CFAssay: Statistics of the Colony Formation Assay

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October 17, 2016

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Contents

1 Overview

The functions in this package provide tools for statistical analysis along with the colony formation assay (CFA) (**?**). These allow fitting of the linear-quadratic (LQ) model for ionizing radiation dependent cell survival curves and ANOVA (analysis of variance) for experimental two-way designs with one dose level of a treatment factor. Maximum-Likelihood (ML) based methods are preferred because, theoretically, parameter estimations of ML for Poisson distributed data come with smaller variances compared to other methods. However, for the sake of comparability also simple least squares (LS) based methods can be optionally used. The ML based methods employ the R-function `glm` for generalised linear modelling, while LS based methods use the R-function `lm`. The functions provided by CFAssay intend to simplify and specialize the general use of that R-functions. The underlying distribution for the ML based methods is Poisson (**?**) and modelling is performed using the link function "log", i.e. cell survival curves are logarithmically linear with "linear" parameters α (per dose unit) and β (per squared-dose unit). In the two-way ANOVA model the dependency of treatment factors is considered as logarithmically additive. Output summaries are adapted from the `glm` or the `lm` functions and use the terminology of quantities in the CFA. An accompanying paper is published in Radiation Oncology (**?**).

1.1 The models

Cell survival S as a function of radiation dose using the so called LQ-model is given by

$$S = S(D) = e^{c+\alpha D+\beta D^2} \tag{1}$$

D is the radiation dose and named `dose` in the code. D^2 is named `dose2` but has not to be set by the user. Coefficients α and β appear as "alpha" and "beta" in the print function of CFAssay. The intercepts $c = log(S0)$ represent the logarithmic plating efficiencies, i.e. surviving fractions of untreated cells in replicated experiments. They correspond to variable

`Exp` which is treated as a factor. Due to the positive formulation (1), parameters c, α and β take negative values in the fit results.

The logarithmically additive 2-way ANOVA with two levels for each of the two factors can mathematically be formulated as

$$S = e^{c+Ax_1+Bx_2+Dx_1x_2} \tag{2}$$

or as nested parametrization

$$S = e^{c+Ax_1+B_0x_2+(B_1-B_0)x_1x_2} \tag{3}$$

There x_1, x_2 take level values 0 or 1 for each of the two factors A or B, where for e.g. 0 means untreated and 1 means treated. D is the factor for potential interaction and is coded as `A:B` in R. In the second, nested parametrization B_0 is the effect of treatment in control cells and B_1 the treatment effect after after applying A. The interaction D is then the difference between B_1 and B_0. In the function `cfa.2way` of CFAssay we use per default the nested version, coded as `A/B` (see "An Introduction to R", Chapter 11 Statistical models in R). c represents again the logarithmic plating efficiencies for each experiment.

1.2 Remark on intercepts c and plating efficiencies

By default CFAssay processes plating efficiencies (PE) from replicate experiments as model parameters, i.e. as intercepts c, controlled by setting the option parameter `PEmethod` to `"fit"`. From a statistical point of view, this appears to be preferable, because likewise the colony counts from treated cells the data from untreated cells are random observations. The shape parameters α and β can be viewed as averaged over the experiments. The conventional normalization method (`PEmethod = "fix"`) that is applied on experimental replicate PE measurements is mathematically equivalent to forcing the mean curve of the data to go through the intercept of each particular replicate curve. In the case of somewhat increased variation between the shape parameters of different experiments, conventional normalization results in a larger dispersion parameter in combination with the ML method. In this case, fitted intercepts c (named PE in the printed output) may deviate from the measured PEs, however, they result in better overall statistics. On the shape parameter values itself, it has little influence. Thus, it is more a matter of scale, what can be visualized in the diagnostic plots using the function `plotExp`.

2 Example: Linear-quadratic model for cell survival curves

The data file contains data sets on irradiation experiments of the two cell lines CAL 33 (**?**) and OKF6T/TERT1. The data set on CAL 33 comprises 4 repeated experiments, and that of OKF6T/TERT1 8 experiments. The workflow shown here is divided in the following three steps: 1. data input and double-check, 2. calculation of cell survival curves for each of the two cell lines separately, 3. comparison test of the curves for the two experiments

2.1 Data input and double-check

First we load the library and read the data into the memory. The data file, expl1_cellsurvcurves.txt, is an unformatted tab-delimited text file and contains the data from the two irradiation experiments.

```
> library(CFAssay)
> datatab <- read.table(system.file("doc", "expl1_cellsurvcurves.txt",
+                                   package="CFAssay"), header=TRUE, sep="\t")
```

The data file contains columns with header names `cline, Exp, dose, ncells and ncolonies`. `cline` distinguishes the curves in the data frame. `Exp` discriminates replicates within each curve. The `dose` column relates to the applied radiation dose, `ncells` to the number of cells seeded and `ncolonies` to the number of counted colonies. The last four names are required by the CFAssay function. The name of the first column (here `"cline"`) is arbitrary and additional columns for the distinction of curves may be contained in the data frame, e.g. pre-treatment.

```
> names(datatab)

[1] "cline"     "Exp"       "dose"      "ncells"    "ncolonies"

> head(datatab, 3)  # First 3 lines

  cline Exp dose ncells ncolonies
1 cal33  e1    0    900       182
2 cal33  e1    1   1800       284
3 cal33  e1    2   3000       323
```

It is advisable to double-check the number of rows, columns and frequencies or cross frequencies of the data with the R functions `dim` and `table`. The output is not shown here.

```
> dim(datatab)
> table(datatab$cline)
> table(datatab$cline, datatab$Exp)
> table(datatab$cline, datatab$dose)
```

2.2 Calculation of cell survival curves

With the CFAssay function `cellsurvLQfit` we calculate the parameters of a linear-quadratic cell survival curve along with quality or goodness-of-fit statistics. For that purpose the data frame `datatab` has to be filtered for data relating to one curve only, because the variable `cline` is ignored by the fit function. With the function `print` the result is shown in three tables, the coefficient table, the observed and fitted plating efficiencies table and a table for analysis of the residual sum of weighted squares in the replicate experiments. In the coefficient table the "t value" column represents values of the t-test against zero of the estimated coefficients ("Estimate") and column "Pr(>|t|)" contains the corresponding p-values. By default the maximum-likelihood method is used and plating efficiencies are fitted as intercepts. Other options can be chosen in the argument list of `cellsurvLQfit` as explained in the help document.

```
>   X <- subset(datatab, cline=="okf6TERT1")
>   dim(X)

[1] 48  5
```

```
>   fit <- cellsurvLQfit(X)

method = ml
PEmethod = fit
       dose       dose2
-0.51937898 -0.02102614
Use 'print' to see detailed results

>   print(fit)

*** Coefficients of LQ-model for cell survival ***
method = ml
PEmethod = fit

Logarithmic plating efficiencies PE fitted as intercepts
see remark in the manual, 1.2
      Estimate Std. Error   t value     Pr(>|t|)
PEe1 -1.606686  0.1061229 -15.13986 1.112338e-17
PEe2 -1.693346  0.1090082 -15.53412 4.781145e-18
PEe3 -2.010377  0.1228551 -16.36380 8.506670e-19
PEe4 -1.869228  0.1165115 -16.04329 1.644185e-18
PEe5 -2.052405  0.1069872 -19.18365 3.828727e-21
PEe6 -2.219654  0.1333357 -16.64711 4.789596e-19
PEe7 -2.434634  0.1455642 -16.72550 4.091214e-19
PEe8 -2.109080  0.1258530 -16.75828 3.830883e-19

Shape parameters alpha and beta
         Estimate Std. Error   t value     Pr(>|t|)
alpha -0.51937898 0.05889260 -8.819088 9.943331e-11
beta  -0.02102614 0.00978985 -2.147749 3.817362e-02

Observed and fitted plating efficiencies (%):
     Experiment   PE PEfitted
PEe1         e1 19.0     20.1
PEe2         e2 17.3     18.4
PEe3         e3 10.0     13.4
PEe4         e4 15.0     15.4
PEe5         e5  9.2     12.8
PEe6         e6 17.0     10.9
PEe7         e7 14.3      8.8
PEe8         e8 11.5     12.1

Residual Deviance: 167.8964
Total residual sum of weighted squares rsswTot: 164.8109
Residual Degrees of Freedom: 38
Dispersion parameter: 4.337128
```

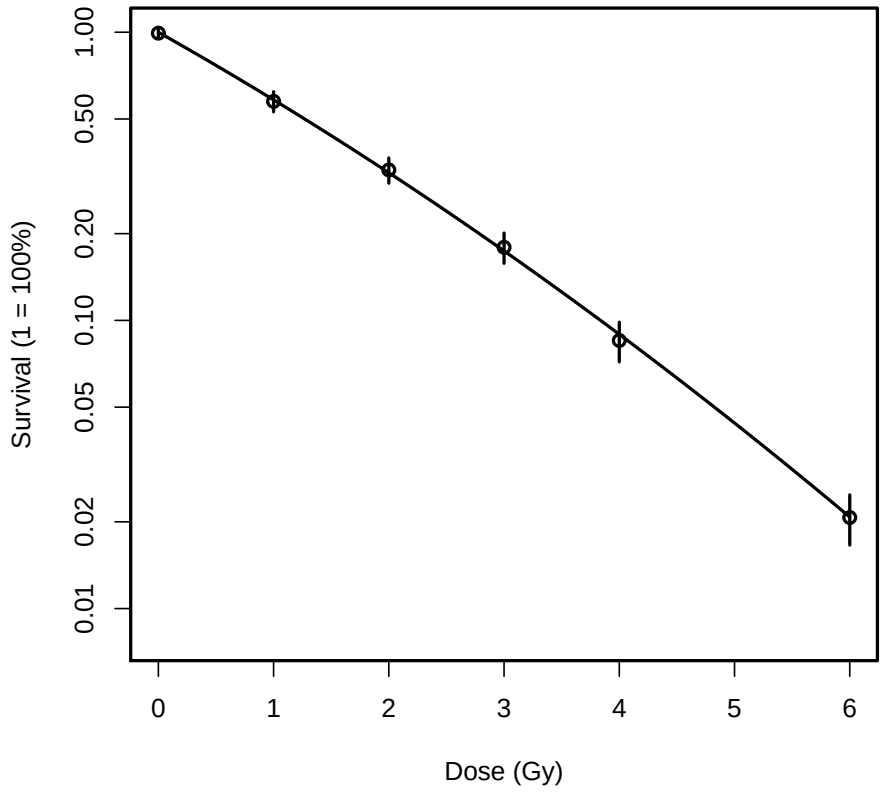
```
Fraction rssw of rsswTot per Experiment
  Experiment  rssw perCent
1         e1  4.84     2.9
2         e2 14.91     9.0
3         e3  9.01     5.5
4         e4  4.51     2.7
5         e5 34.77    21.1
6         e6 52.22    31.7
7         e7 41.09    24.9
8         e8  3.46     2.1
```

If the dispersion parameter is high, experimental data may have to be removed or replaced. An appropriate cut-off depends on experience and may vary between different labs. For the example data, where plating efficiencies were fitted, we recommend a cut-off of 9.0, which corresponds to 3 Poisson standard deviations. With fixed plating efficiencies a cut-off of 12.0 may be appropriate. For the pure Poisson distribution the expected value of the dispersion parameter is 1.0.

A plot of the mean curve is generated with `plot`. Values of plotted mean survival fractions and error bars are shown with functions `sfpmean` and `pes`.

```
>   plot(fit)
>   S0 <- pes(X)$S0
>   names(S0) <- pes(X)$Exp
>   sfpmean(X, S0)
```

```
           dose_0     dose_1     dose_2     dose_3     dose_4      dose_6
SF    0.99275669 0.57563672 0.33274302 0.17935702 0.08517535 0.020704208
stdev 0.03731008 0.04618487 0.03341709 0.02160074 0.01340983 0.004103207
```



With `plotExp` diagnostic plots for each experiment are generated. Here we plot them into a pdf-file.

```
>   pdf("okf6TERT1_experimental_plots.pdf")
>           plotExp(fit)
>   dev.off()
```

The procedure is repeated for the other cell line, "cal33". The result is not shown here.

```
>   X <- subset(datatab, cline=="cal33")
>   dim(X)
>   fit <- cellsurvLQfit(X)
>   print(fit)
>   plot(fit)
>   plotExp(fit)
```

2.3 Comparison of the two cell survival curves

The two linear-quadratic cell survival curves are compared with the CFAssay function `cellsurvLQdiff`. The required argument `curvevar` is set to `"cline"`, which is the name of the column in `datatab` which distinguishes the two curves to be compared. The function uses an ANOVA test for comparison of two model fits. In "Model 1", which corresponds to the

Null-hypothesis, the dose coefficient (alpha) and the dose-squared coefficient (beta) are independent of the two curves. In "Model 2" the coefficients are different. Detailed results are printed with function `print`.

```
>   fitcomp <- cellsurvLQdiff(datatab, curvevar="cline")

*** Overall comparison for two linear-quadratic cell survival curves ***
Compared curves: cal33 okf6TERT1
method: ml
PEmethod: fit

Test used: F-test
            F   Pr(>F)
values 7.3509 0.001463 **
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
Use 'print' to see detailed results

>   print(fitcomp)

Overall comparison test for coefficients alpha and beta of LQ-models
=====================================================================
method = ml
PEmethod = fit

12 PEs fitted as intercepts. To look at, use simple R print function.
Null hypothesis (Model 1): one set of shape parameters alpha and beta for all data
-------------------------------------------------------------------------------
         Estimate  Std. Error   t value     Pr(>|t|)
alpha -0.39893900 0.047865098 -8.334653 1.687495e-11
beta  -0.03434671 0.007828292 -4.387510 4.906364e-05

Goodness-of-fit values
        Residual Deviance: 317.2604
        Total sum of squared weighted residuals rsswTot: 319.7611
        Residual Degrees of Freedom: 58
        Dispersion parameter: 5.513123

Alternative hypothesis (Model 2): two sets of shape parameters alpha and beta
-------------------------------------------------------------------------------
                          Estimate  Std. Error   t value     Pr(>|t|)
alpha:curvescal33     -0.26831918 0.062588637 -4.287027 7.206618e-05
alpha:curvesokf6TERT1 -0.51937898 0.059669207 -8.704305 5.439622e-12
beta:curvescal33      -0.04892355 0.010058934 -4.863691 9.739225e-06
beta:curvesokf6TERT1  -0.02102614 0.009918948 -2.119795 3.846873e-02

Goodness-of-fit values
        Residual Deviance: 251.804
```

```
        Total sum of squared weighted residuals rsswTot: 249.3271
        Residual Degrees of Freedom: 56
        Dispersion parameter: 4.452269

Analysis of Variance Table and F-test
Model 2 versus Model 1
  Resid. Df Resid. Dev Df Deviance      F   Pr(>F)
1        58     317.26
2        56     251.80  2   65.456 7.3509 0.001463 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

The two curves are plotted with different colors in one plot, using the option `add=TRUE`. Further annotations can be added by the user to the plot with the R functions `legend` and `text` as needed.

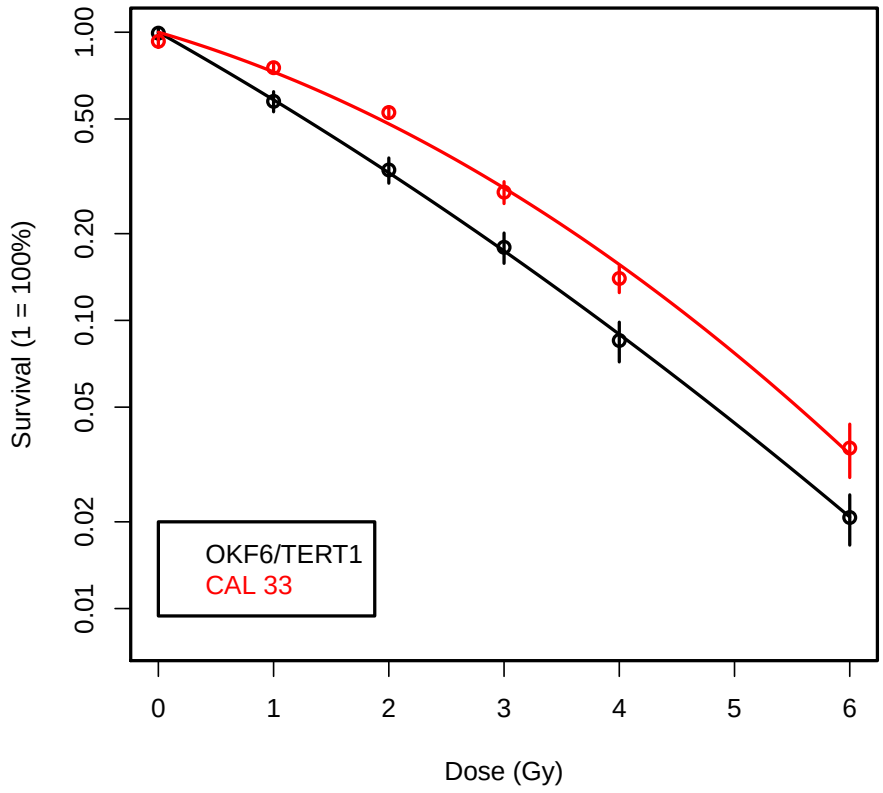
```
> plot(cellsurvLQfit(subset(datatab, cline=="okf6TERT1")), col=1)

method = ml
PEmethod = fit
       dose       dose2
-0.51937898 -0.02102614
Use 'print' to see detailed results

> plot(cellsurvLQfit(subset(datatab, cline=="cal33")), col=2, add=TRUE)

method = ml
PEmethod = fit
       dose       dose2
-0.26831918 -0.04892355
Use 'print' to see detailed results

> legend(0, 0.02, c("OKF6/TERT1", "CAL 33"), text.col=1:2)
```



3 Example: ANOVA for experimental two-way design

In this section a two-way ANOVA is demonstrated for the human oesophageal adenocarcinoma cell line OE19 which was treated with the chemotherapeutic drug cisplatin/5-FU before and after siRNA transfection. The results were previously published in (**?**). Of special interest was a potential interaction, i.e. chemosensitisation between the siRNA transfection and the drug effect.

3.1 Data input and double-check

First the data are read into memory.

```
> datatab <- read.table(system.file("doc", "exp2_2waycfa.txt", package="CFAssay"),
+                       header=TRUE, sep="\t")
```

The data file contains columns with header names `Exp, x5fuCis, siRNA, ncells and ncolonies`. `x5fuCis` and `siRNA` stand for the drug and biological treatment, respectively. They take values 0 for control or 1 for treated. The names of the other columns are as in the cell survival curve example.

```
> names(datatab)
```

```
[1] "Exp"       "x5fuCis"   "siRNA"     "ncells"    "ncolonies"

> head(datatab, 3) # First 3 lines

  Exp x5fuCis siRNA ncells ncolonies
1 e1a       0     0   1000       750
2 e1a       0     1   1000       546
3 e1a       1     0   1000       316
```

Again, number of rows and columns and frequencies or cross frequencies of the data may be checked with R functions `dim` and `table` (output not shown).

```
> dim(datatab)
> table(datatab$x5fuCis)
> table(datatab$siRNA)
> table(datatab$Exp, datatab$x5fuCis)
> table(datatab$Exp, datatab$siRNA)
```

3.2 ANOVA model

Statistical analysis is performed with CFAssay function `cfa2way`, using parametrisation option `"A/B"` corresponding to formula (3). In the argument list `A` and `B` have to be set as shown. Maximum-likelihood method is default, but least-squares can be chosen optionally. The output shows the result of a test for interaction.

```
>   fitcomp <- cfa2way(datatab, A="siRNA", B="x5fuCis", param="A/B")

*** Two-way ANOVA for factors A and B with interaction ***
A= siRNA , B= x5fuCis
Test for interaction: F-test
           F  Pr(>F)
values 9.831 0.01202 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
Use 'print' to see detailed results
```

Detailed results are shown with function `print.cfa2wayfit`. In the output `A0:B1` and `A1:B1` correspond to B_0 and B_1 in formula (3).

```
> print(fitcomp, labels=c(A="siRNA", B="x5fuCis"))

*** Logarithmic linear two-way ANOVA for factors A and B with interaction ***
================================================================================
A= siRNA , B= x5fuCis
Postscript digits for A or B: 0 inactive, 1 active
surv_percent = exp(Estimate)*100

Null hypothesis (Model 1): no interaction
----------------------------------------
```

```
     Estimate Std. Error    t value     Pr(>|t|) surv_percent
A1 -0.4237844 0.06366051  -6.656944 5.660071e-05         65.5
B1 -1.1187559 0.07738539 -14.456939 4.980588e-08         32.7

Goodness-of-fit values
        Residual Deviance: 77.99569
        Total sum of squared weighted residuals ssqwresTot: 75.87275
        Residual Degrees of Freedom: 10
        Dispersion parameter: 7.587275

Alternative hypothesis (Model 2): interaction
-------------------------------------------
parametrization: A/B
        Estimate Std. Error    t value     Pr(>|t|) surv_percent
A1    -0.3484218 0.05266374  -6.615972 9.746888e-05         70.6
A0:B1 -0.9757699 0.07170380 -13.608343 2.619891e-07         37.7
A1:B1 -1.3432352 0.09470322 -14.183627 1.832336e-07         26.1

Goodness-of-fit values
        Residual Deviance: 37.15596
        Total sum of squared weighted residuals ssqwresTot: 37.38767
        Residual Degrees of Freedom: 9
        Dispersion parameter: 4.154185

Analysis of Variance Table and F-test
Model 2 versus Model 1
  Resid. Df Resid. Dev Df Deviance     F  Pr(>F)
1        10     77.996
2         9     37.156  1    40.84 9.831 0.01202 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Diagnostic plots for repeated experiments are printed to pdf.

```
>   pdf("TwoWay_experimental_plots.pdf")
>     plotExp(fitcomp, labels=c(A="siRNA", B="x5fuCis"))
>   dev.off()
```