

CFAssay: Statistics of the Colony Formation Assay

Herbert Braselmann

May 4, 2016

Research Unit Radiation Cytogenetics, Group Integrative Biology
Helmholtz Zentrum München

`braselm@helmholtz-muenchen.de`

Contents

1 Overview	1
1.1 The models	2
1.2 Remark on intercepts c and plating efficiencies	2
2 Example: Linear-quadratic model for cell survival curves	3
2.1 Data input and double-check	3
2.2 Calculation of cell survival curves	4
2.3 Comparison of the two cell survival curves	6
3 Example: ANOVA for experimental two-way design	9
3.1 Data input and double-check	9
3.2 ANOVA model	10

1 Overview

The functions in this package provide tools for statistical analysis along with the colony formation assay (CFA) (Franken et al., 2006). 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 (Frome and Beauchamp, 1968) 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 (Brasemann et al., 2015).

1.1 The models

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

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

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 $c = \log(S_0)$ 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

$$S = e^{c+Ax_1+Bx_2+Dx_1x_2} \quad (2)$$

or as nested parametrization

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

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 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 (Bauer et al., 2010) 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 rswTot: 164.8109

Residual Degrees of Freedom: 38

Dispersion parameter: 4.337128

Fraction rsw of rswTot per Experiment

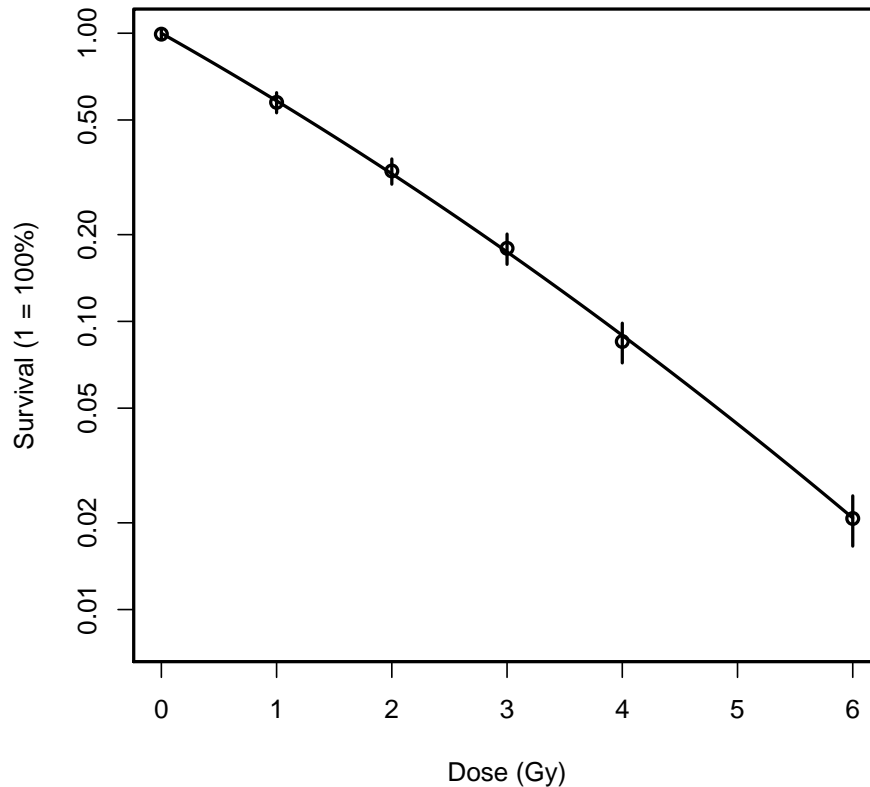
	Experiment	rsw	perCent
1	e1	4.84	2.9
2	e2	14.91	9.0
3	e3	9.01	5.5
4	e4	4.51	2.7
5	e5	34.77	21.1
6	e6	52.22	31.7
7	e7	41.09	24.9
8	e8	3.46	2.1

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 (α) and the dose-squared coefficient (β) 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 rswTot: 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)
```

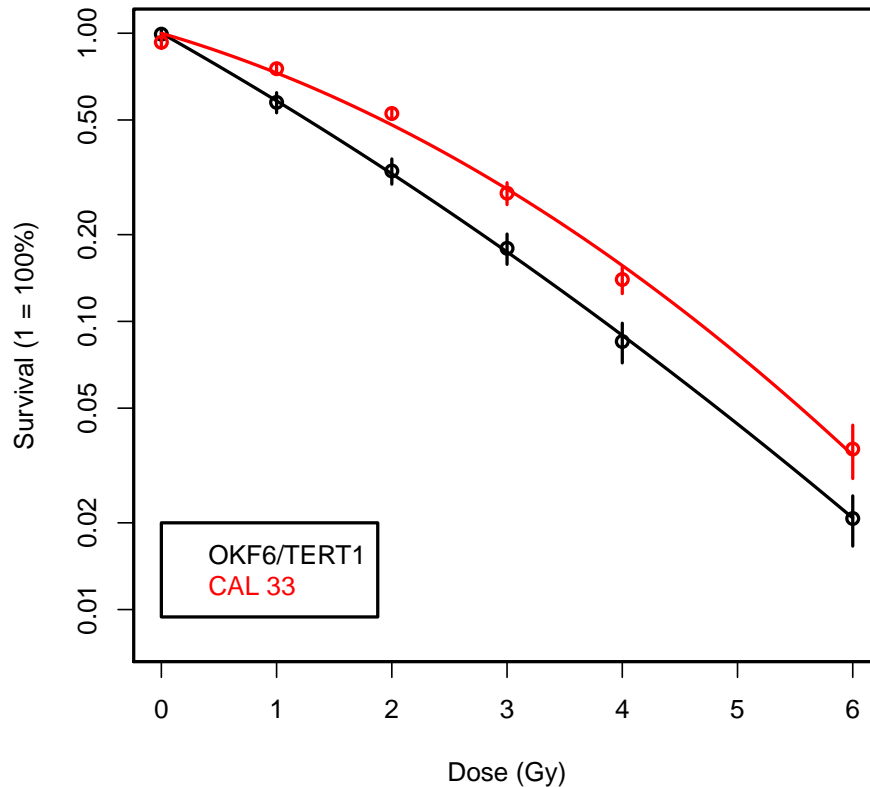
```
method = ml
```

```
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 (Aichler et al., 2013). 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()
```

References

- Aichler, M., Elsner, M., Ludyga, N., Feuchtinger, A., Zangen, V., Maier, S. K., Balluff, B., Schöne, C., Hierber, L., Braselmann, H., et al. (2013). Clinical response to chemotherapy in oesophageal adenocarcinoma patients is linked to defects in mitochondria. *The Journal of pathology*, 230(4):410–419.
- Bauer, V. L., Hieber, L., Schaeffner, Q., Weber, J., Braselmann, H., Huber, R., Walch, A., and Zitzelsberger, H. (2010). Establishment and molecular cytogenetic characterization of a cell culture model of head and neck squamous cell carcinoma (hnscc). *Genes*, 1(3):388–412.

- Braselmann, H., Michna, A., Heß, J., and Unger, K. (2015). Cfassay: statistical analysis of the colony formation assay. *Radiation Oncology*, 10(1):1.
- Franken, N. A., Rodermond, H. M., Stap, J., Haveman, J., and Van Bree, C. (2006). Clonogenic assay of cells in vitro. *Nature protocols*, 1(5):2315–2319.
- Frome, E. L. and Beauchamp, J. J. (1968). Maximum likelihood estimation of survival curve parameters. *Biometrics*, pages 595–605.