Quantifying Calcium concentration in living cells

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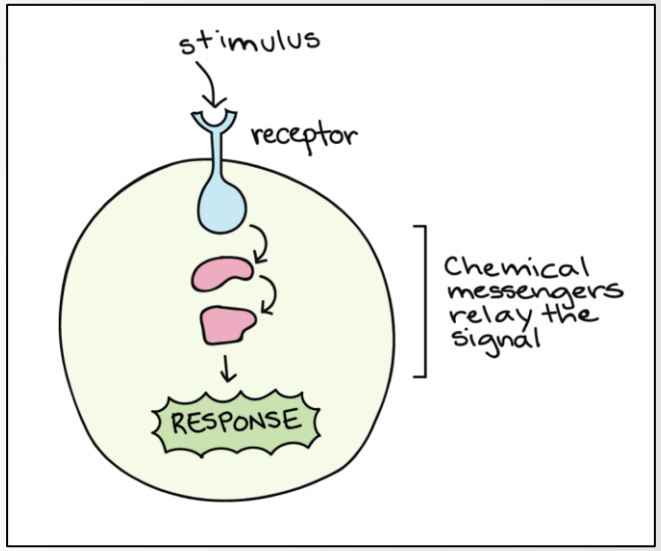
Cell signalling

- **Cell signalling** is the cells ability to detect and react to external stimuli
- Signalling regulates cell metabolism and tissue homeostasis
- The stimulus is transmitted inside the cells through a chain of **chemical messengers**



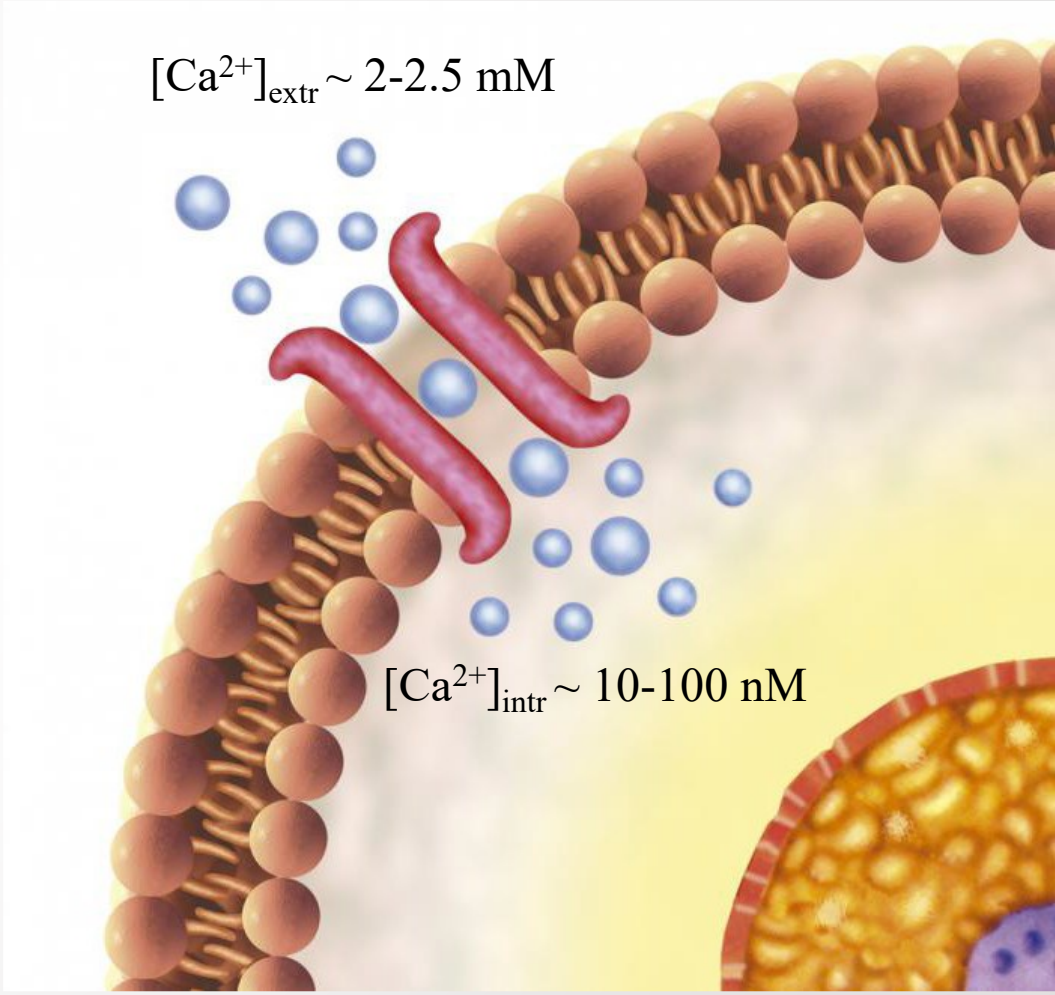
Ca^{2+} is a fundamental chemical messenger

Ca^{2+} signalling

- The intracellular concentration of Ca^{2+} in cytosol $[Ca^{2+}]_{intr}$ is very low compared to extracellular concentration $[Ca^{2+}]_{extr}$ (4 - 5 orders of magnitude)

- Stimuli open channels for Ca^{2+} and allow Ca^{2+} extracellular to flow into the cytosol, raising intracellular Ca^{2+} concentration ($[Ca^{2+}]_{intr}$ gradient)

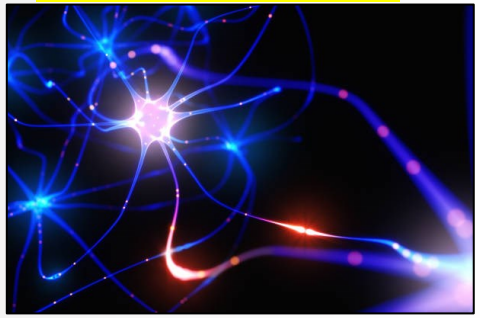
- Ca^{2+} ions bind to some proteins in the cell changing their activity providing a response to a stimulus



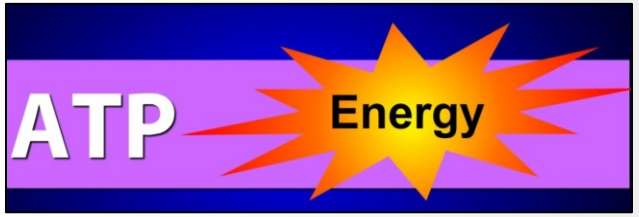
Ca^{2+} signalling

Many significant processes are regulated by **gradient Ca^{2+} concentration** between the intracellular and extracellular environment.

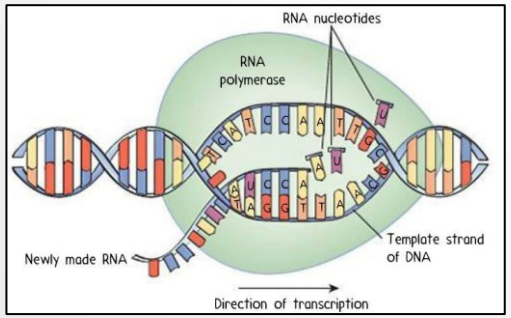
Neurotransmission



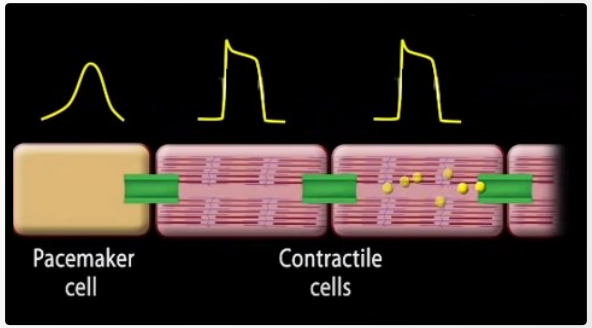
ATP production



Gene transcription



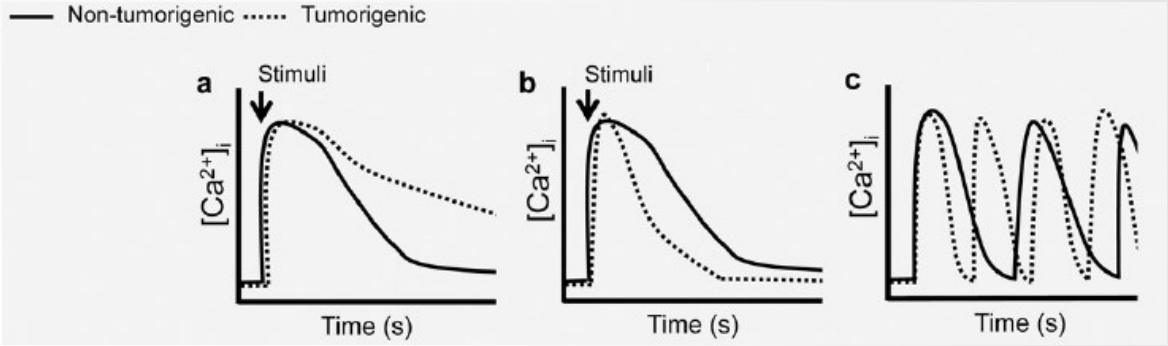
Cardiac regulation



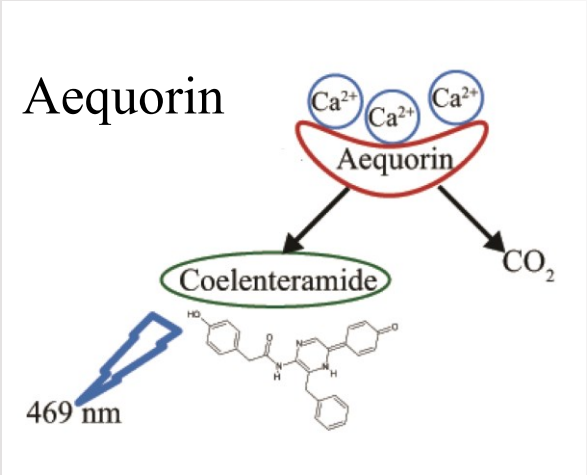
Ca^{2+} signalling

The reconstruction of the intracellular Ca^{2+} concentration kinetics is a powerful diagnostic tool

Teneale, A. *et al*., *Bioch. et Biophys. Acta*, 1848 (2015) 2502–2511



a: pancreatic cancer cells b:colon cancer cells c: breast cancer cells



The reconstruction of the Ca^{2+} signal is made possible using fluorescent labels or bioluminescent proteins, offering significant advantages:

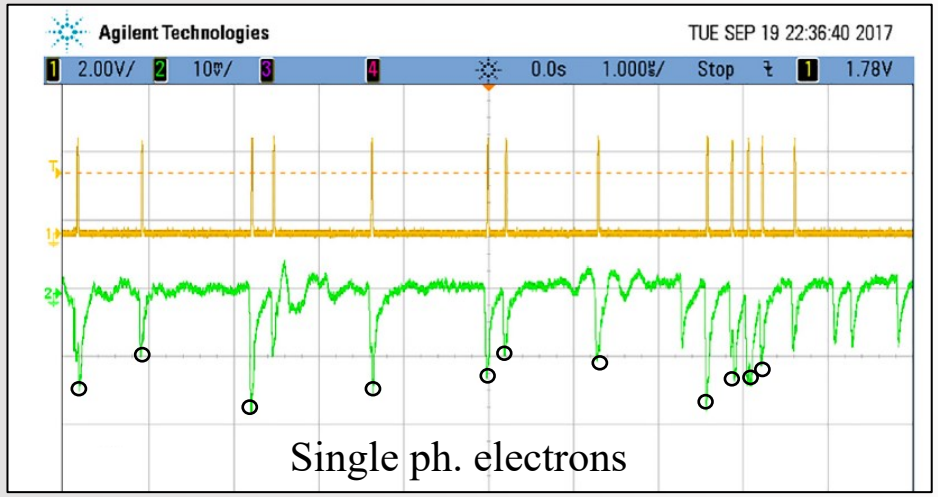
- Selective intercellular distribution
- Wide dynamic Ca^{2+} concentration range (100 nM-100μM)
- High signal to noise ratio (compared to standard fluorescent dye)
- Low Ca^{2+} buffering effect

Among bioluminescent proteins, Aequorin is the work-horse.

Aequorin emission features

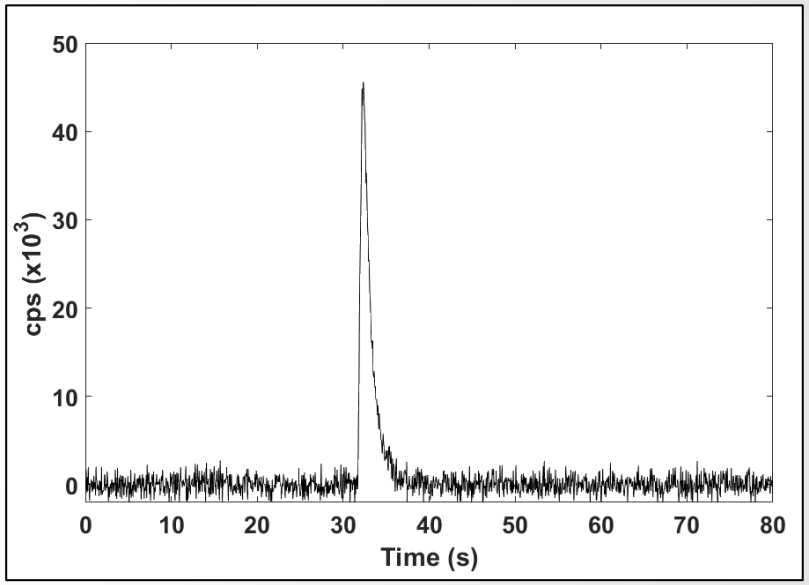
The light emitted by Aequorin is a trail of single photons

- λ_{em}: 469 nm
- Signal lenght ÷ 1 – 40 s



Single ph. electrons

Counts in gated windows of 50ms

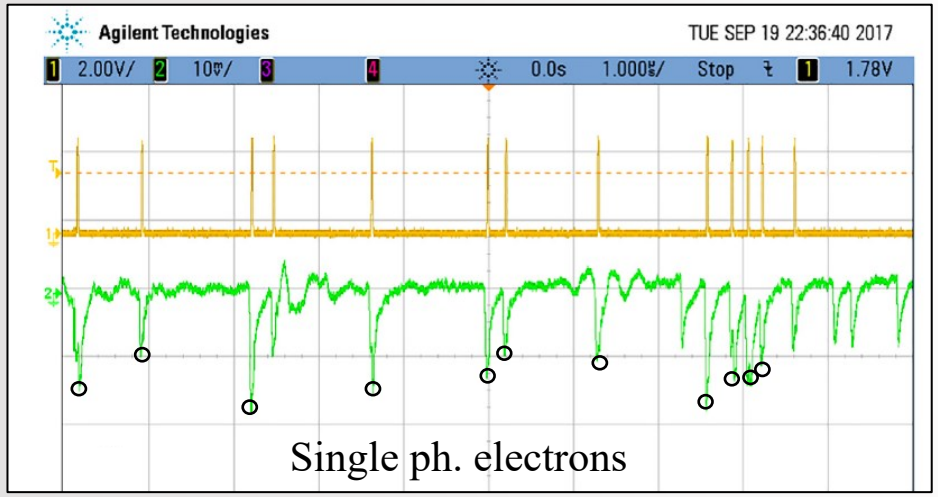


A detector with single photon sensitivity is required

Aim

The light emitted by Aequorin is a trail of single photons

- λ_{em}: 469 nm
- Signal lenght ÷ 1 – 40 s



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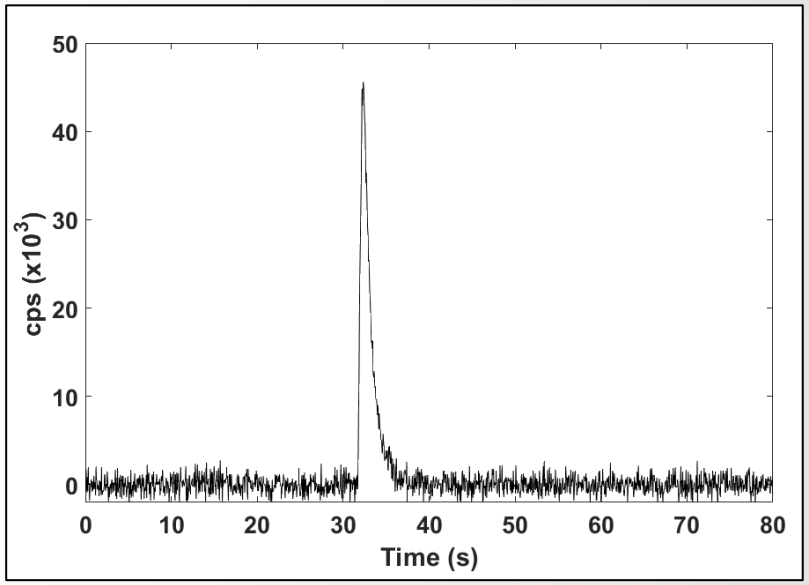
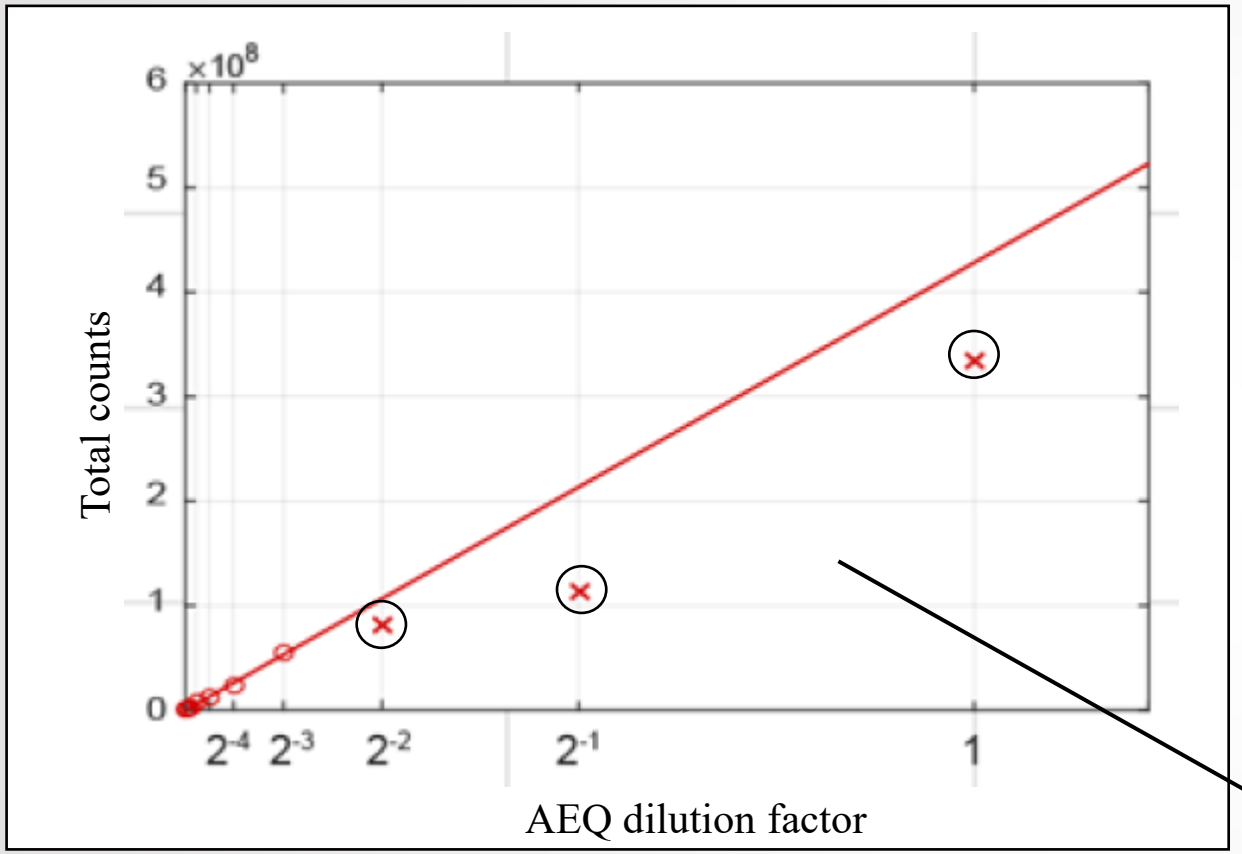


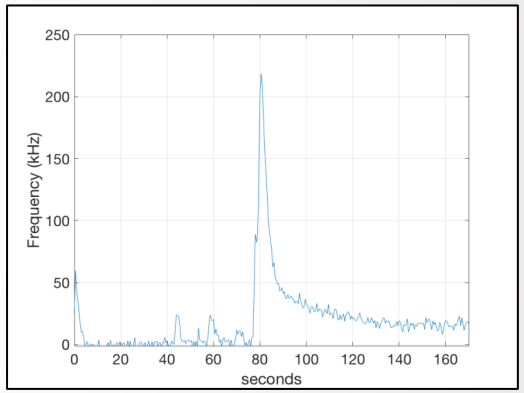
Develop a SiPM based instrument that could be an alternative to PMT based apparatus, offering comparable or better performance, ruggedness and portability.

Previous experiments



Lomazzi *et al*., *ACS Sens.* 2020, 5, 2388−2397
Lomazzi *et al*., *Nucl. Inst. & Meth. Sect. A* 2020, 979, 1644-93

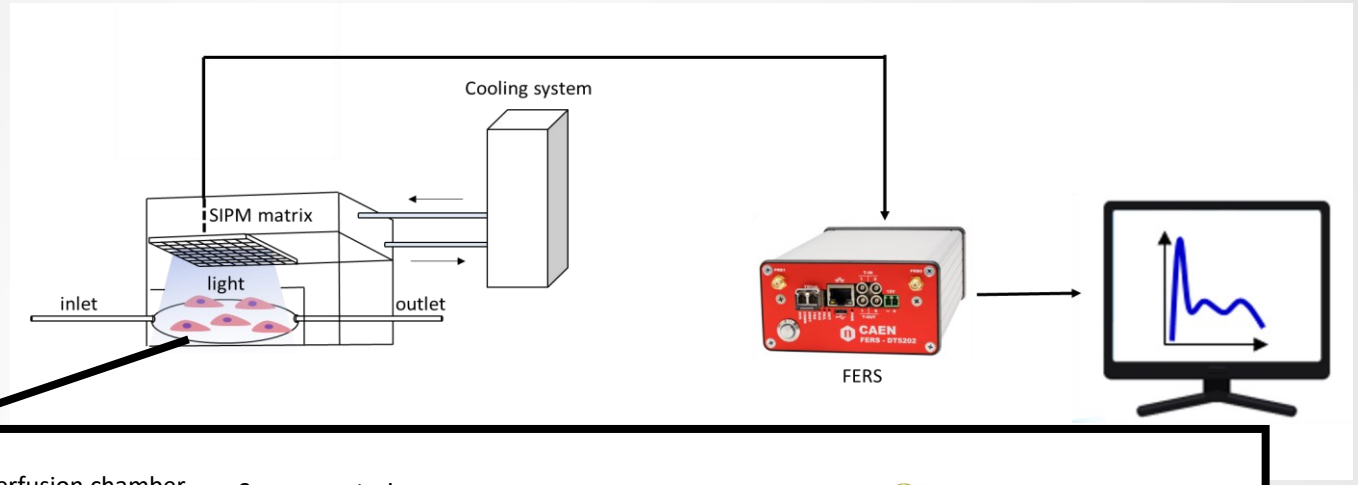
- First prototype based on Single SiPM (6x6mm^2) has been demonstrated to be able to detect bioluminescence signals in living cells

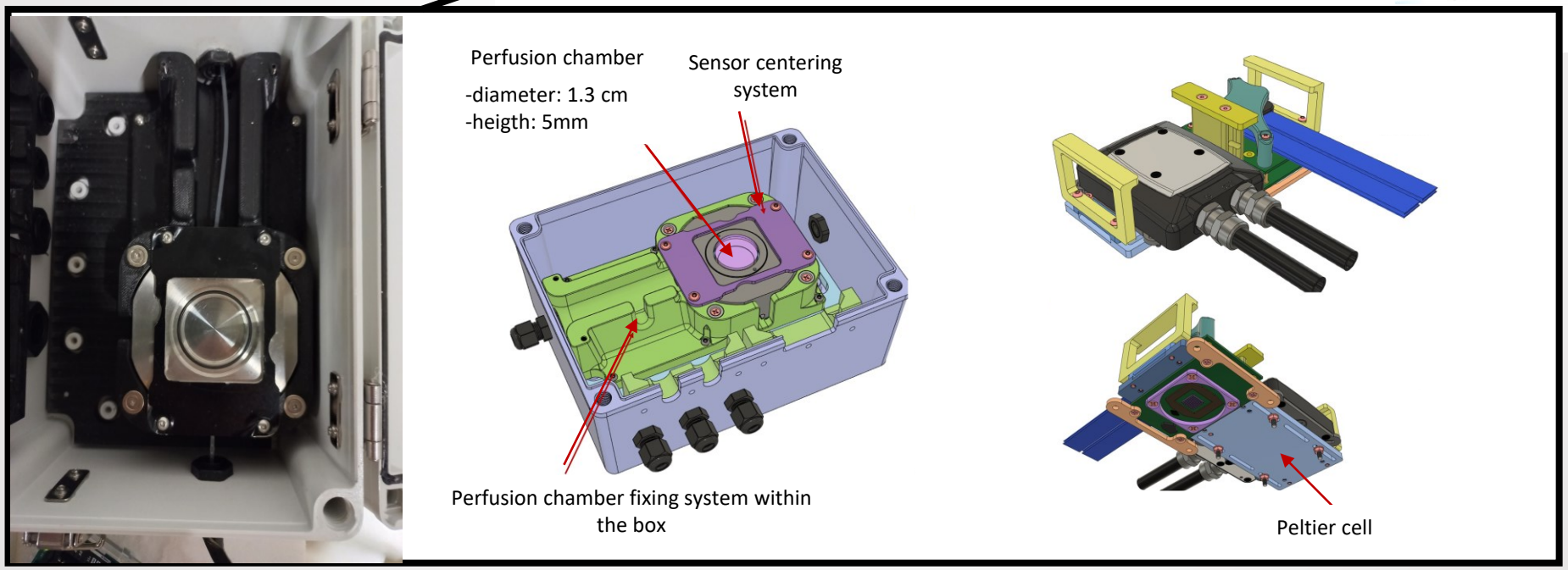


Problem

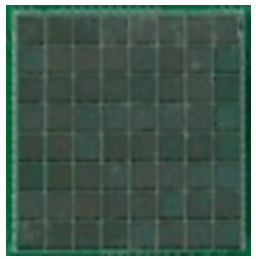
- Linearity response limited at 3 MHz due to pile-up effects.

Experimental setup

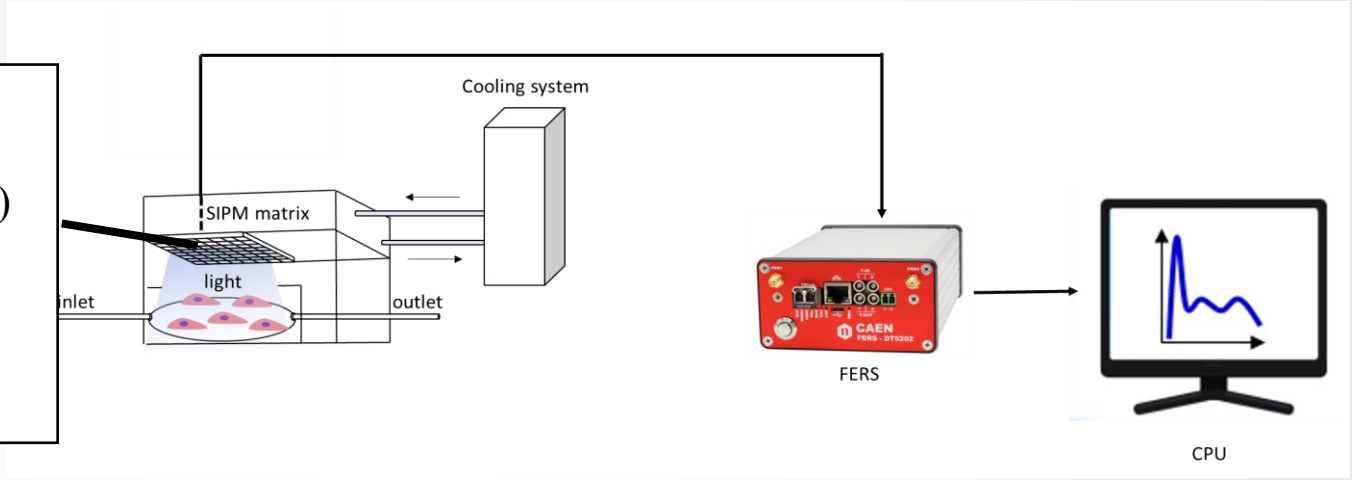




Experimental setup



Matrix of 64 SiPM
by Hamamatsu (S13615-1050)
Each SiPM:
Size: 1 x 1 mm^2
Cell pitch: 50 μm
Gain = $1.5*10^6$



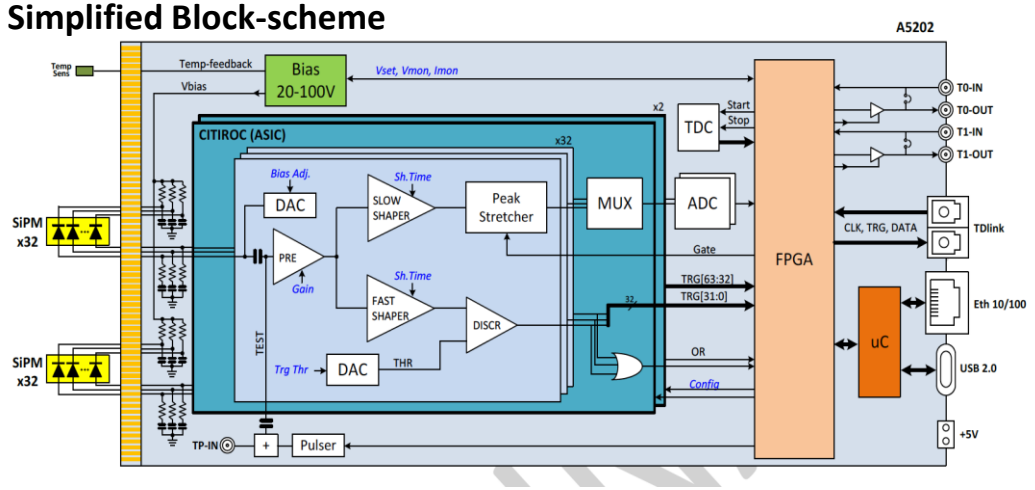
Single SiPM (6x6mm^2) VS a matrix of SiPMs

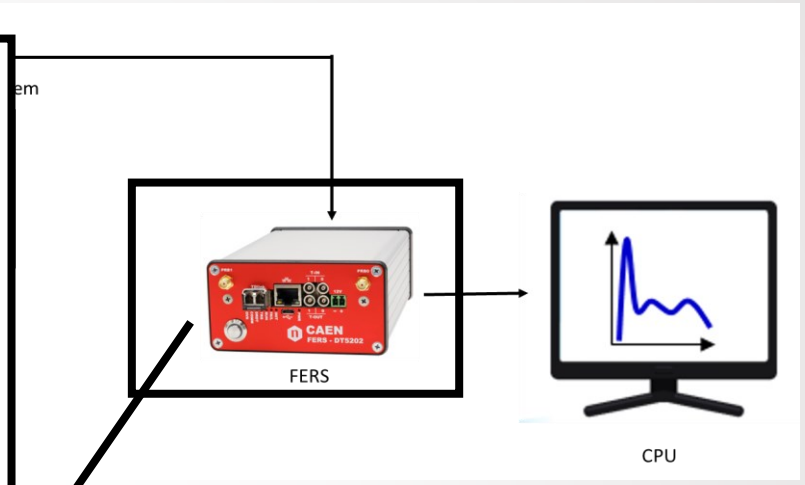
- Minimum Detectable Signal (MDS) is limited by Dark Count Rate (DCR)
- Linearity in counting is limited by the pile-up probability

The indipendent readout of 1x1mm^2 SiPMs improves the linearity range allowing for larger acceptance. The price to pay is the system complexity

Experimental setup

Simplified Block-scheme





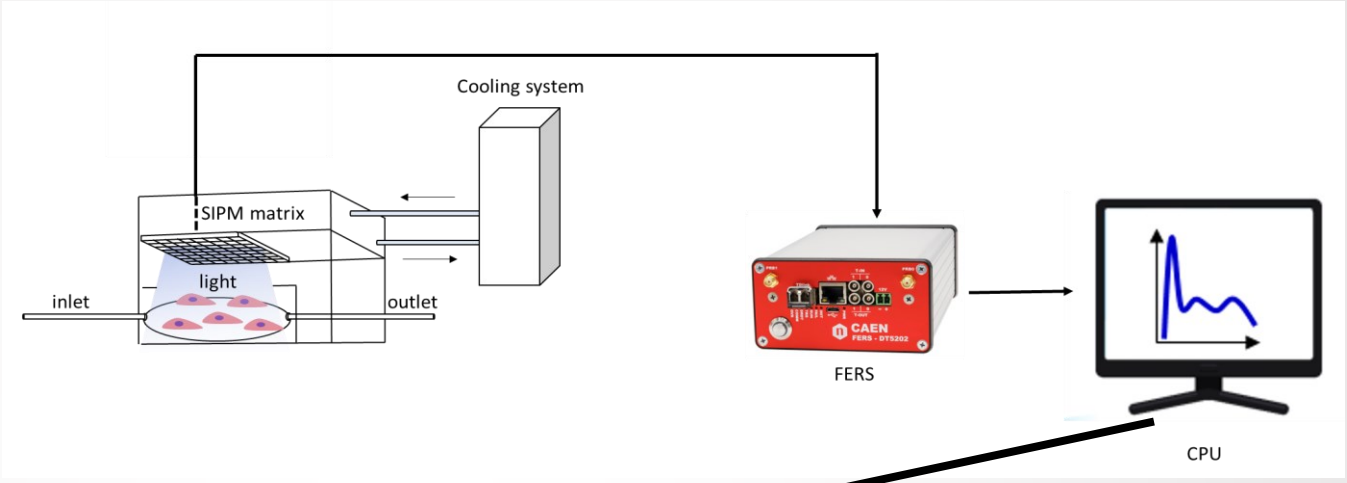
https://www.weeroc.com/products/sipm-read-out/citiroc-1a

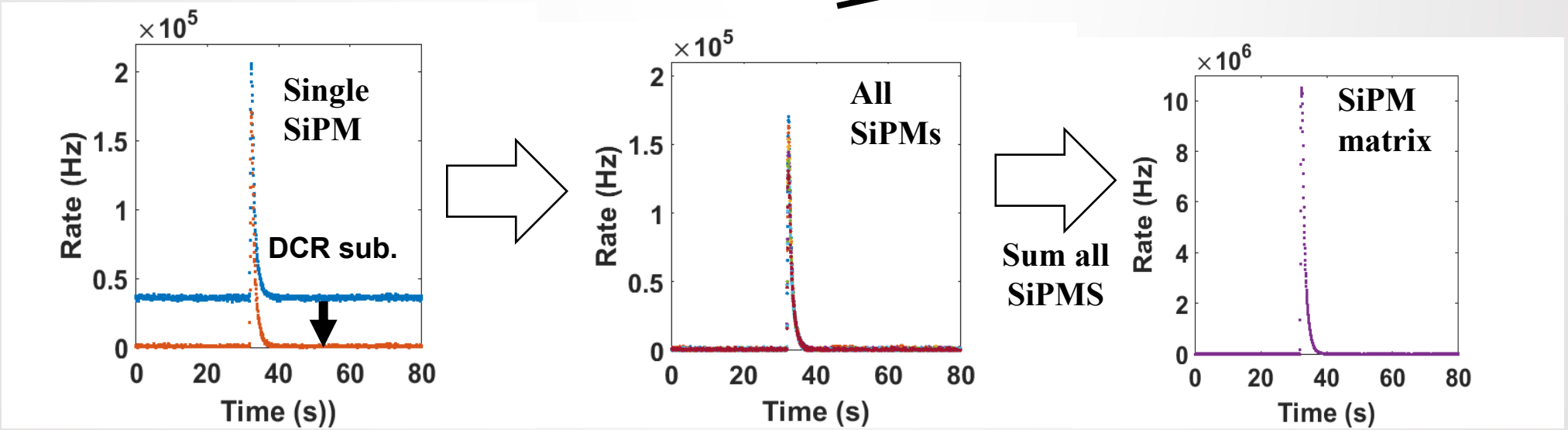
https://www.caen.it/products/dt5202/

Citiroc 1A embedded in DT5202 readout board

- 64 SiPM readout with single channel HV adjust and temp. compensation
- 1 HV power supply (20 – 100V) with temperature compensation
- Single ph-e counting capability
- Maximum counting rate for each channel: 20 MHz

Data processing



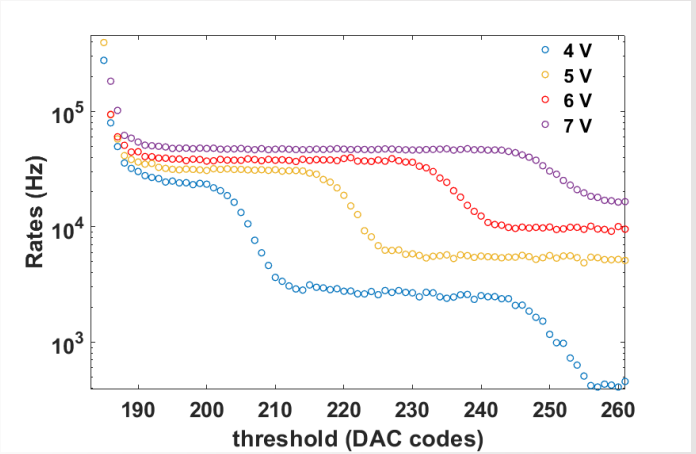


Working point definition

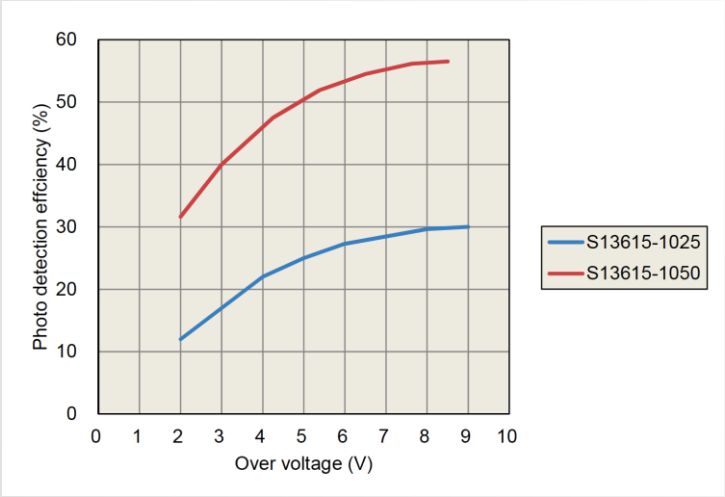
The system operates in counting with a threshold set at 0.5 ph-e

- The SiPM characteristics are largely affected by the over-voltage setting:
  - Higher over-voltage = higher gain (larger plateau that allows for stable condition)
  - Higher over-voltage = higher PDE
  - Higher over-voltage = higher stocastic effects (i.e. DCR, Crosstalk and After Pulse)

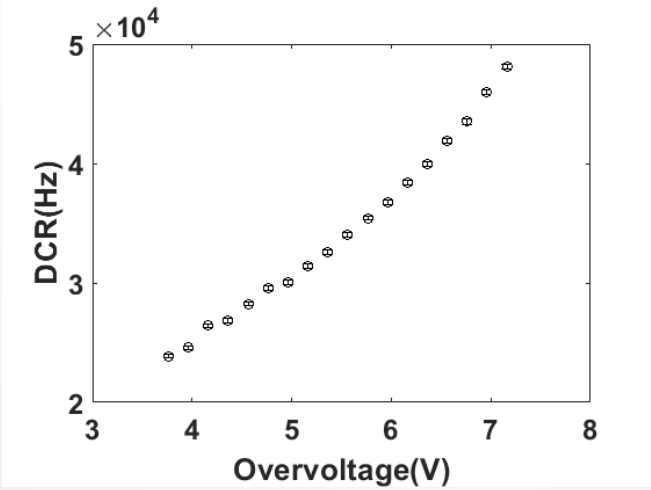
Staircase VS Over Voltage



PDE VS Over Voltage



DCR VS Over Voltage



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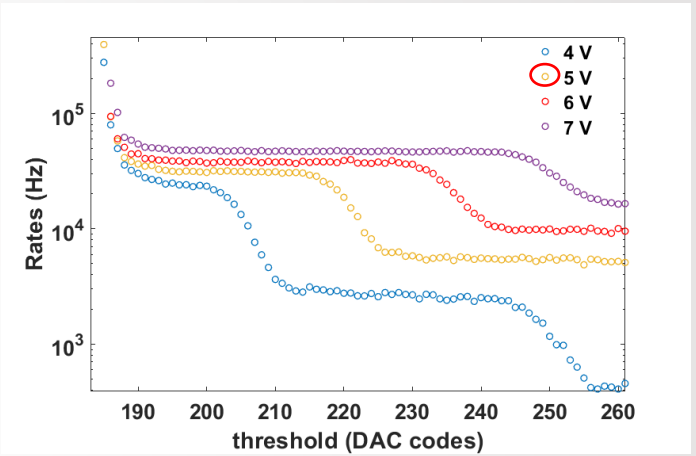
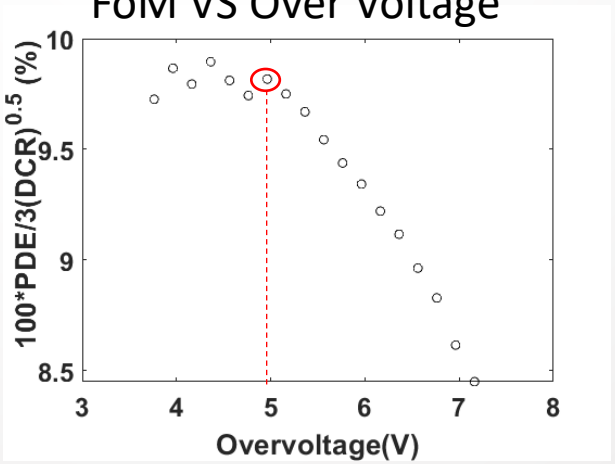


The chosen working point is a compromise of all these effects

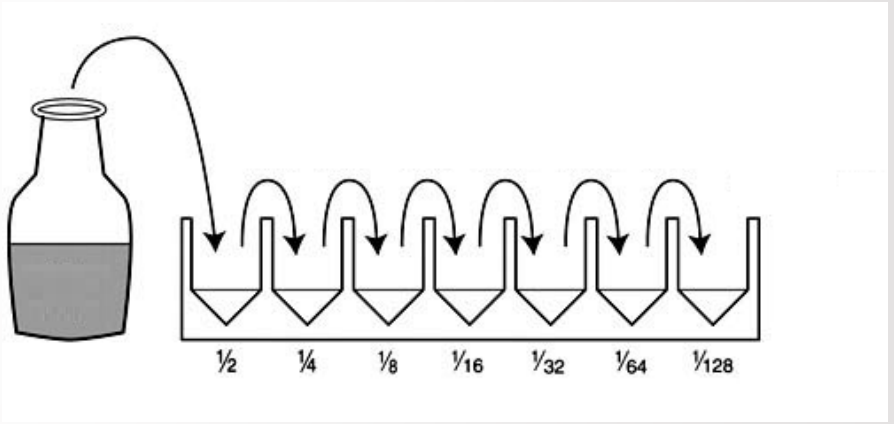
- MDS = $3\sqrt{DCR}$
- FoM = $\frac{\text{PDE}}{\text{MDS}}$

FoM VS Over Voltage



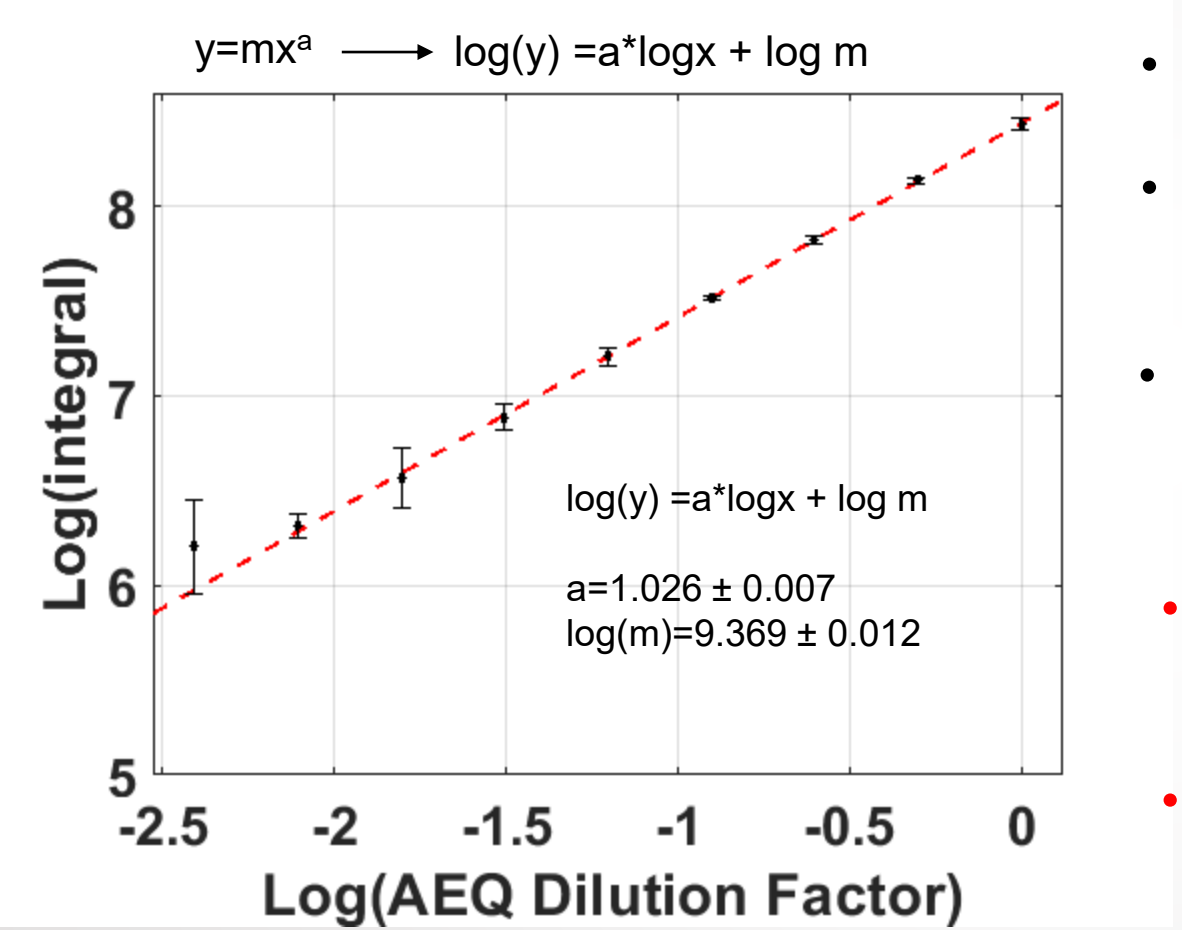
System linearity

- The linearity response is assessed using a cell lysate* obtained from cytosolic aequorin (cyt-AEQ)-transfected HeLa cells, i.e., namely cells engineered to produce Aequorin
- The aequorin concentration is diluted according to a geometric progression of common ratio 2 exploring a domain of ~3 orders of magnitude
- Aequorin was burned by injecting high concentrated Ca^{2+} solution, to ensure that all Aequorin binds to $Ca^{2+}$and emits light.



* A liquid containing suspended components of cells whose membrane has been previously destroyed

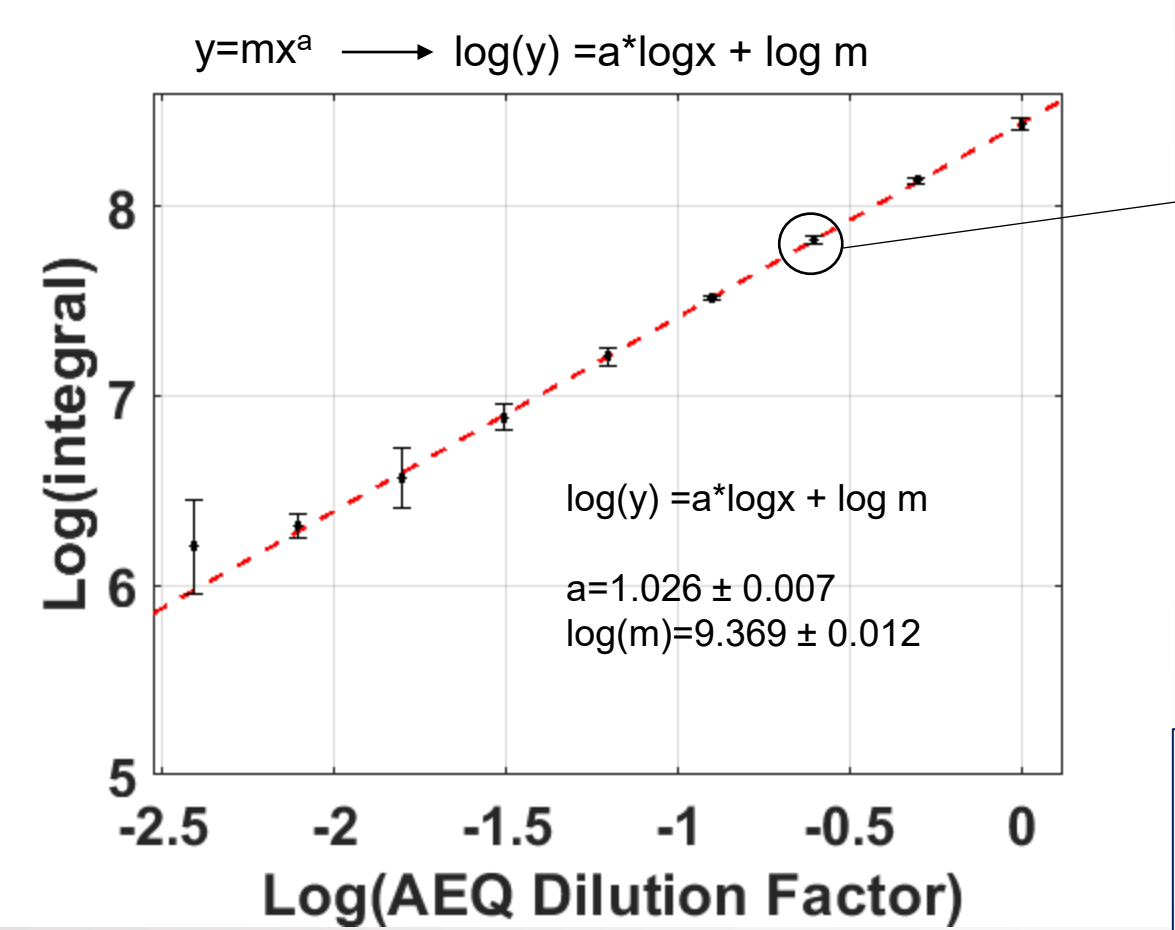
System linearity

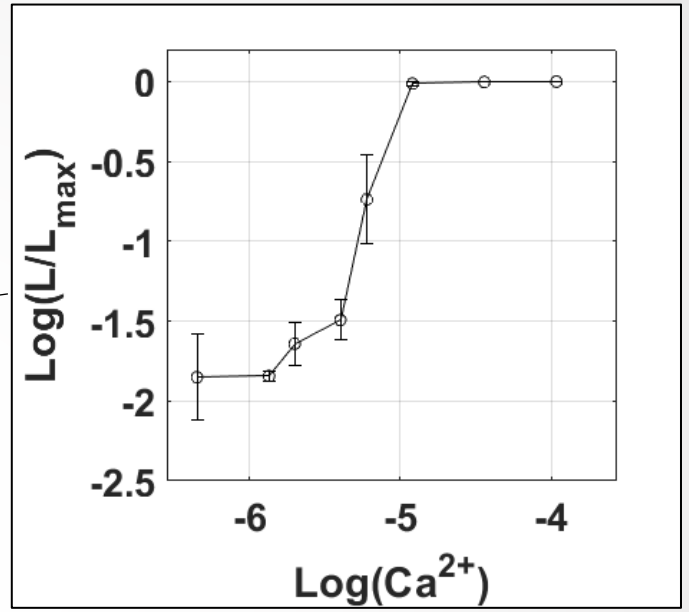


- Linearity range: (300 kHz-125 MHz)
- For a dilution factor <1/512, the signal of each SiPM is below the MDS (≈2 kHz)
- The upper limit could be further extended exploiting pile-up correction techniques

- **Linearity range in line with the typical rates of a luminometer (500 kHz – 5MHz)**
- **Upper limit extended by ≈ 25 times with respect to the single SiPM based system**
(Lomazzi *et al.*, *ACS Sens.* 2020, 5, 2388−2397 **)**

Building-up the calibration curve



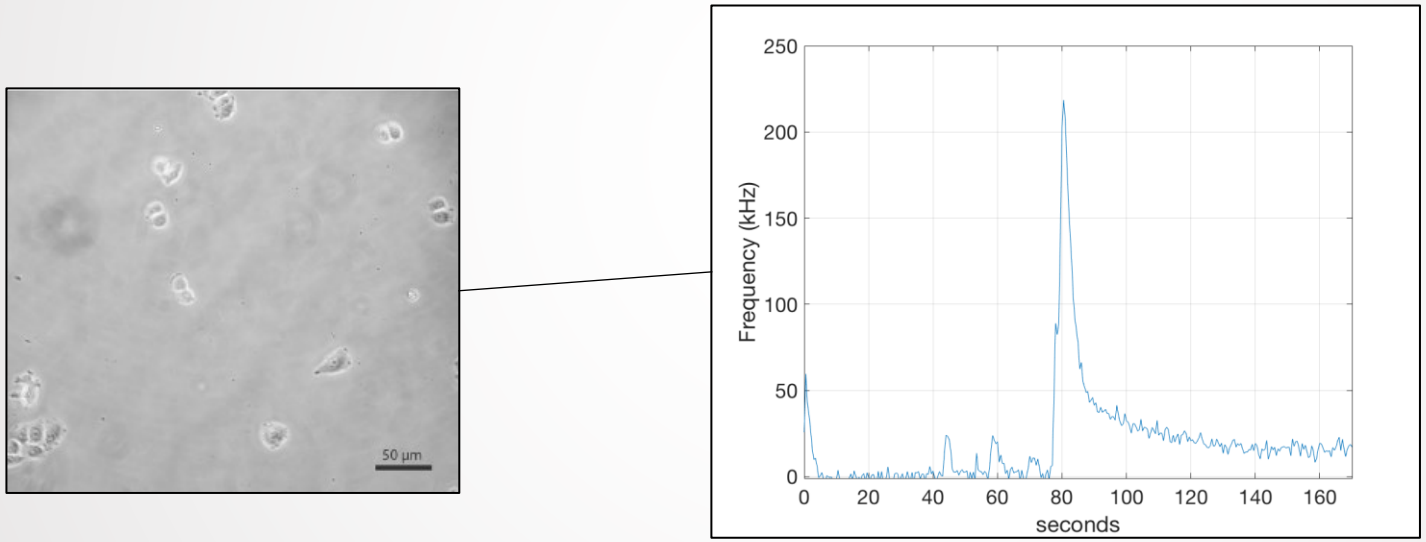


- Maximum variation of $Log(L/L_{max})$ Ca^{2+} concentration between 1-10 µM .

- Different types of engineered Aequorin allow to measure the calcium concentration in different ranges

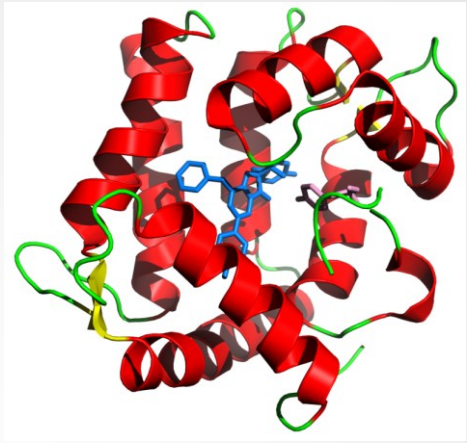
Future perspectives

We are already now to perform measurement on cells by exploiting our system!

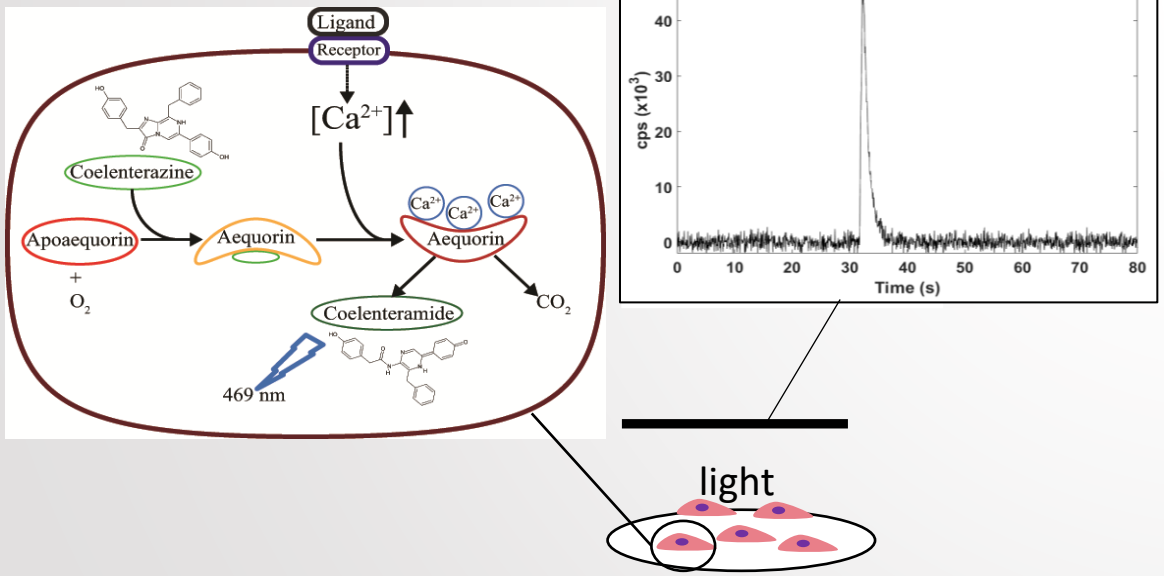


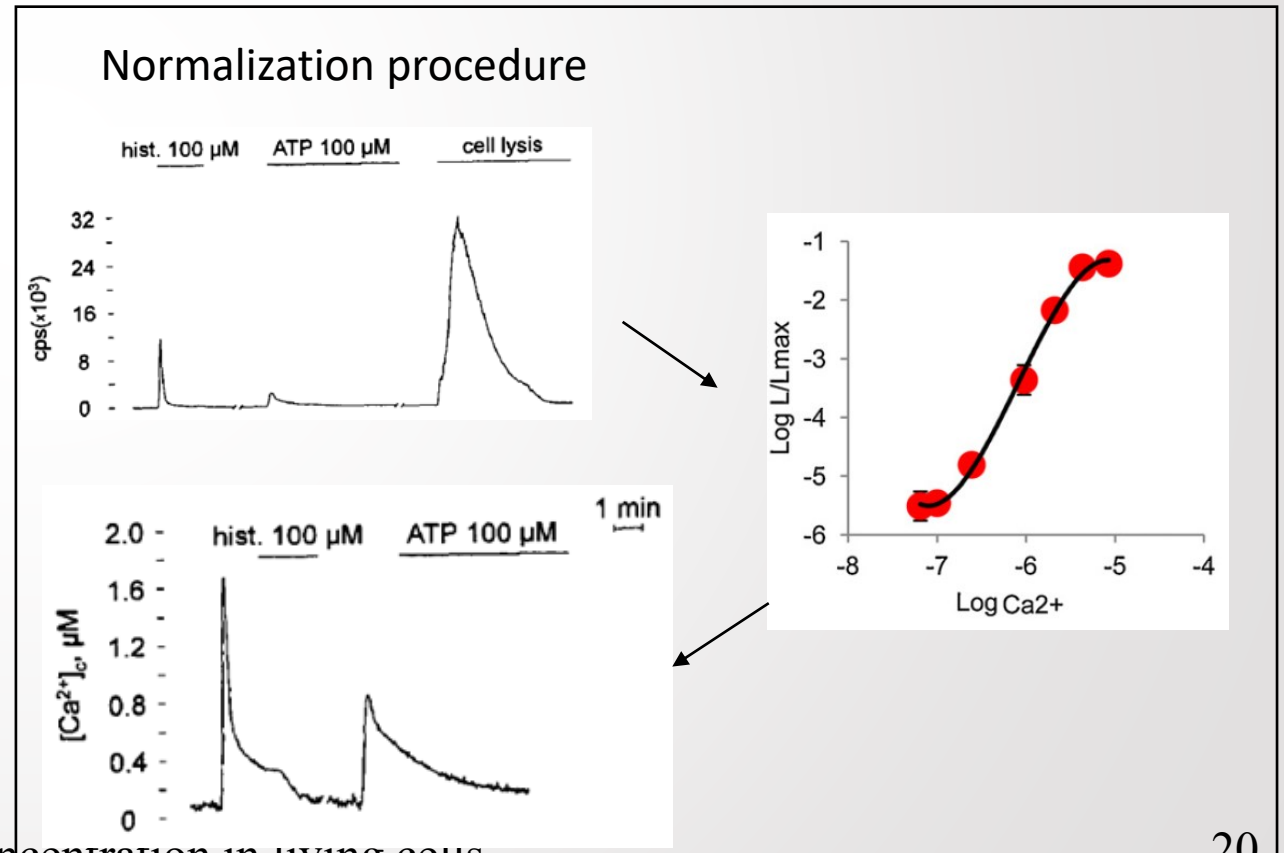

thank you

Aequorin: an useful bioluminescence sensor to measure calcium transients concentrations in living cells

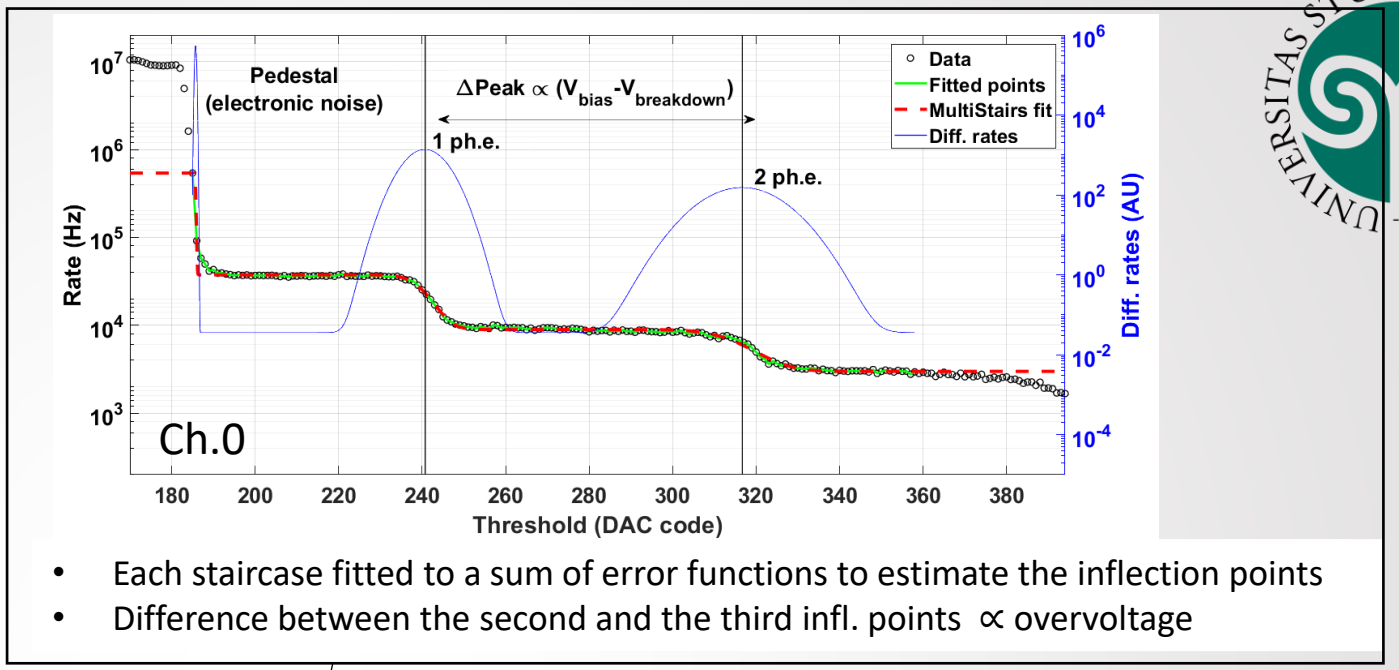


- Standard biotechnology methods for cellular Aequorin expression
- Wide dynamic range
- High signal-to-noise ratio
- Low Ca^{2+}-buffering effect

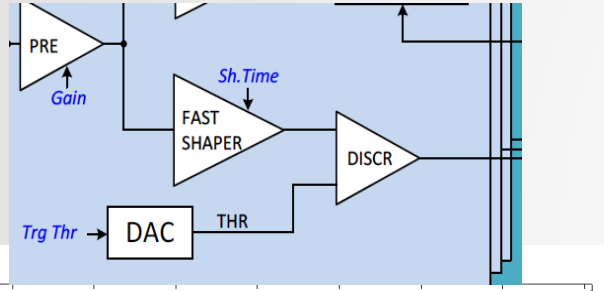


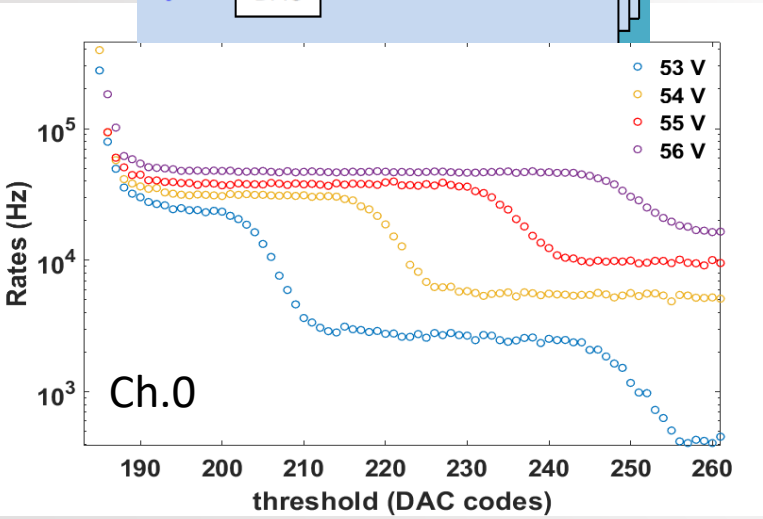


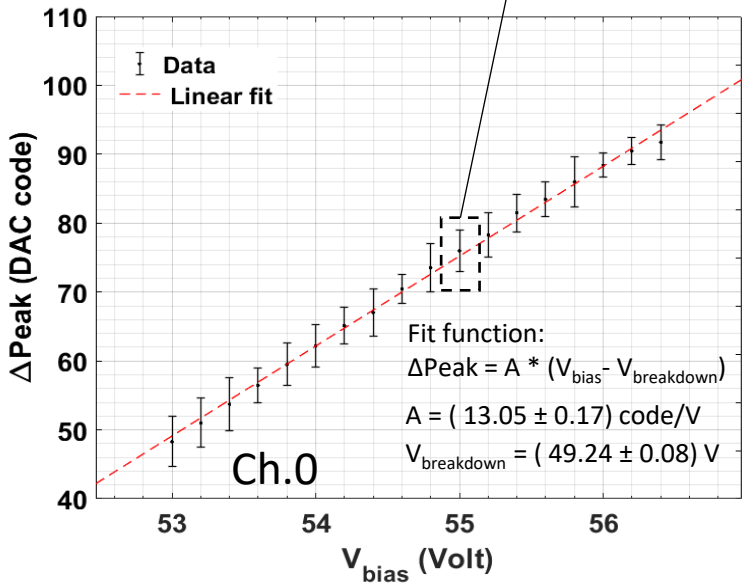
Matrix SiPMs breakdown voltage estimation from"Staircase" data at sensor T=15°C



- Each staircase fitted to a sum of error functions to estimate the inflection points
- Difference between the second and the third infl. points ∝ overvoltage

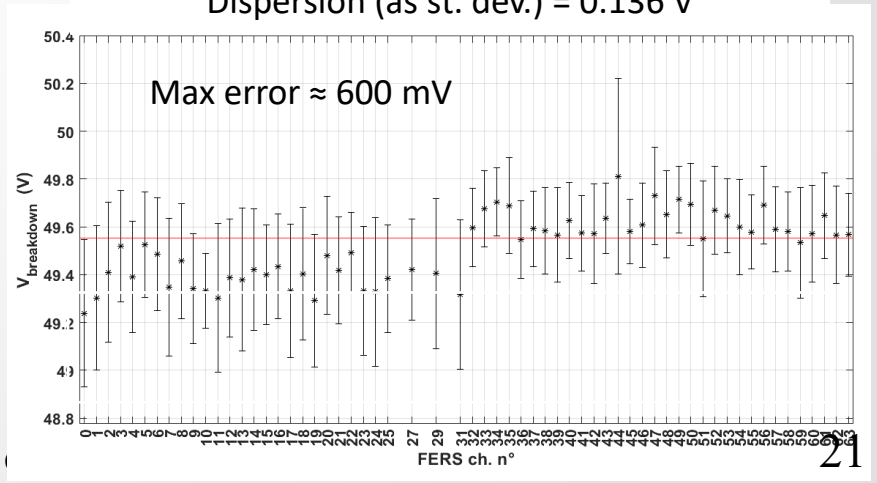






This procedure applied to all channels to estimate bias voltage for each SiPM of the matrix

Matrix Av. Breakdown Voltage = 49.511 V
Dispersion (as st. dev.) = 0.136 V



The calibration curve

The emitted light is proportional to bound Ca^{2+}. The calibration curve is required to extract the overall Ca^{2+}concentration from the detected light

- The calibration strategy
  - Starting with a fixed (unknown) dilution of AEQ, a series of known Ca^{2+} concentrations are injected in the sample (the 1st peak is referred to the detected light)
  - The second injection (high Ca^{2+} concentration) is required to burst all the remaining AEQ
  - 1st signal / total signal is only related to the Ca^{2+} concentration
  - The two injections are close in time to avoid unbinding kinetics between Ca^{2+} and AEQ