantifiable signal (Eggeling et al., 2015; Zhang et al., 2015). Application of biosensors for the detection and quantitation of selected metabolites allows for high-throughput screening of producer strains (Schallmey et al., 2014). In an optimal case, the sensor allows for real-time, non-destructive analysis of key metabolites.

Metabolic sensors can work on different levels; they can report for example changes in promoter activity, protein or RNA expression, or concentration of a specific metabolite. By combining information from different levels, changes in the dynamics of the metabolism can be observed. Information about the pathway dynamics can reveal important aspects that would not be detectable or accessible with end metabolite analysis alone. Moreover, the sensors are often more sensitive and specific compared to conventional analyses methods. In this study, we introduce a biosensing system working on two levels: the system directly detects the presence of molecules of interest, alkanes, by a transcription factor mediated sensing, as well as provides information about the dynamic intermediate compounds, aldehydes, by an enzyme-metabolite interaction. The system can be applied for studying both the alkane degradation as well as synthesis, depending on the cell context. To highlight the modularity, the system was constructed using a bacterial strain Acinetobacter baylyi ADP1, which has not been employed for alkane synthesis before.

Microbial alkane production is a highly interesting option for the replacement of fossil fuels and chemicals (Schirmer et al., 2010; Choi & Lee, 2013). However, current titers are too low for practical applications. One of the reasons for the low titers is the inefficient flux from acyl-CoA to alkanes through a toxic aldehyde intermediate. Several strategies have been tested in order to improve the production (Andre et al., 2013;

Wang & Lu, 2013), but the work is hindered by the lack of time- and cost-effective methods for the efficient screening of potential genetic modifications and producer strains. Thus, intracellular alkane biosensors could greatly facilitate the development of an efficient alkane-producing microbial factory (Kang & Nielsen, 2016).

Various whole-cell sensors for alkane detection have been developed (Sticher et al., 1997; Minak-Bernero et al., 2004; Reed et al., 2012; Zhang et al., 2012). These sensors are based on the induction of either a luciferase (LuxAB) or the green fluorescent protein (GFP) by an artificially constructed or naturally alkane-responsive promoter. However, those sensors have only been applied in the context of extracellular alkane detection. Recently Wu et al. (Wu et al., 2015) developed an alkane sensor for the detection of intracellular alkanes produced by the sensor strain. In the sensor, the alkanes produced by the cells activate the expression of GFP under an artificially constructed alkane-inducible promoter. This was the first demonstration of in situ alkane detection within the producer strain using a biosensor. The aforementioned reporters are based on a transcriptional activation of a reporter gene, and consequently show relatively slow responses.

Previously, we developed a simple and robust sensor for extracellular alkanes based on the novel synthetic biology chassis A. baylyi ADP1 (Santala et al., 2012). This sensor relied on two important aspects of A. baylyi metabolism; first, its natural ability for efficient alkane uptake and degradation, and second, the generation of a specific intermediate of the alkane degradation pathway. The intermediate, long-chain aldehyde, is a specific substrate for a light-producing bacterial luciferase LuxAB, thus eliminating the need for the addition of external substrate or development of synthetic induction systems.

In the current study, we wanted to improve our LuxAB-based alkane sensor by adding a second sensory level, allowing the detection of alkanes through a reporter transcript output, mediated by a ligand responsive transcription factor. This was achieved by placing a gfp gene under a native alkane-inducible promoter (Ratajczak et al., 1998a). As a result, the twin sensor simultaneously detects alkanes and alkane degradation/biosynthesis intermediates (long chain aldehydes), offering insights into the dynamics of the metabolism. The twin sensor was employed for monitoring the natural alkane degradation route as well as a heterologous alkane biosynthesis pathway.

Materials and Methods

Bacterial strains

The wild type A. baylyi ADP1 (DSM 24193) was used in the study. For plasmid construction and maintenance, Escherichia coli XL1-Blue (Stratagene, USA) was used.

Genetic modifications

The molecular work was carried out using established methods. The reagents and primers were purchased from ThermoFisher Scientific (USA) and used according to the manufacturer's instructions. Antibiotics were used in concentrations of 25 µg ml^{-1} for chloramphenicol and 50 µg ml^{-1} for kanamycin and spectinomycin, when appropriate. Transformation of A. baylyi was carried out as described previously (Metzgar et al., 2004). All genome modifications were confirmed with PCR and sequencing.

The genetic cassette for the introduction of gfp to the genome of A. baylyi under the alkane-inducible promoter was assembled sequentially by restriction cloning using a previously described integration cassette iluxAB_Cm^r/pAK400c as the backbone (Santala et al., 2011a). The construct contains a gfp gene and a kanamycin resistance marker flanked by the upstream and downstream regions of AlkM. The upstream flanking region contained the alkane-inducible promoter. The 3' and 5' flanking regions were PCR-amplified from A. baylyi genome with primers tI01 & tI02 and tI09 & tI10, respectively (Table 1). A gene encoding superfolder GFP (sfGFP) was amplified from the Biobrick collection (BBa_I746915) with primers tI03 and tI04 and the kanamycin resistance marker from plasmid pBAV1K-T5-gfp (Bryksin & Matsumura, 2010) with primers tI07 and tI08. A. baylyi was transformed with the cassette and strains characterized based on their ability for n-alkane utilization. For the external alkane detection, a strain capable of alkane degradation was used (indicating that the integration of the cassette did not knock out the alkM gene). For internal alkane production, a strain with a functional alkM knockout was used in order to prevent the degradation of the synthesized alkanes.

For the introduction of the luxAB genes into the genome, a previously described cassette was employed (Santala et al., 2011b). This cassette is designed to knock out the ACIAD3383-3381 genes, thereby removing the endogenous fatty acyl-CoA reductase and pyruvate dehydrogenase activities. The luxAB genes were cut from the iluxAB_Cm^r/pAK400c plasmid using NdeI and XhoI restriction enzymes and cloned to the cassette.

For the alkane producing strain, a construct was prepared where aar (Synechococcus elongatus PCC7942_orf 1594) and ado (S. elongatus PCC7942_orf 1593) genes are under T5/lac promoter in an integrative cassette. The integrative region of the plasmid pIM1463 (Murin et al., 2012) was PCR amplified in two parts with primers (tI24, tI25, tI26 and tI27) designed in such a way that the gusA gene becomes replaced by a Biobrick accepting cloning site (EcoRI-XbaI-SpeI-PstI). Aar and ado genes were codon-optimized and purchased from Genscript (USA) with appropriate restriction sites and ribosomal binding sites and placed under the promoter using standard methods. The construct contained flanking sites to facilitate the integration into a prophage region in the genome of A. baylyi ADP1.

Luminescent and fluorescent measurements

For the induction with external alkanes, the cells were grown overnight in the LB medium (5 g l^{-1} NaCl, 5 g l^{-1} yeast extract, 10 g l^{-1} tryptone) supplemented with 0.4% glucose. The measurement was done in 96-well plate, with 200 µl of the overnight culture and approximately 40 mM of alkane added. Between the measurements, the plate was incubated in 30°C and shaking. Optical density (Multiscan Ascent, Thermo Labsystems, Finland; wavelength 540 nm), fluorescence (Fluoroskan Ascent FL, Thermo Labsystems, Finland) using the wavelengths 485 nm (half band width (HBW) 14±2 nm) for excitation and 538 nm (HBW 25±3 nm) for emission, and luminescence (Wallac 1420 Victor2, Perkin Elmer, Finland; 1 s measurement time) measurements were performed every 15 minutes.

For the measurement of internally produced alkanes, the cells were grown on Luria-Agar (LA) (5 g l^{-1} NaCl, 5 g l^{-1} yeast extract, 10 g l^{-1} tryptone, 15g l^{-1} Agar-agar) on 48-well plate inside Xenogen In Vitro Imaging System (IVIS® Lumina, Caliper Life Sciences, USA) at 30°C. Luminescence and fluorescence (excitation: 445-490 nm, emission: 515-575 nm) signals were measured every hour. To investigate the possible overlap of the emission wavelength of the luciferase with the excitation profile of the GFP, the fluorescence emission was also measured without the excitation (the excitation filter was closed). Any "autofluorescence" obtained in this setting would result from the luciferase exciting the GFP, but the signal was found to be negligible compared to the actual fluorescent signal. The relative fluorescence signal was calculated by dividing the fluorescence of the sensor strain with the fluorescence of wild type A. baylyi ADP1 grown in the same conditions. For confirming the alkane production, GC-MS analysis was carried out (Supplementary File 1).

For the visualization of the fluorescence from single colonies, the sensor cells were grown overnight in LB, diluted and plated on LA plates with 100 µM isopropyl β-D-1-thiogalactopyranoside (IPTG). From the control plates, the IPTG was omitted. The plates were incubated in 30°C for 24 hours and visualized with a Safe Imager™ Blue Light Transilluminator (ThermoFischer Scientific, USA).

Results and Discussion

To detect the presence of alkanes by the reporter system, a native alkane-responsive promoter (P_{alkM}) of A. baylyi was exploited in the construction of the sensor. The promoter is activated in the presence of alkanes by a transcriptional activator AlkR (Ratajczak et al., 1998a). For monitoring the levels of specific intermediates, long-chain aldehydes, a constitutively expressed bacterial luciferase LuxAB was employed. The bacterial luciferase uses aldehydes as a substrate in the reaction producing a fatty acid molecule and measurable light signal (bioluminescence).

The twin sensor for monitoring of the alkane degradation in A. baylyi (designated as EAS for External Alkane Sensor) is presented in Figure 1 (Left). The sensor was created as follows: A synthetic gene cassette was

constructed consisting of the P_{alkM} promoter (sequence immediately upstream of alkM), a gfp gene and a kanamycin resistance marker. The cassette was flanked by homologous regions facilitating its integration to the genome by homologous recombination (Elliott & Neidle, 2011). LuxAB was introduced using another genetic cassette, containing the lux A and B genes under constitutive promoter and flanking regions facilitating the integration to the genetic locus replacing the genes ACIAD3381-ACIAD3383 thus eliminating the native aldehyde synthesis (Santala et al., 2011a).

Because luxAB is constitutively expressed, the detection of aldehydes by luminescence is not dependent on transcription or the presence of an inducer. Thus, the luminescence response is instant and dynamic. In contrast, the production of fluorescence signal is a slow process and the detected signal is cumulative in nature, due to the working mechanism of the reporter including induction event and protein expression as well as the relatively high stability of GFP.

The response of the twin sensor to external n-alkanes of various chain lengths is presented in Figure 2. The alkanes induce transcription from the alkane-responsive promoter, which can be observed as the increase in the fluorescence signal. As the alkane degradation pathway activates, the formation of long-chain aldehydes is seen as the increase in the luminescence signal. Induction of the luminescence and fluorescence signals behave differently with varying alkane chain lengths. This can be attributed to the different chain-length preferences of LuxAB and the AlkR-P_{alkM} induction system. Among the tested alkanes, dodecane (C12) yields the highest signals in both luminescence and fluorescence, being consistent with our earlier results (Santala et al., 2012). In the previous studies (Ratajczak et al., 1998a; Zhang et al., 2012), octadecane has been observed as the strongest inducer of AlkM expression. The differences can be due to different experimental settings or reporter systems in the genetic level. For example, the differences in solubility between the alkanes with different chain lengths might cause variation between the experimental setups. With octadecane (C18), the fluorescence induction is stronger than the luminescence, whereas with tetradecane the luminescence induction is stronger. For the luminescence signal, the differences in the induction can be partially explained with the substrate specificity of the luciferase; for example, tetradecanal (C14) is preferred over longer carbon chains by LuxAB, (Colepicolo et al., 1989) explaining its relatively higher activity. The variations in the luminescence and fluorescence signal ratios in response to alkanes of different chain lengths further emphasize the importance of monitoring metabolic pathways at multiple levels.

Apart from the detection of the external alkanes, we wanted to use the sensor for studying the alkane biosynthesis. A. baylyi exhibits relatively high tolerance towards hydrocarbon metabolites and naturally accumulates large amounts of acyl-CoA and aldehyde derived hydrocarbons as storage compounds, thus making it an appealing host for microbial alkane production (Santala et al., 2011b; Santala et al., 2012).

Although A. baylyi has been previously employed as a robust host for genetic investigations, metabolic engineering, and synthetic biology (de Berardinis et al., 2009; Murin et al., 2012; Santala et al., 2014a; Santala et al., 2014b; Kannisto et al., 2015), further research is required to investigate its potential for industrial use.

For the detection of internally produced alkanes, a different version of the twin sensor was used and a genetic module harboring the elements for alkane biosynthesis was added to the sensor strain (Fig. 1, Right). In this sensor strain, alkane degradation is prevented by a knock-out of alkM, which encodes for alkane hydroxylase and is essential for alkane degradation in A. baylyi (Ratajczak et al., 1998b). Alkane biosynthesis has been achieved in E. coli by the expression of two cyanobacterial genes, aar and ado, (Schirmer et al., 2010) which encode enzymes with the activities of fatty acyl-ACP reductase and aldehyde-deformylating oxygenase, respectively. We applied the two widely used components to investigate alkane biosynthesis in A. baylyi. The sensor strain was transformed with a construct containing ado and aar under a lac/T5 promoter and homologous regions to facilitate the integration of the cassette to the genome. To our knowledge, this is the first time when a naturally alkane degrading strain is engineered to produce alkanes. In this twin sensor/producer strain, designated as IAS (for Internal Alkane Sensor), the same reporter elements LuxAB and GFP allow the monitoring of long-chain aldehydes produced by Aar and alkanes produced by Ado, respectively (Fig. 1, Right). Similarly to EAS strain, the gene cassette eliminating ACIAD3381-3383 was used for the insertion of luxAB. The ACIAD3383 gene encodes for a native fatty acyl-CoA reductase of A. baylyi. It was desirable to remove this activity in order to eliminate the endogenous fatty aldehyde production. The ACIAD3381 encodes for a pyruvate dehydrogenase, a deletion of which has been associated with potentially higher wax ester production (Santala et al., 2011b). Thus, it seemed possible that its deletion could be beneficial also for alkane production. ACIAD3382 encodes for a homocysteine synthase, and its deletion is neutral in terms of growth and lipid production (Santala et al. 2011b); the ACIAD3382 gene was deleted for practical reasons to allow the deletion of ACIAD3383 and ACIAD3381 with a single cassette. It is also notable that LuxAB consumes aldehyde molecules in the light-producing reaction. However, the fatty acid molecules produced in the reaction are recycled by the cells, which somewhat alleviates the possible effect on alkane production. Furthermore, we have previously used a similar detection system for the analysis of wax ester production pathway, and have found that the luciferase does not have a significant influence on the production (Santala et al. 2011a). Thus, the overall effect of the LuxAB activity on the production of alkanes can be considered negligible.

In order to study the alkane biosynthesis, Aar and Ado were expressed under an IPTG-inducible promoter with different concentrations of the inducer. The luminescence and fluorescence signals were measured in real time during the cell growth. In the presence of the inducer, a response was seen in the luminescence and fluorescence signals, indicating the production of alkanes via the aldehyde intermediate (Fig. 3). In both

signals, a positive correlation was seen with the IPTG concentration and the signal strength, indicating a dynamic behavior of the sensor. The alkane production of the Ado-Aar expressing strain was also confirmed with GC-MS analysis (Supplementary File 1). A moderate induction (10 µM IPTG) of Aar alone (for control) resulted in luminescence but not fluorescence production, as expected. However, with higher inducer concentrations the expression of Aar resulted in inconsistent cell growth, potentially caused by the toxic effect of long-chain aldehydes.

Biosensors can speed up the development of efficient producer strains by allowing for high-throughput screening of different genetic modifications. Currently, practical technologies allowing the selection of single cells in a high-throughput manner are limited to fluorescence activated cell sorting (FACS), which requires special instrumentation. Thus, it would be highly convenient to be able to screen the best phenotypes directly from colonies grown on plates. To assess the applicability of our twin sensor for high-throughput screening, individual cells were plated on agar plates and the signal strength was evaluated from single colonies. The fluorescence was readily detected from single colonies grown in the presence of the inducer (Fig. 4), suggesting the sensor would be amenable to high-throughput screening directly on plates.

A major challenge in metabolic engineering is to balance the metabolic pathway in a way that the flux is directed to the desired product and accumulation of potentially toxic intermediates is minimized (Jones, Toparlak et al. 2015). One option is to screen for various natural or modified enzymes and their expression levels to find a combination that results in maximal product formation. In the alkane biosynthesis pathway, the fatty aldehyde production must be carefully balanced in order to provide ADO with sufficient substrate load while avoiding the accumulation of the fatty aldehydes, which can cause toxic or inhibitory effects (Cao et al., 2016). The twin configuration of the sensor enables the detection of both the intermediate and the end-product, which allows more targeted optimization of the production pathway than a sensor working on a single level. The optimal production strain should show high fluorescence to luminescence ratio, indicating that aldehydes are effectively converted to alkanes and do not accumulate in the cells. It is well known that ADOs are relatively slow enzymes, with k_{cat} values approximately 1 min^{-1} (Marsh & Waugh, 2013). Various strategies have been attempted to increase the turnover rate, including the removal of inhibition (Andre et al., 2013) and improving the substrate availability by scaffolding (Sachdeva et al., 2014). Another approach to improving the alkane synthesis could involve protein engineering of ADOs. The sensor presented here could serve as an efficient tool for screening the variants.

In this study, we developed and characterized a novel twin biosensor for the analysis of alkane metabolism. The sensor can be applied for the detection of both external and internally produced alkanes in real time. The twin configuration of the sensor makes it possible to simultaneously detect signals from two different

levels, offering insights into the dynamics of the metabolism. Furthermore, this is the first report describing alkane production in the promising synthetic biology chassis A. baylyi. The presented sensor system holds potential for speeding up the metabolic engineering work aiming for efficient alkane production.

Author Contributions

T.L., S.S. and V.S. designed the study. T.L. performed the molecular and microbiological work, analyzed the data and wrote the manuscript. S.S. and V.S. supervised and coordinated the study. All authors approved the final version of the manuscript.

The authors declare no competing financial interests.

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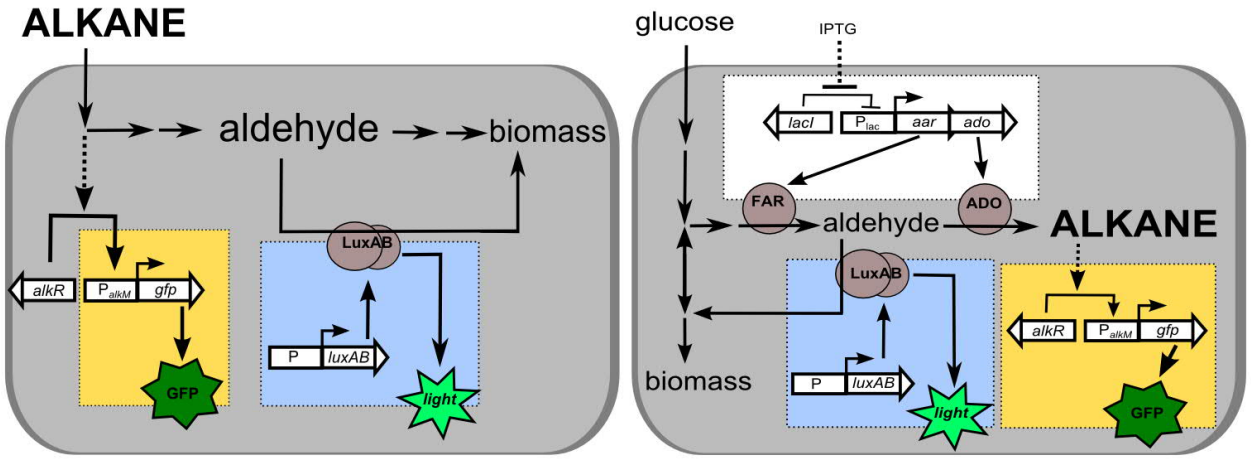

Figure 1. Twin-layer sensors for monitoring alkane degradation and synthesis. The sensors can be used for the monitoring alkane degradation (left) or biosynthesis (right). The twin sensor components encompass a constitutively expressed luxAB encoding for bacterial luciferase, which produces bioluminescence upon interaction with the aldehyde intermediate (blue module), and an alkane inducible reporter consisting of the transcription factor AlkR and the cognate promoter P_{alkM} regulating the expression of GFP (yellow module). For alkane biosynthesis (right), a module containing the activities of a fatty acyl-CoA reductase (FAR) and an aldehyde-deformylating oxygenase (ADO) under inducible promoter system (lacI-Plac) was added.

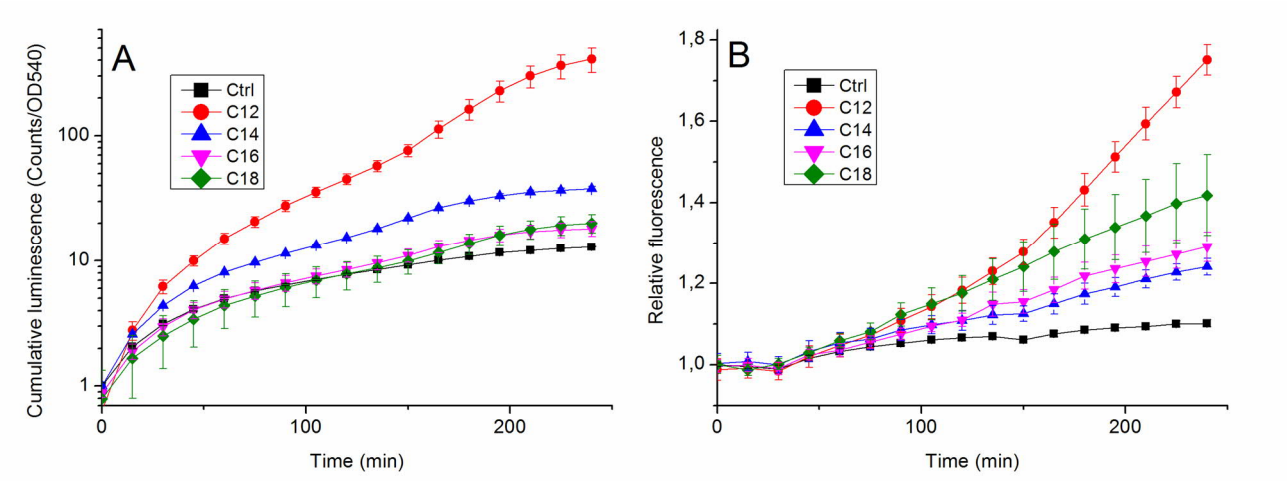

Figure 2. Luminescent and fluorescent responses of sensor cells to external alkanes. The EAS sensor cells (expressing LuxAB constitutively and GFP under an alkane-inducible promoter) were cultivated in LB overnight, divided into 96 well plate and aliphatic alkanes with carbon chain lengths of 12-18 added (final concentration 40mM). Glucose (40mM) was added to the control samples. The luminescent (A) and fluorescent (B) responses were monitored every 15 minutes. Relative fluorescence is defined as the fold change of the fluorescence signal divided by the OD_{540} of the sample. The luminescence signal is presented relative to the optical density. The optical densities are provided in Supplementary Table 1. The signals are presented as average of three replicates. Error bars represent standard deviation.

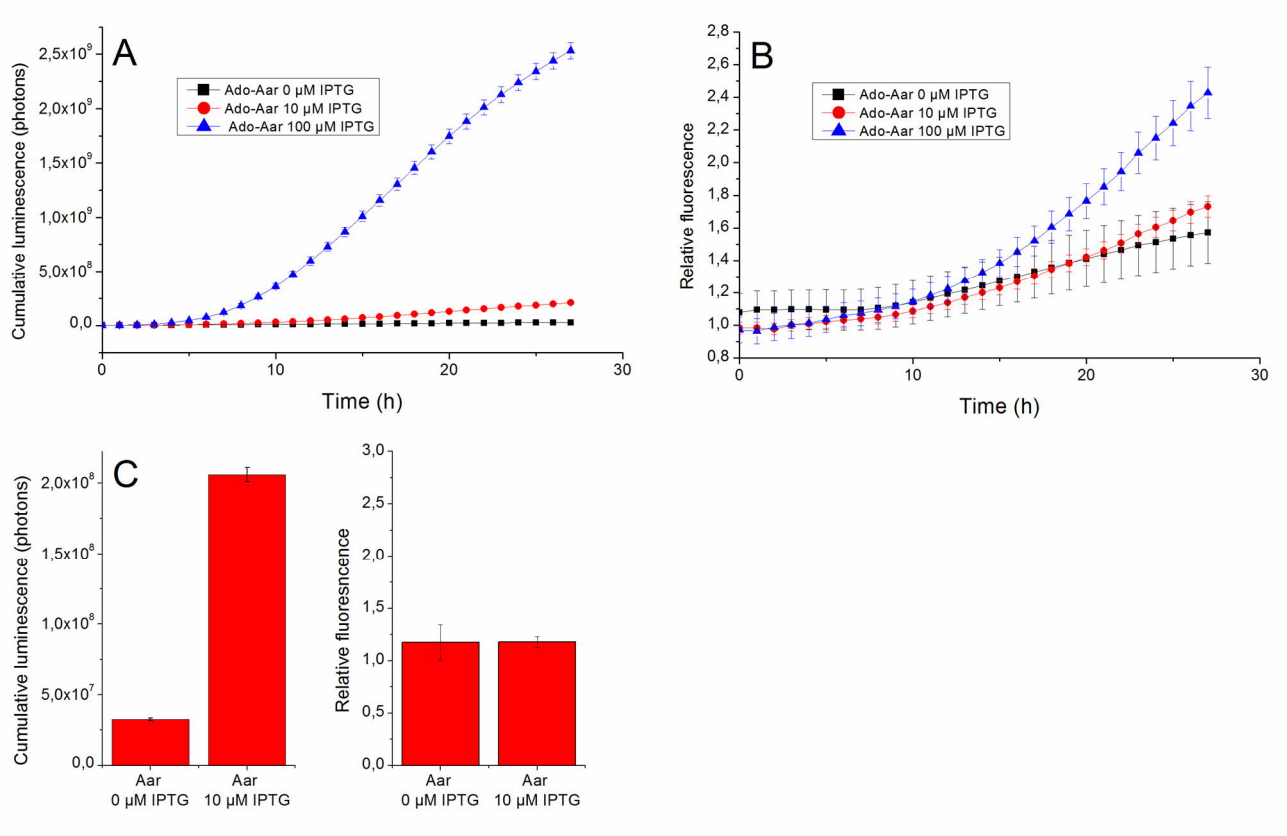

Figure 3. Monitoring the biosynthesis of alkanes. The IAS sensor cells (ADP1ΔACIAD3381-3383::luxAB, ΔalkM::gfp, harboring the alkane synthesis element with ado and aar under the IPTG-inducible lac/T5 promoter) were grown on LA plates in the presence of different IPTG concentrations. The constitutively expressed LuxAB measures the production of the aldehyde intermediate, while the expression of GFP is dependent on the activation of the alkane-inducible promoter P_{alkM} by alkanes. For control, responses of sensor cells expressing only Aar and not Ado were measured (C). The signals are presented as average of three replicates. Error bars represent standard deviation. Relative fluorescence was calculated by dividing the fluorescence signal of the sensor cells with the fluorescence signal of wild type A. baylyi ADP1 cells grown in the same conditions.

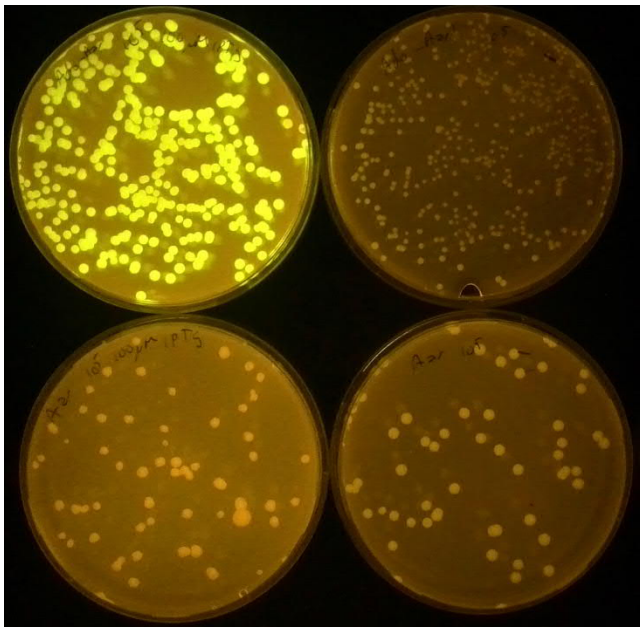

Figure 4. Detection of fluorescence from single colonies on agar plate. The alkane-producing sensor cells were plated from liquid culture on agar plate with 100 µM IPTG (top left) or without IPTG (top right) and incubated for 24 hours. The bottom row is the sensor cells with only Aar and no Ado, with and without IPTG. The plates are illuminated with a blue light transilluminator.