JULY

Week 1 :

Monday :

Attended the 2nd edition of the French-American workshop, organized by GIANT innovation campus (http://www.giant-grenoble.org/en/) (Grenoble, France). Attended several scientific talks, discussed career opportunities and presented our iGEM poster to a group of French and American researchers.

The following remarks were made by the audience :

- The goal of our project is not clearly presented in our poster
- Too much text and not enough keywords
- The graphs must be improved (units were sometimes missing)
- The team has to think more about the implications and consequences of the project
- Applications of the projects must be underlined more clearly

Tuesday :

***pQE30::KillerRed* construction :**

Extraction of *KillerRed* from the *pBabe-KillerRed* eukaryotic vector by PCR, using the following primers, enabling the addition of BamHI and KpnI at the 5' and 3' ends of the *KillerRed* gene, respectively. The restriction sites are highlighted in yellow.

Name: KR_left_pQE
TAATTCCGGATCCATGGGTTCAGAGGGCGGC (Tm=67∞C for 31 bases)
Restriction Site: (BamH1)

Name: KR_right_pQE
CAGGTACCTTAATCCTCGTCGCTACCGATGG (Tm=66∞C for 31 bases)
Restriction Site: (Kpn1)

Protocol available here.

10 µL of PCR products were subsequently analyzed by gel electrophoresis (1.2 % agarose, 30 min, 135 V). The results of the migration, on which a 700 bp DNA band is clearly visible, demonstrate the success of the amplification of *KillerRed*.

The rest of the PCR product was purified using the QIAprep® PCR Purification Kit (#28104, Qiagen, Venlo, Netherlands) and titrated using the nanodrop spectrophotometer available in the lab (final concentration : 95.8 ng/µL). Results of migration and titration are available in the figure below.

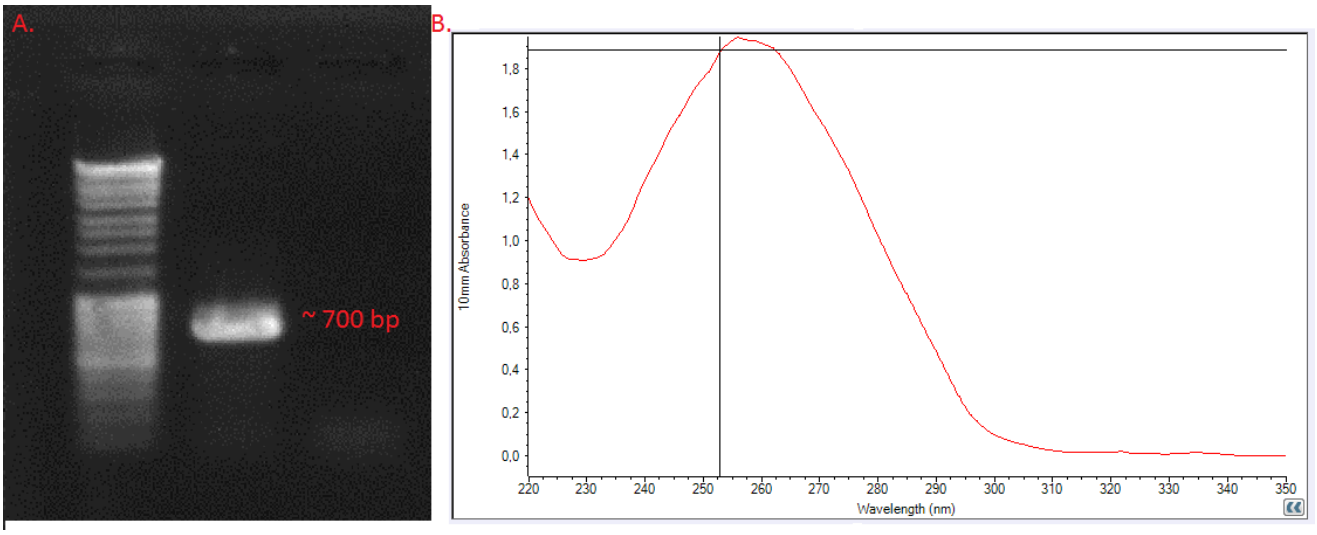

Fig X. Results of migration (A.) and titration (B.) of the KillerRed PCR product.

Restriction of the *pQE30::αSNAP* vector for isolating the *pQE30* plasmid backbone from the gene of non interest *αSNAP*, using BamHI and KpnI restriction enzymes (protocol available here). Migration on a 1.2% agarose gel (130V, 30 min).

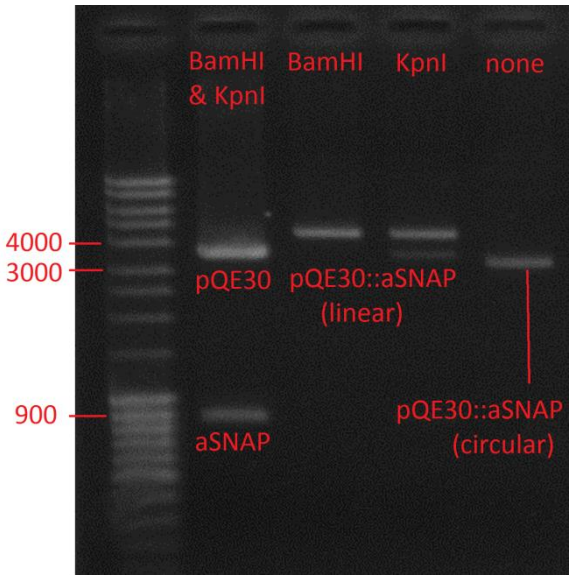

Fig X. Results of the gel migration aiming to isolate pQE30 vector backbone from αSNAP

The pQE30 vector was subsequently purified using the QIAquick Gel Extraction Kit (#28704, Qiagen, Venlo, Netherlands) and titrated (final concentration : 17 ng/µL).

XL1-blue MRF' cells (ref 200301-81, Stratagene, La Jolla, California, USA) were grown ON at 37°C, in LB medium for transformation with the product of *pQE30-KillerRed* ligation.

Wednesday :

***pQE30::KillerRed* Construction :**

Digestion of the *KillerRed* PCR product with BamHI and KpnI (protocol available here). Subsequent migration on 1.2 % agarose gel (30min, 135V). The results given by the positive control *pQE30::αSNAP* suggest that the restriction of the insert was a success.

The *KillerRed* insert was further purified using the QIAquick® PCR Purification Kit (#28104, Qiagen, Venlo, Netherlands). Final concentration : 96 ng/µL.

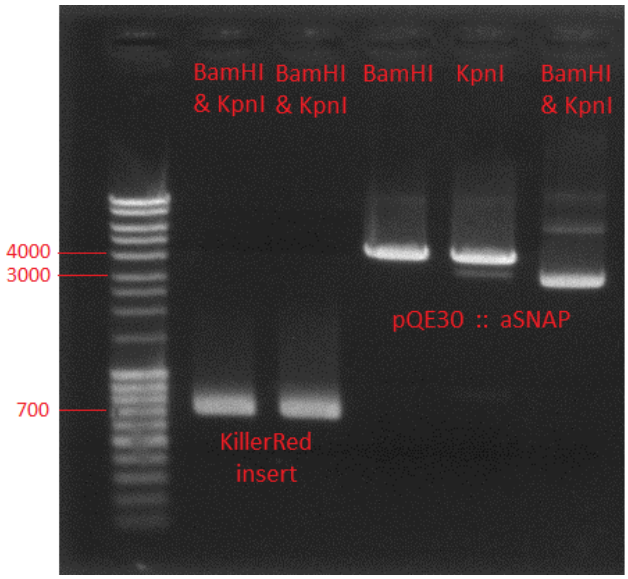

Fig X : results of the migration of the KillerRed insert digested with BamHI and KpnI

The restricted *pQE30* vector and *KillerRed* insert were subsequently ligated (protocol). For increasing the probability of success of the reaction, several experimental conditions were tested:

Ratio vector:insert	Amount of T4 DNA ligase (Weiss unit)	Finale volume (µL)
1:3	2	20
1:3	1	20
1:3	1	10
1:0	2	20

Table X : summary of the experimental conditions used for ligating pQE30 and KillerRed

XL1-blue cells (0D600 = 0.5) were further transformed with 5 µL of the ligation product, using the TSS2x method (protocol). Transformed cells were plated on agar plates (100µg/µL Ampicillin) and incubated ON, at 37°C. The rest of the ligation product was incubated 10 min at 70°C for T4 DNA ligase inactivation and stored at -20°C.

Thursday :

- Results from Wednesday plates :

PHOTO PETRI DISHES STEWART

From left to right, top row :

Ratio vector:insert	Amount of T4 DNA ligase (Weiss unit)	Final volume (µL)	#Colonies
1:3	2	20	53
1:3	1	20	22
1:3	1	10	27
1:0	2	20	0

From left to right, bottom row : transformation with pQE30::αSNAP (positive control) and dH20 (negative control).

The growth of several colonies on the 3 first plates of the top row suggests that the ligation was successful. Furthermore, all the experimental conditions tested appear to be suitable for the ligation reaction. Note that the 1:3 vector:insert molar ratio with 2 units DNA ligase and a final volume of 20µL gave the most important number of colonies (53). For this reason, this set of experimental conditions will be used in the future ligation performed during the project.

- PCR performed on the ligation products with the COM3' and COM3' primers, enabling to amplify any insert present in the pQE30 vector backbone (**protocol**).

July, 31st, 2013

1) Study of KillerRed-expressing cell fluorescence and OD600 under illumination :

Modelisation meeting with Hide
FRANCOIS SHOULD COMPLETE THAT

Manip with fake M9 : échec : cells were stuck at OD600 = 0,05

August 1st
Preparation of new M9 medium : detail composition of the new medium

Isolation of pRep4, mCherry, KillerRed from M15 cells, using the QIAprep® Spin Miniprep Kit (#27104, Qiagen, Venlo, Netherlands). Final concentration :

KillerRed-containing M15 cells were grown for 6h (37°C, 200 rpm) in LB medium, supplemented with 100 µg/mL Ampicillin and 50 µg/mL Kanamycin. The culture further undertook a 100x dilution in fresh M9 medium, supplemented with antibiotics. This M9 culture was incubated ON at 37°C, 200 rpm.

August, 1st.

The *KillerRed*-containing M15 cells, cultured ON in M9 medium supplemented with antibiotics (until OD600 = 3.3), were diluted in fresh M9 medium until OD600 = 0,074. OD600 and fluorescence measurements (540/630 nm) were then performed every hour, and KillerRed protein expression was induced by addition of 1mM IPTG at OD600 = 0.34. At OD600 = XXX, the sample was split into two different Erlenmeyers. The first one was kept in the dark while the second one being illuminated with white light for 150 min (SOURCE POWER and Bandwidth).

ADRIEN's CURVES

M15 cells seem to enter their exponential phase, but their growth is considerably slowed down as soon as the sample is split into the two different Erlenmeyers. One hypothesis suggests that this sudden change in environmental conditions is stressing cells, thus slowing down their division.

The experiment will be redone soon, starting the culture in the morning in two distinct Erlenmeyers.

Transformation of BW25113 with both pRep4 and KillerRed plasmids using the TSS2x method (**protocol**). Transformed cells were plated on LB-agar, supplemented with both Ampicillin and Kanamycin.

August 2nd.

- Result of the transformation : it fails.

No colony are visible on the agar plate, which suggests that not any cell was able to integrate both plasmids. The transformation will soon be reperformed, but in two steps (transformation of the cells with KillerRed, followed by an additional transformation with pRep4).

Monday, August, 5th :

1) Culture of M15-KR in 4mL LB (Amp + Kana) : growth from 10 am to 6 pm and then transfer in 4 mL M9 minimum medium (Amp + Kana). The M9 culture was further kept at 37°C, 200 rpm until Tuesday.

2) Preparation of M9 minimum medium.

3) Transformation of BW25113 (DO = 0,395) with :

    a. pQE30::KR
    b. Adrien's vector Backbone (reference : high copy plasmid with Amp resistance)

4) Experiment : are the M15 cells growing in M9 medium ?

2 Erlenmeyer (1 dark, 1 transparent), were filled with 22 mL M9 medium, supplemented with 50 µg/mL Kanamycin, 100 µg/mL Ampicillin and 1mM IPTG. M15 cells were diluted until OD600 = 0.02. OD600 and fluorescence were then measured every 1h for 4 h. OD600 saturated at 0.04, which means that cells are apparently not growing. 3 hypotheses can explain this phenomenon. First of all,

when when added during the inoculation, IPTG might disturb cell growth due to KillerRed phototoxicity. The other hypothesis is that the incubator temperature regulation module is misfunctionning.

To check the second hypothesis, the experiment were performed again using one new Erlenmeyer filled with 20 mL M9, supplemented with 1mM IPTG, Kanamycin and Ampicillin. Cells were inoculated at 0,05 and the sample was incubated in the other incubator, available in the lab (37°C, 200 rpm).

20h47 → 0,094
21h42→ 0,106
22h24→0,112

Automated OD600 measurements were eventually performed every 10 min for 6h, using the Tristar microplate reader available in the lab. This was tried to check whether or not the OD saturation at very low values was due to a prolonged lag phase in M9 medium. Indeed, cells were inoculated from a LB culture, and the switch from one medium to another could be responsible for a prolonged lag phase, during which cells are adapting to their new culture medium. BW25113 E. coli bacteria were added to the 96-well plate, to study and compare their growth to M15 cells in M9 minimum medium.

5) Isolation of the pRep4 plasmid from M15 cells, using the QIAprep® Spin Miniprep Kit (#27104, Qiagen, Venlo, Netherlands) and titrated (final concentration : 160 ng/µL) .

This is performed for further transforming KillerRed-containing BW25113 cells with the pRep4 plasmid. Indeed, using those cells with Pr. Voigt's plasmids (pJT122, etc.) seems a good idea. BW25113 are first XXXX (ask Pierre), and are also well known in the Hans Geiselmann lab with which our student team is collaborating.

Demain : traiter données cell growth Tristar

- Préparer boite Amp + Kana
- Préparer 2 Erlenmeyer de M15 M9 avec ANTIBIO et IPTG (dilution au 100 eme, puis plus until OD600 = 0,05)
- Faire Manip dans l'incubateur pourri, avec alu seulement.
    - En effet, lors de la première manip avec Xl1-blue KR, seul de l'alu était utilisé et 2 Erlen était utilisés. Aucun pb au niveau de l'Erlen « dark », ce qui sous-entend que l'alu est OK et qu'il n'y a pas de fuite de lumière suffisamment importante pour perturber l'OD de la culture dans le noir.
- Préparer Erlen Nico M9 M15 KR

Tuesday :

- Result of the transformation:

The transformation did not work. Some of BW25113 possibly transformed with KillerRed and ADRIEN's backbone were kept ON at RT, without any antibiotics. They were centrifuged, resuspended in 200 µL LB medium and plated on two fresh agar plates, supplemented with ampicillin.

- KillerRed characterization :

M15 cells, cultured ON in M9 medium, were at OD = 2.46. Cells were diluted in 2*25 mL fresh, prewarmed M9 medium, supplemented with antibiotics and 1mM IPTG, until 0D600B = 0,035, in two

different Erlenmeyers. OD600, fluorescence measurements and cell plating (50 µL of 1/10000 cell solution on LB-agar) were then performed every 1h.

A new culture of M15-KillerRed cells was started in LB medium (with antibiotics).

A new experiment was started, using the same conditions, but with LB medium instead of M9. It's time now to produce some consistent results for modeling, and we are wasting a lot of time by optimization of the experiment with M9 medium.

The few past experiments make me think that cell growth is inhibited in response to IPTG induction in M9 medium. Indeed, M15 cells are able to grow in M9 medium until OD600 = 2,4/3,3 (37°C, 200 rpm). They stay stuck at OD600 =0,1 when induced during the inoculation. Besides, one experiment has shown that M15 cells' exponential growth is suddenly interrupted when adding IPTG to the medium. This M9 minimum medium may offer a better dynamic when studying E; coli cell growth, but it doesn't seem to be suitable when it comes to deal with IPTG induction.

Protocol for tomorrow :
30 µL of clone 0 glycerol stock were added to 4 mL LB supplemented with antibiotics and IPTG.

In the morning : prepare 2 Erlenmeyers filled with LB, IPTG, antibiotics. Inoculate at 0,02. Let grow and trigger the light at OD = 0,2, for 1 h.

07/08/13 :

Comparison of the growth of BW25113 and M15 E. coli strains in LB medium, M9 medium, with our without IPTG. The experiments carries out in M9 medium were not successful: M15 cells do not grow when KillerRed production is induced by IPTG addition.

Cells were grown in the same conditions (LB/M9, IPTG or not, BW, M15) in 3mL, in 50 mL Falcon tubes.

10h30 : inoculation of M15 cells (KR) in LB medium (3mL LB + 3µL Kan + 3µL Amp + 30µL glycerol stock). Inoculation of BW-KR in LB medium (from 1 clone of the Monday transformation in 3mL LB + 3µL Amp).

Desinfection of the incubator with 70% ethanol.

09/07/13 :
Results of growth of BW and M15 in LB/M9, supplemented or not with 1mM IPTG :
M15 cells display a better growth curve that BW and will then be kept for characterizing KillerRed. It seems that BW are disturbed by KillerRed expression in every medium, with or without IPTG.

Criteria :

1) Growth in IPTG
2) "Best exponential phase"

Winner : M9 medium supplemented with IPTG, where M15 cells are growing.

On both LB and M9 supplemented with IPTG, it seems that M15 cells need some time to get use to the presence of IPTG (note that the transfer from LB to M9 trigger a lag phase, but very quick, which means that cells do not need to much time to get use to M9 from LB). The idea is thus to culture M15 cells in LB for a few hours, and then to transfer it into M9 saturated with IPTG and to incubate the sample ON, 37°C. In the morning, cells can then be diluted to 0.02-0.04 in fresh M9 medium, supplemented with IPTG, for characterizing KR.

This was done today, using the culture grew ON in M9 IPTG (M15 cells). Cells were resuspended in 50 mL fresh M9 medium, supplemented with AB (yesterday there were AB as well) and IPTG 1mM. They grew pretty well, as expected, but did not fluoresce at all…

Can growth in 1mM IPTG for extended period trigger the inability to produce KR ?
After a few hours without fluo, the mother solution of M15 cells growing in M9 medium with saturated IPTG was used for fluorescence measurements. The level of fluorescence was low, but still higher that the "background fluorescence" observed for the new culture. It means that those cells were really M15 cells and not something else.

Note that no mistake was made when preparing the falcon tubes containing the 8 diffferent cultures yesterday (painstakingly performed, step by step : M9+fer+traces/LB→AB Kana and Amp→ 1mM IPTG.

Grow cells at a high level of IPTG is a good idea. First of all, cell growth is unlikely to be disturbed by IPTG induction. Then, we are sure to have a high enough level of KillerRed to kill cells when illuminating with ligh, even in early exponential phase.

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M15 cells were incubated 6-7 hrs in 3mL LB medium, supplemented with 2X Amp and 1X Kana. They were then transfer in prewarmed M9 medium, supplemented with AB (200 µL in 3mL M9) and incubate 14-15h at 37°C, 200 rpm. (090813). Clone 3 did not grow in LB medium.

In the morning, cells were observed with the microscope. They did not fluoresce and looked a bit stressed. 4 Erlenmeyer were prepared. 600 µL of each solution was added to Erlemeyers filled with pre warmed M9 medium, supplemented with Kanamycin 1X and Ampicillin 2X. Fluo measurements and OD610 measurements were further performed on a regular basis (every 1.5 hrs).

Cells seem to both grow and produce fluorescence, which is good news.

The growth very slow : back to LB tomorrow and screw M9.

The half-life could not be measured because the fluorescence go on increasing. Maturation time ?

- ➔ Inefficiency of the AB ? Try tomorrow with a lethal coktail

New experiment this time for measuring KR half life :
E. coli grew ON in LB + 2x ampi + 1x Kana were supplemented with 1mM IPTG and incubated for 5h at 37°C. The experiment was further performed as yesterday, except that the microwells were filled with 300 µL medium, to prevent evaporation. Duration of the acquisition : 90 cycles of 10 min = 900 min.

11/08/13

The half-life experiment did not give any consistent results. It seems that the increase in fluorescence that is observed is just an IPTG induction curve. Today, new experiment :

Supplement a M15-KR saturated solution (containing a lot of KillerRed) with 10X Kana, 10X amp, 10X Cam, 10X Spectinomycin. DO not add IPTG. Input in the spectrophotometer and perform a fluo acquisition every 10 min.

New experiment aiming to characterize KillerRed :
A saturated red culture of M15 KillerRed was diluted in LB, supplemented with 1mM IPTG, 2X Amp and 1X Kana, to OD600 = 0.03. The usual procedure was further followed.
Cells do not grow, and this underlines and interesting phenomenon : KillerRed is highly disturbing cell growth when present in high quantity in the cell. This is confirmed by the fact that BW cells were not growing. Those cells do not have any LacI repressor and were then expressing too much KillerRed in exponential phase. In our case, it seems that the pRep4 plasmid is not as effective in stationary phase than in exponential phase. For this reason, cells do not grow today : the culture was started from E. coli saturated cells, that contains already an enormous amount of intracellular KillerRed. Note that in this experiment comparing growth rate in LB and M9 medium, cells were not at saturation when they were rediluted at the beginning of the experiment.
Cells finally manage to grow, but lost their plasmid : OD exploded from 0.08 to 3.4 in 2h, but fluorescence did not increase. KillerRed must have a pretty long half-life, reason for which the fluorescence of the culture stay stable. At high protein concentration and in presence of IPTG, they manage to loose their plasmid, by for instance, overproducing beta-lactamase, responsible for Ampicillin degradation.

New idea : it seems that this time again, cells take a long time to grow, even in LB medium. It is likely to be due to the high amount of KillerRed contained in each cell (Fluo/OD~8000). Having a constant amount of KR/cell is good since it allows to mimic a constitutive promoter. However, it is worth studying which conditions are optimal for both growing cells and being able to kill them. We should then find a compromise between cell growth rate and the amount of KR/cell. This is gonna be investigated through the following experiment :

IPTG gradient : 1mM, 0.5mM, 0.25mM, 0.125mM, 0.0625mM

Well 1-5 : Saturated culture of E. coli M15-KR diluted to 0.02, with various amount of IPTG added (triplicates→ 15 wells)
Well 6-10 : exponential culture of E. coli M15-KR diluted to 0.02, with various amount of IPTG added (triplicates→ 15 wells).

This should further be repeated with M15-mCherry or m15-mRFP.

Preparation of the solutions :
SATURATED : M15KR cells were grown ON in 10mL LB, supplemented with KANA 1X and Ampi 2X. They were further diluted 10X in 10mL fresh LB, with the same amount of Antibiotics.

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The same experiment as yesterday was performed. According to the first results given by the spectrophotometer, there is no difference between the fluorescence of the cells that were cultured 24h in about 5-7mL LB, supplemented with Kanamycin 1X and Ampicillin 2X and those cultured from a glycerol stock this morning (in 10mL of the same LB). This shed the light on one important phenomenon : cells grow more and express more KillerRed when cultured in more important volumes. Then, it seems that this experiment is gonna be suitable for investigating the possibility of

starting KillerRed experiment from cells expressing a high amount of KillerRed proteins (those grew in 10mL ON for instance). However, it will give.

Then, protocol adviced : start from a glycerol stock a culture

13/08/13

According to the analysis of the results of the previous ON experiment performed on a 96 well plate, aiming to study the effect of [KR] on cell growth :

Cells grow better when they are precultured in a small volume of LB, supplemented with the right AB (Amp 2X, Kana). This protocol is then likely to be followed in the future experiments.

A calibration curve, giving a relationship between SL at 490 nm and real OD600 was performed for M15-KR cells.

Results of the kinetics : best for low concentrations in IPTG.
Best growth (no lag phase) for cells that are precultured and rediluted when in exponential phase.

The kinetics from this night also provided information about KR half-life : ~100h. The fluorescence of M15 non fluorescent cells must also be measured at stationary phase, at the same OD (background substraction).

14/08/13

New experiment aiming to study the physiological effects of KillerRed on cell growth.
Cells were cultured in the morning from glycerol stocks :

1) M15-KR 30 µL + Amp 2x + Kana in 10 mL LB
2) mCherry-KR 30µL + Kan + CamR

➔ in 50 mL Falcon tubes

2 times 5 Erlenmeyers were prepared, filled with 25mL LB supplemented with the right AB for mCherry and KR. IPTG Gradient : 0.2mM-0.05mM-0.025mM-0.0125mM-0mM. Those Erlen were kept at 37°C while cells were gorwing in their preculture.

Resuspension at 0.02uA.

Dilution 5x pour mesure de DO à 610nm.

Conclusion : best concentration = 0,05mM IPTG !

Until 17/08/13 :
Test with low intensity : no effects, but good growth.
Extra experiment, simple, with only 3 Erlenmeyers. Cells were cultured from glycerol stocks (mCherry and KillerRed), with the right AB. They were further resuspended at 0.08 in 25mL LB-IPTG-AB. Pb : the initial DO was too high → the ratio Fluo/DO was going to be high only in late exponential phase/stationary phase (repeatability problem, considering the previous experiments that were run).

17/08/13 :
Test of Propidium Iodide on m15-KR cells.

A saturated solution of M15-KR was split in two different Falcon tubes, one of those being supplemented with 10x Kanamycin, 10x Ampicillin, 10x Chloramphenicol. Cells were stained after 30 min with PI :

1) centrifugation of 200µL cells (1min at 13200 rpm)
2) incubation 5 min at 37°C with 200µL 0.05mg/mL PI
3) Centrifugation (1min at 13200 rpm)
4) Resuspension in 200 µL LB and transfer into the 96 well plate for fluorescence measurements at 540/630nm

Results after 30 min
1) Without AB : 8090 (KR) and 8982 (KR + PI)
2) With AB : 8010 (KR) and 8982 (KR + PI)

It seems to be a good technique for investigating cell viability when illuminating cells with KR. GOOD !

A new experiment was launched with KillerRed :
1) Glycerol stock : 30µL in 10mL LB, supplemented with AB (Amp2x and Kan1x or Cam1x and Kan1x) (done for both KillerRed and mCherry)
2) Resuspension in prewarmed LB (25mL), supplemented with 0,05mM IPTG and AB at 0.03OD
3) First measurements (t0) 15min after induction with IPTG.

Results (manip KillerRed 170813 (first test acridine orange)-sheet1

18/08/13

A new experiment was launched with KillerRed :
1) Glycerol stock : 30µL in 10mL LB, supplemented with AB (Amp2x and Kan1x or Cam1x and Kan1x) (done for both KillerRed and mCherry)
2) Resuspension in prewarmed LB (25mL), supplemented with 0,05mM IPTG and AB at 0.03OD
3) First measurements (t0) 15min after induction with IPTG.

When inoculated, KR was at OD1.2 and mCherry at OD0.7. They were diluted to OD0.03

Tests with IP were not successful since the pellet that we get in exponential phase is very small. Too many cells are lost during the different washes and the pellet is sometimes discarded by mistake when removing supernatant.

However, microscopy acquisition were performed at t) tfinal-1. It seems that there are still some living cells in the illuminated KR sample (they are looking good and fluoresce, meaning that they are likely not to have been completely bleached yet, and hence, killed.

At t-1, a IP test was performed on the KillerRed sample (5min centri + 15min incubation with 0.05mg/mL IP + Three wash with PBS (5min each, 300 µL).

Week 1 September : Sunday, Monday, Tuesday Wednesday : Kinetics with M9 medium
Cells were precultured ON in M9 medium, supplemented with Amp and Kan (10mL M9 in a 50 mL Falcon tube). The day after, cells were resuspended in different Erlenmeyers at 0,015. OD610, Fluo and cell plating were subsequently performed every hour for 12 hours.

Good results, that should be fitted soon by François. The number of colony is also significantly decreasing. The proof of concept for KR induced cell death is verified !