Data analysis for enhanced calcium ion mobilization in osteoblasts on amino group containing plasma polymer nanolayer (Python)

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Abstract: The following Jupyter notebook encodes parts of the data analysis that was done for the above mentioned article [4]. The idea of this notebook is to illustrate how Jupyter notebooks can be employed to make data analyses reproducible and interactive. To this end, the notebook first load and inspect the data visually, then defines the main functions to re-create figures and tables from the original article. Finally interactive notebook elements will be employed to allow further data exploration.

1 Introduction

The main purpose of the experiment and the data analysis at hand is to investigate the mechanisms of cells on a material surface in the cell-biosystem interactions. To this end, the influence of external factors of **material coatings** to **morphological** and **structural** characteristics of the surrounding tissue are explored in order to establish a design for new biofunctional implants, and thus improve the integration in the tissue.

Cells react directly or indirectly to their environment, such as physico-chemical properties of implant surfaces. It is, however, not yet fully understood how these external signals and stimuli are transmitted into the cell and are finally translated in a cell-specific way. A further step to understand the specific response of cells at the molecular level is the investigation of intracellular signaling cascades. An important "second messenger" in the cells are the calcium ions (Ca^{2+}) which regulate important cell signaling pathways. One option to analyze the mobilization of intracellular Ca^{2+} is Ca-imaging [3].

Briefly, the vital cells were stained with a calcium indicator (Fluo-3 acetoxymethyl ester). The Fluo-3 dye combined with the intracellular Ca^{2+} ions induces fluorescence that can be detected via confocal laser scanning microscopy (LSM780, Carl Zeiss). This fluorescence profile of emitted light upon laser excitation (488 nm) is then time-dependently recorded by 240 cycles á 2 s (time 480 s) (software Zenblack, Carl Zeiss). To activate the release of intracellular Ca^{2+} (from intracellular stores, the endoplasmic reticulum), adenosine 5'-triphosphate (ATP) was added even after the 90th cycle.

Previous cell biological studies have shown an improvement in initial cell attachment, spreading, and regulation of cells' physiology due to a chemically modified surface - a nanolayer of plasma polymerized allylamine with positive charge carriers (PPAAm) [2, 1]. In our reconstruction analysis, Ca^{2+} mobilization measurements were performed in human bone cells (osteoblasts) (i) for the cell line MG-63 [5] on titanium (Ti) substrates with PPAAm - Ti + PPAAm and bioactive collagen type-I - Ti + Col, bare Ti substrates - Ti; tissue culture plastic - IBIDI), as well as (ii) to establish the results for primary osteoblasts (HOB) compared to MG-63 cells on Ti+PPAAm vs. bare Ti. The study demonstrated that the positively charged PPAAm layer resulted in an improved global intracellular Ca^{2+} mobilization after adenosine 5'-triphosphate (ATP) stimulus in human osteoblasts [5].

Using the modular analysis-software (Zen blue, Zeiss) with the function "Mean ROI", a region of interest (ROI, same size) was placed in the cell, and the fluorescence intensity of the Ca^{2+} signal was analyzed in 10 cells. The measured fluorescence intensity is exported as tabular data to a Microsoft Excel file. The original publication used a manual process to ETL (extract, transform, load), which is time consuming and error prone, and analyzed the data with a graphical tool for data analysis. While these tools allow the effective and efficient statistical data analysis, they are generally not traceable as the analysis is mainly driven by point and click interaction.

The objective of this document is to reproduce parts of the data analysis of the original publication [4] in order to illustrate possible advantages with respect to traceability and reproduciblity. To this end, the Python programming language is used to resemble the original data analysis.

2 Preparation of the computational environment

The following code cell loads necessary packages that extend the functionality of the core Python 3 environment. The **xlrd** package provides functionality in order to load the proprietary file format of Microsoft Excel. It is used in combination with the **pandas** package that provides the data type *DataFrame* which is used for handling the data. The **numpy** and **scipy** packages are used for the statistical analysis. The **matplotlib** package is used in order to plot the data.

[1]:

```
%matplotlib inline

import xlrd
import numpy as np
import pandas as pd
from scipy import stats
import matplotlib.pyplot as plt
```

Beside loading neccessary packages, it is of interest to document the runtime environment including the version of all loaded packages and the active system configuration. This is done in the following by employing the watermark extension.

[2]:

```
%load_ext watermark
%watermark -i -v -h -m -iv -g -r -w
```

```
matplotlib 3.1.1
pandas     0.25.1
```

```
scipy      1.3.1
xlrd       1.2.0
numpy      1.17.2
2019-11-19T14:41:35+01:00

CPython 3.7.3
IPython 7.8.0

compiler   : GCC 8.3.0
system     : Linux
release    : 4.19.0-6-amd64
machine    : x86_64
processor  :
CPU cores  : 8
interpreter: 64bit
host name  : jupyter
Git hash   : 2bf7dd2fb63a829cb633aabaff69247a4aca9a07
Git repo   : git@gitlab.elaine.uni-rostock.de:INF/paper/P191115-Jupyter-MS.git
watermark 2.0.1
```

3 Define Helper Functions

3.1 Load Excel Files

As the data is distributed over different files, in the following we define functions to read a list of files and merge their content appropriately. For each Excel file in the list, the data is loaded, the first row is omitted and all columns that contain either the word `Time` or the word `IntensityMean` in the column name are selected. Then the names are replaced and the data are merged by row index.

[3]:
```
def read_measurement_files(filenames: list, type_name: str):
    data = None
    for idx, file in enumerate(filenames):
        df = pd.read_excel(file, skiprows=[1])
        # select only columns with Time and IntensityMean
        df = df[df.columns[df.columns.to_series().str.
 ↪contains('Time|IntensityMean')]]
        df.columns = ['Time'] + ['R%i_%i' % (colidx, idx+1) for colidx in␣
 ↪range(1, len(df.columns))]
        data = df if idx == 0 else pd.concat([data, df.loc[:, df.columns !=␣
 ↪'Time']], axis=1)
    data['Type'] = type_name
    return data
```

3.2 Summarize the Measurement Data

In order to provide a generalized statement for each cycle, for each row, the data is summarized by mean value and the standard error of the mean.

[4]:

```
def summarize_measurement(data: pd.DataFrame):
    selection = data.drop(['Time','Type'], axis=1)
    data['mean'] = selection.mean(axis=1)
    data['sem'] = selection.sem(axis=1)
    return(data)
```

3.3 Plot Measurement Data

To plot the data, first it is transformed from wide (different measurements of the same variable are represented by columns) to long format, where all measurement data, independent from the repetition is represented in the same column. For both the original data and the row wise summary the data is then grouped by the surface type (variable Type) and plotted. For the row wise summary the mean is provided by a line and the standard error of the mean by a colored region around the mean.

[5]:

```
def plot_measurement_all(data: pd.DataFrame):
    selection = data.drop(['Type','mean','sem'], axis=1)
    twothirds = selection.drop(['Time'], axis=1).max().max() * 2/3
    selection.plot.line(x='Time', legend=False, marker='.')
    plt.vlines(3, ymin = 0, ymax = twothirds, linestyles='dotted')
    plt.text(x=3, y=twothirds+5, s='ATP')
    plt.xlabel('Time [min]')
    plt.ylabel('Mean fluoresence intensity')

def plot_measurement_summary(data: pd.DataFrame):
    selection = data[['Time', 'Type','mean','sem']]
    maxMean = selection['mean'].max()

    for t in selection.Type.unique():
        sel = selection[selection.Type == t]
        plt.plot(sel['Time'], sel['mean'])
        plt.fill_between(sel['Time'], sel['mean']-sel['sem'],␣
 ↪sel['mean']+sel['sem'],alpha=0.1)
    plt.vlines(3, ymin = 0, ymax = maxMean, linestyles='dotted')
    plt.text(x=3, y=maxMean+5, s='ATP')
    plt.xlabel('Time [min]')
    plt.ylabel('Mean fluoresence intensity')
    plt.legend(title='Type', labels=selection.Type.unique())
```

4 Load and Inspect Measurement Data

Four different conditions for the surface have been investigated: * Bare titanium substrate (`Ti`) * Titanium substrate modified by amino functionalization with plasma polymerized allylamine (`Ti+PPAAm`) * Titanium substrate modified by immobilization of a bioactive collagen type-I layer (`Ti+Col`) * Standard tissue culture plastic (`IBIDI`)

For each of the conditions, three different samples were taken. For each of the samples, the global Ca^{2+} fluorescence signal from individual cells was measured and recorded by use of the ZEN software (ZEISS efficient Navigation, ZEN 2011 SP4, black edition). This was done for 240 cycles with a duration of 2s per cycle. After the 90th cycle ATP was added in order to stimulate the cells' endoplasmic reticula. For each such time series, at least ten cells were observed for the entire time span and the mean fluorescence intensity was determined. This procedure yielded in three data frames with 240 measurements per cell and at least ten measurements per cycle.

The data was then converted into a table structure and stored in three different Excel files per condition. In the following the filenames are provided accordingly.

[6]:
```
ppaam_fn = ["../_Data/Fig. 5_MG63_19.07.2016_PPAAm_n1.xlsx",
            "../_Data/Fig. 5_MG63_19.07.2016_PPAAm_n2.xlsx",
            "../_Data/Fig. 5_MG63_19.07.2016_PPAAm_n3.xlsx"]

ti_fn = ["../_Data/Fig. 5_MG63_19.07.2016_Ti_n1.xlsx",
         "../_Data/Fig. 5_MG63_19.07.2016_Ti_n2.xlsx",
         "../_Data/Fig. 5_MG63_19.07.2016_Ti_n3.xlsx"]

ticol_fn = ["../_Data/Fig. 5_MG63_Col_n1.xlsx",
            "../_Data/Fig. 5_MG63_Col_n2.xlsx",
            "../_Data/Fig. 5_MG63_Col_n3.xlsx"]

ibidi_fn = ["../_Data/Fig. 5_MG63_IBIDI_n1.xlsx",
            "../_Data/Fig. 5_MG63_IBIDI_n2.xlsx",
            "../_Data/Fig. 5_MG63_IBIDI_n3.xlsx"]
```

4.1 Titanium surface

Load the base measurement data for the Ti surface and provide a preview to the first rows of the dataset.

[7]:
```
ti = read_measurement_files(ti_fn, 'Ti')
ti_summary = summarize_measurement(ti)
ti.head(n=6)
```

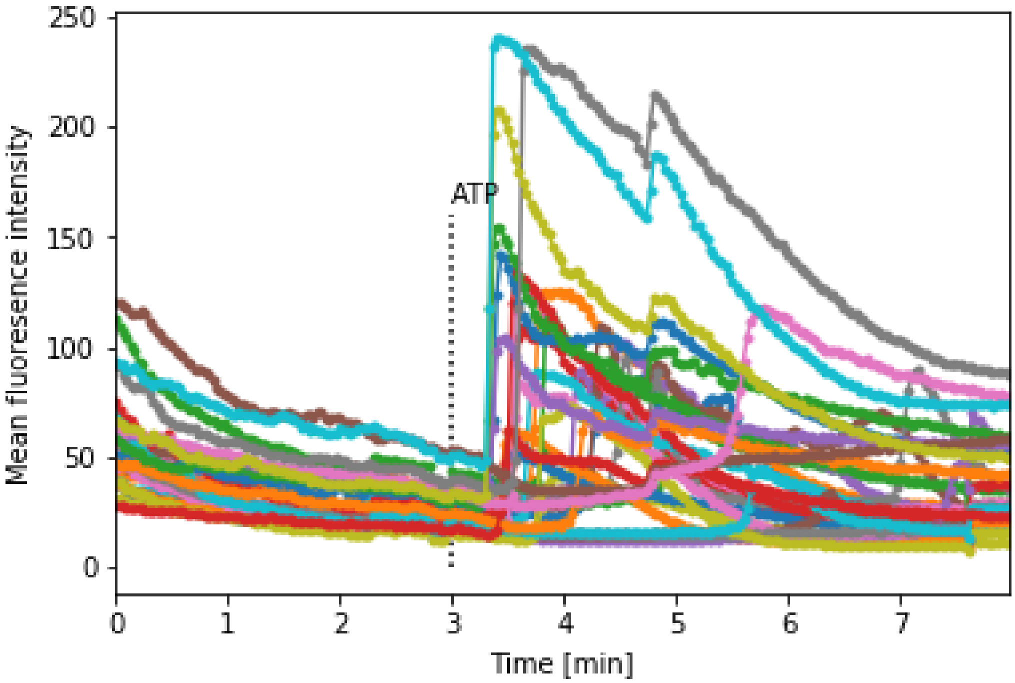
[7]:
```
       Time       R1_1       R2_1       R3_1       R4_1       R5_1       R6_1  \
0  0.000000  46.724508  41.204120  61.086799  49.620561  46.633628  56.501838
1  0.033333  47.425760  41.162921  59.473779  49.358879  46.361062  55.518382
2  0.066683  47.516995  40.814607  58.828210  48.740187  47.511504  55.996324
```

```
3  0.100050  46.649374  41.205993  57.909584  48.957009  47.660177  55.799632
4  0.133367  46.973166  41.400749  56.844485  48.800000  46.851327  54.345588
5  0.166733  46.069767  40.949438  56.065099  49.345794  46.723894  55.163603

        R7_1       R8_1       R9_1  …       R4_3       R5_3        R6_3  \
0  53.880435  47.160221  31.318426  …  28.382138  64.847222  120.590909
1  52.876812  46.856354  31.128801  …  27.253294  64.269444  120.114370
2  51.094203  47.075506  31.187835  …  27.412884  62.806944  119.005865
3  50.663043  47.572744  30.899821  …  27.120059  61.900000  117.187683
4  50.378623  47.430939  30.572451  …  26.459736  60.809722  115.790323
5  49.932971  47.889503  29.568873  …  26.931186  59.987500  115.285924

        R7_3       R8_3       R9_3      R10_3 Type       mean       sem
0  63.167647  92.656695  66.343658  92.756757   Ti  57.753450  4.062095
1  62.911765  91.414530  65.308260  92.302987   Ti  56.870557  3.974362
2  62.054412  87.141026  63.859882  91.891892   Ti  55.962700  3.877056
3  61.683824  85.226496  63.205015  90.017070   Ti  55.249254  3.781677
4  62.110294  82.109687  62.874631  89.513514   Ti  54.454818  3.691801
5  61.457353  81.566952  63.485251  90.816501   Ti  53.805348  3.660207

[6 rows x 34 columns]
```

Plot the individual time series for the Ti surface. The addition of ATP is marked by a dotted line after 90 cycles.

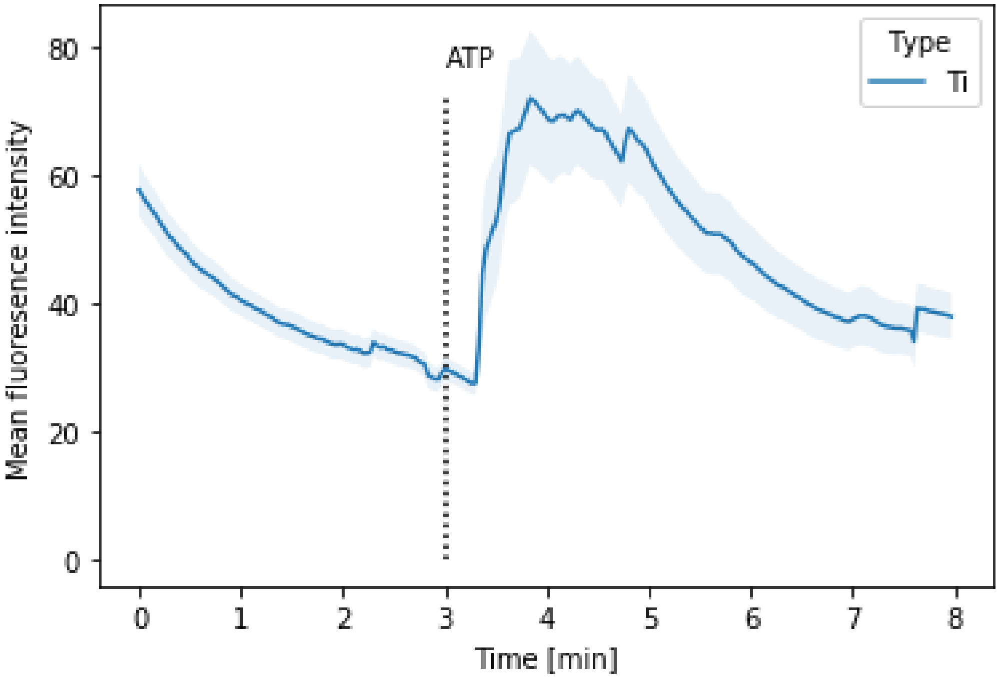
[8]:

```
plot_measurement_all(ti)
```



Plot the mean and the standard error of the mean for the time series of the Ti surface. Again, the dotted line represents the time of ATP addition. From the plot it can be observed that after providing ATP, the mean fluorescence in the observed cells increases. As the actual amount of the increase varies for the different cells, it is of interest to investigate the mean values of all cells.

```
[9]: plot_measurement_summary(ti_summary)
```

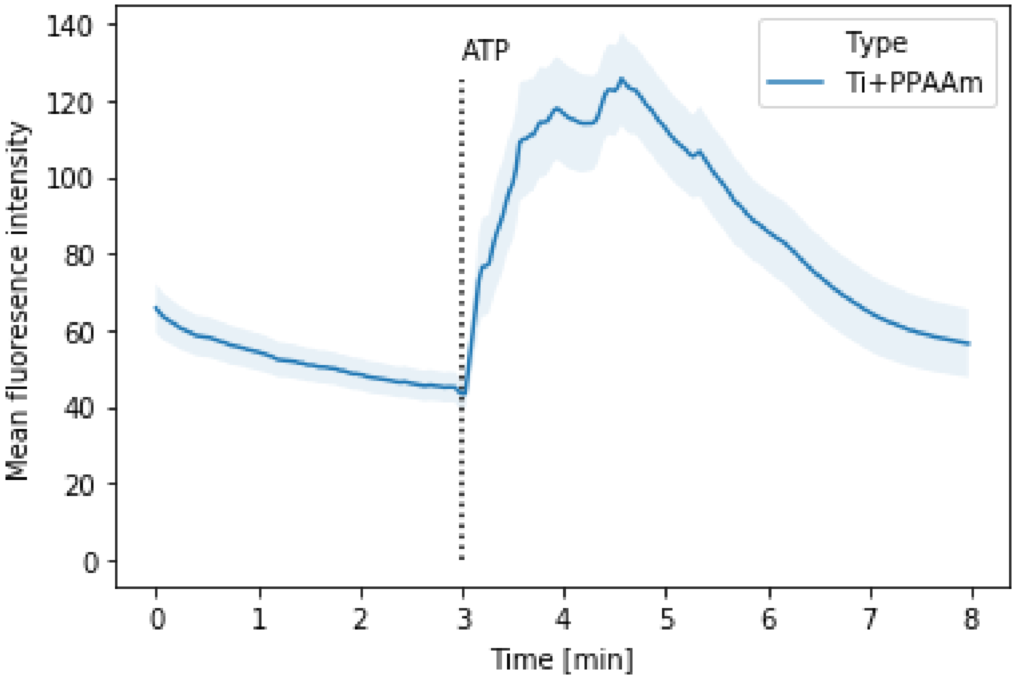


Again, it can be observed that the mean fluorescence increases shortly after providing the ATP. The amount of actual increase differs as can be seen from the standard error of the mean in the time between three and five minutes. After 5 minutes, the mean increase of the intensity decreases steadily.

4.2 Plasma polymerized allylamine modified Ti surface

When considering the Ti+PPAAm modified surface, a similar, but more distinct increase is expected.

```
[10]: ppaam = read_measurement_files(ppaam_fn, 'Ti+PPAAm')
      ppaam_summary = summarize_measurement(ppaam)

      plot_measurement_summary(ppaam_summary)
```



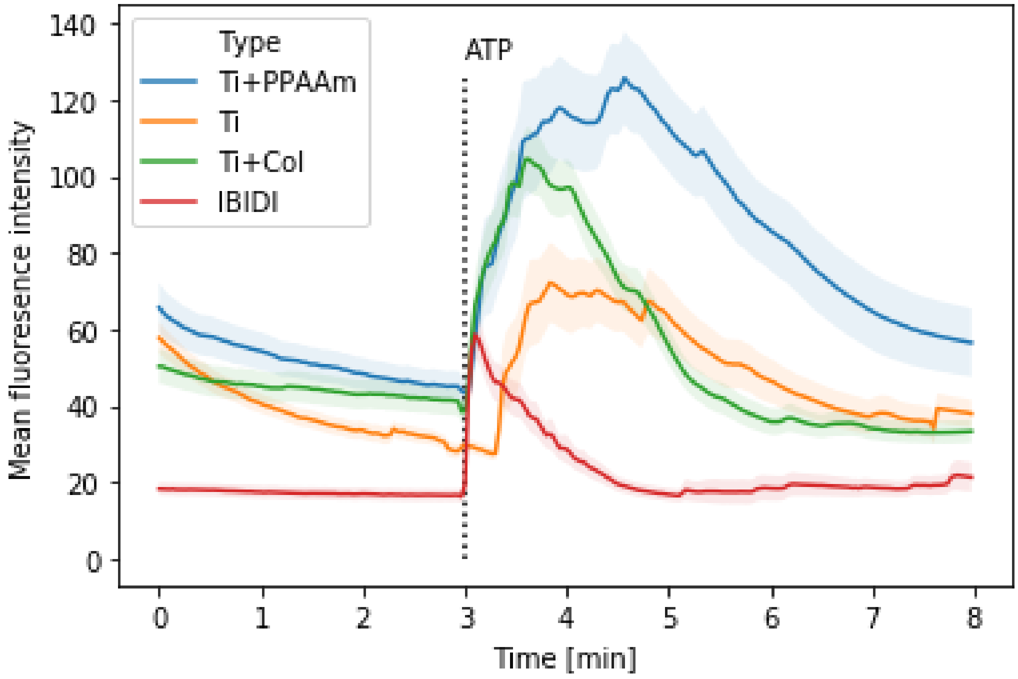
From the above plot, it can be observed that the increase of the mean fluorescence intensity increases directly after providing the ATP. In addition the actual amount of increase is much higher than for the bare Ti surface.

4.3 Comparing the different conditions

By comparing the different conditions, the difference in the reaction of the cells on the differently modified surfaces is more distinct.

To this end, we first load the both remaining datasets and create a new data frame from all four measurement tables. In the direct comparison, it can be observed that the cells on all surfaces, except for the bare Ti surface, directly react on the ATP addition. Also, it can be observed that the reaction of the both, `Ti+PPAAm` and `Ti+Col` are more intensive.

[11]:
```
# read measurement data for Ti collagen surface
ticol = read_measurement_files(ticol_fn, 'Ti+Col')
ticol_summary = summarize_measurement(ticol)

# read measurement data for the tissue culture plastic
ibidi = read_measurement_files(ibidi_fn, 'IBIDI')
ibidi_summary = summarize_measurement(ibidi)

# merge all measurements to the same table
all_data = pd.concat([
    ppaam_summary[['Time', 'Type', 'mean', 'sem']],
    ti_summary[['Time', 'Type', 'mean', 'sem']],
    ticol_summary[['Time', 'Type', 'mean', 'sem']],
    ibidi_summary[['Time', 'Type', 'mean', 'sem']]
], ignore_index=True)
plot_measurement_summary(all_data)
```



In order to determine the difference of the mean fluorescence intensity of the different conditions before and after ATP provision, in the following a table is created that provides the mean and the standard error of the mean for all measurements. First, the raw data for all conditions is loaded, transformed from wide (one column per cell time series) to long format (all measurement in one column). The variable `Region` is then omitted and the data is grouped into data **before** and **after** ATP provision (variable `CaSignal`). Then, the mean and standard error of the mean is calculated for each combination of surface (variable `Type`) and `CaSignal` and transformed into a table.

From the table, it can be observed that, indeed, the mean fluorescence intensity when using Ti+PPAAm surface increased much.

[12]:
```
summary_data = pd.concat([
    ppaam.drop(['mean', 'sem'], axis=1),
    ti.drop(['mean', 'sem'], axis=1),
    ticol.drop(['mean', 'sem'], axis=1),
    ibidi.drop(['mean', 'sem'], axis=1)
], ignore_index=True)
summary_data.loc[all_data['Time'] < 180/60, 'CaSignal'] = "Basal Level"
summary_data.loc[all_data['Time'] >= 180/60, 'CaSignal'] = "After ATP"
```

```
summary_data_melt = pd.melt(summary_data, id_vars=['Time', 'Type', 'CaSignal'])
summary_data_melt = summary_data_melt.drop('variable', axis=1).groupby(['Type',␣
 ↪'CaSignal']).agg(
    mean=pd.NamedAgg(column='value', aggfunc=np.mean),
    sem=pd.NamedAgg(column='value', aggfunc=stats.sem)
).T

statistics = pd.DataFrame()
statistics.index.name = 'CaSignal'
for cols in summary_data_melt.columns.values:
    statistics.loc[cols[1], cols[0]] = "%.1f±%.1f" % (
        np.round(summary_data_melt.loc['mean', cols], decimals=1),
        np.round(summary_data_melt.loc['sem', cols], decimals=1),
    )
statistics[['Ti', 'Ti+PPAAm', 'Ti+Col', 'IBIDI']].sort_index(ascending=False)
```

```
[12]:                    Ti   Ti+PPAAm    Ti+Col     IBIDI
      CaSignal
      Basal Level  38.8±0.3   52.0±0.5  44.5±0.4  17.2±0.2
      After ATP    49.9±0.6   89.1±1.0  55.2±0.6  23.6±0.3
```

5 Summary

The above analysis demonstrated how biological and medical data analyses can be performed in a reproducible and comprehensible manner by the use of Jupyter Notebooks. While the documentation was written using Markdown and Latex, the data analysis itself was written using the Python programming language. As this Notebook contains the original results and is at the same time executable, it can be (1) easily re-executed, (2) changed, or (3) extended to other experiments.

6 Acknowledgements

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References

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