

Scratch Assay

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1 Aim

Investigate whether migration of the B16-F1-Luc2-BR2 melanoma cell line is impaired in the presence of beta-hydroxybutyrate (BHB).

2 Reagents

- B16-F1-Luc2-BR2 cells.

These were generated as follows:

- B16 cells were obtained from American Type Culture Collection (ATCC).

- To facilitate quantitative measurement of tumor growth, they were modified as described previously [1].
The cells were stably transfected with the gene encoding luc2 (luciferase) using the pGL4.51 [luc2/CMV/Neo] vector (Promega Corp, Madison, WI) and FuGENE6 Transfection Reagent (Roche Applied Science, Indianapolis, IN) following conditions specified by the manufacturer.
- They were then injected into the right ventricle of a mouse.
These animals were sacrificed when bioluminescence was detected in the brain.
- Cells metastatic to the brain were recovered put into culture.
- (R)-(-)-3-hydroxy butyric acid sodium salt (Sigma-Aldrich, St. Louis, MO).
- Media: DMEM (Gibco[®]) + 10% FCS + 600 ug/mL G418 + 1x glutamine. pH 7.4.
FCS = fetal calf serum.
- PBS = phosphate buffered saline.

3 Equipment

- 6-well plastic culture plate (Falcon[®])
- Multi-channel pipette
- Sharpie[®] permanent marker
- Image J software. Freely available from <http://imagej.nih.gov/ij/>.

4 Methods

4.1 Preparation

Incubate the cells at 37 C, in 5% CO₂, overnight.

4.2 -1 Hours

1. Ensure the cells have reached 95% confluency.
2. Attach 3 consecutive 200ul pipette tips to the multi-channel pipetter and 'scratch' each well.
3. Draw a line with the marker perpendicular to the scratches in each well.
4. Gently rinse wells with PBS and replace media with either:
 - DMEM + 10% FCS with normal saline.
 - DMEM + 10% FCS with 10mmol/L BHB.

4.3 0, 3, 6, 20 hours

1. For each well: take a photograph above and below the perpendicular 'scratch' line.
2. Return the cells to the incubator.

4.4 Analysis

1. Select the 'scratch area' using the 'free hand' tool.
2. Obtain the 'area density' of the scratch area. This reflects the distance between the cellular elements on either side of the original scratch. As the cells migrate, this decreases over time.

5 Results

The key to the data is shown in table 1.

5.1 Key

```
library("xtable")
options("xtable.booktabs"=TRUE)
print(xtable(k1,
  align=c(rep("c", 1), rep("l", 4)),
  caption="Key to data",
  label="tab:key"),
  include.rownames=FALSE)
```

name	long_name	Values	Values...explanation
tx	treatment	m	mock (no treatment/ control)
		b	BHB 10 mmol/L
w	well		1 to 3
PTL	position to line	a	above
		b	below
ℓs	location of scratch	ℓ	left
		c	center
		r	right
h	hours after start of experiment		
d	density	NA	not available

Table 1: Key to data

Results of the image density for each well and scratch, for each time point (0, 3, 6 and 20 hours) are given below. A sample of the data to indicate the layout is given in table 2.

5.2 Data

```
xtable(head(d1),
  caption="Sample data",
  label="tab:data")
```

	tx	w	PTL	ℓs	h	d
1	m	1	a	ℓ	0	4654583
2	m	1	b	ℓ	0	4975494
3	m	1	a	c	0	5623493
4	m	1	b	c	0	5896831
5	m	1	a	r	0	7087319
6	m	1	b	r	0	7129416

Table 2: Sample data

5.3 t-Tests

We assume that the density is normally distributed at each time point.

The results of a t-test performed at each time point are shown in table 3.

For the calculation, we perform a one-sided test. That is, we test whether cell migration was slower i.e. we test whether the density is lower in the wells that were incubated without BHB.

We assume unequal variances for the samples, and the test is calculated using the standard Welch-Satterthwaite equation:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}} \quad (1)$$

where \bar{x} is the mean, s^2 is the variance and n is the size of each sample. The null hypothesis is that the difference in means, $\bar{x}_1 - \bar{x}_2 = 0$.

The degrees of freedom for the test is calculated from:

$$df = \frac{(\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2})^2}{(\frac{s_1^2}{n_1})^2/(n_1 - 1) + (\frac{s_2^2}{n_2})^2/(n_2 - 1)} \quad (2)$$

```
t1 <- d1[, t.test(d ~ tx=="b", alternative="less", by=h)]
xtable(data.frame("time"=unique(t1[[1]]),
                  "p_value"=unique(t1[[4]])),
        caption=paste0(t1$method[1], ". 1-sided.
Null hypothesis: mock less than BHB.
Density (percentage of starting value) by treatment."),
        label="tab:tt")
```

	time	p_value
1	0	0.07
2	3	0.04
3	6	0.03
4	20	0.03

Table 3: Welch Two Sample t-test. 1-sided. Null hypothesis: mock less than BHB. Density (percentage of starting value) by treatment.

5.4 Standard error

For plotting, we first calculate the standard error at each time point:

$$SE_{\bar{x}} = \frac{s}{\sqrt{n}} \quad (3)$$

where s is the sample standard deviation ($s = \sqrt{\text{variance}}$).

```
stdErr <- function(x) sqrt(var(x, na.rm=TRUE)) / sqrt(length(x))
d1 <- d1[, "mean" := mean(d, na.rm=TRUE), by=list(tx, h)]
d1 <- d1[, "SE" := stdErr(d), by=list(tx, h)]
```

5.5 Density over time

Here we plot the change in density over time.

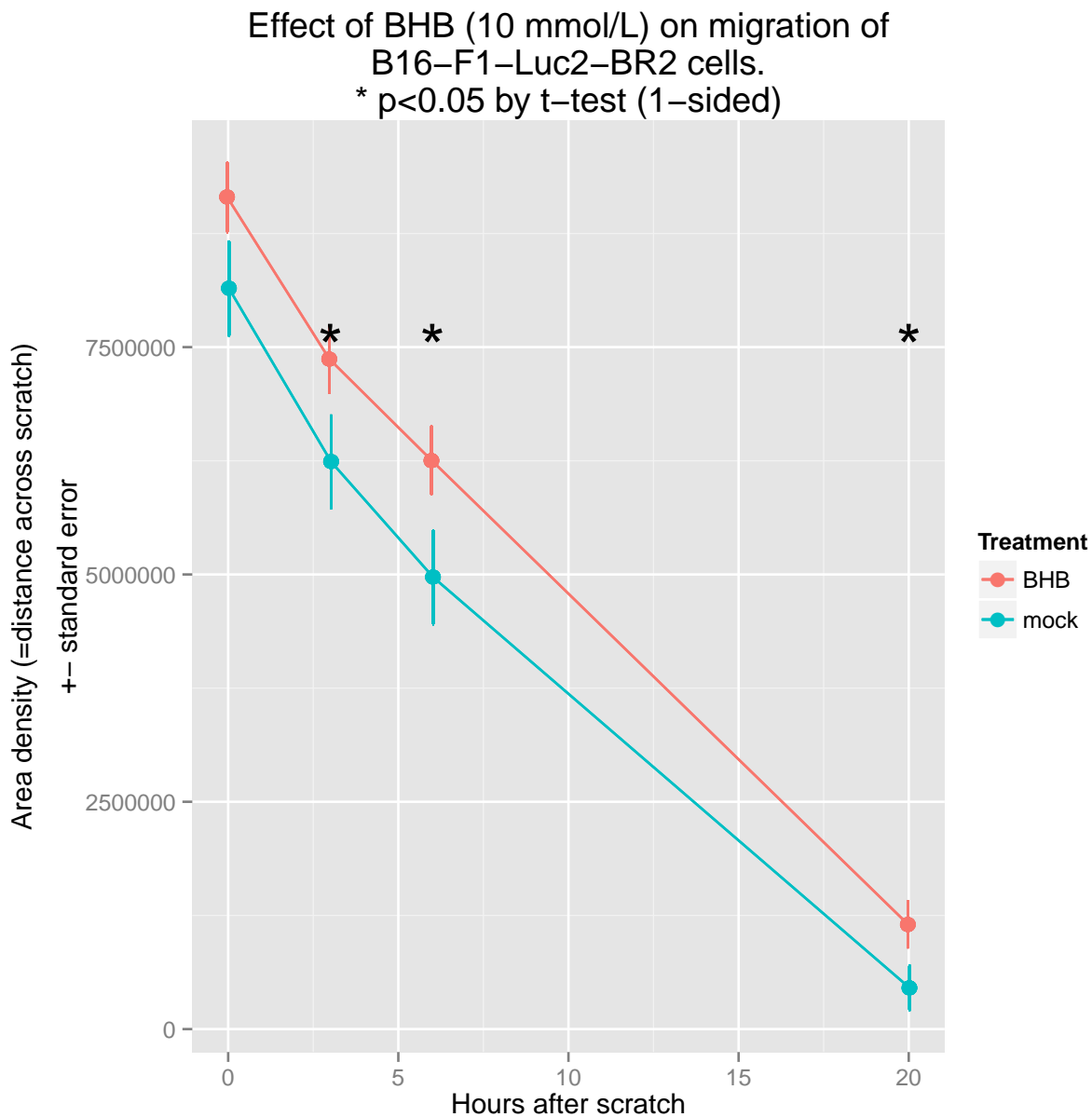
A linear decrease over time is evident. The density of the wells with BHB begins at a slightly higher value, but the difference becomes more pronounced over time.

```

suppressPackageStartupMessages(library("ggplot2"))
pd1 <- position_dodge(0.1)
ggplot(d1, aes(x=h, y=mean,
               color=factor(tx, levels=c("b", "m"),
               labels=c("BHB", "mock")))) +
  geom_line(position=pd1) +
  geom_errorbar(
    aes(ymin=mean-SE, ymax=mean+SE), width=.1, position=pd1) +
  geom_line(position=pd1) +
  geom_point(position=pd1, size=3) +
  annotate("text", x=c(3, 6, 20), y=7.5e6, label="*", size=10) +
  ggtitle("Effect of BHB (10 mmol/L) on migration of
B16-F1-Luc2-BR2 cells.
* p<0.05 by t-test (1-sided)") +
  theme(plot.title = element_text(size=15)) +
  xlab("Hours after scratch") +
  ylab(expression(atop("Area density (=distance across scratch)", "+- standard error")))+
  labs(color="Treatment")

## ymax not defined: adjusting position using y instead
## ymax not defined: adjusting position using y instead
## ymax not defined: adjusting position using y instead

```



5.6 Express as percentage of starting value

A more intuitive graph expressed the density as a percentage of the starting value for each sample. Thus time=0 is 100% and subsequent values are taken in comparison to this.

The calculations are shown below. Again, we calculate the standard errors for each timepoint by treatment group.

```
d1 <- d1[, "psv" := (d1[, mean, by=list(tx, h)][, mean] /
  d1[h==0, mean, by=tx][, mean]) * 100]
d1 <- d1[, "psv" := 100 * d1[, d] / d1[h==0, d]]
d1 <- d1[, "psvM" := mean(psv, na.rm=TRUE), by=list(tx, h)]
d1 <- d1[, "psvSE" := sqrt(var(psv, na.rm=TRUE)) /
  sqrt(length(psv)), by=list(tx, h)]
```

5.7 Check t-tests again

t-tests are again significant as we can see.

```
t1 <- d1[!h==0, t.test(psv ~ tx=="b", alternative="less"), by=h]
xtable(data.frame("time"=unique(t1[[1]]),
                  "p_value"=unique(t1[[4]])),
        caption=paste0(t1$method[1], ". 1-sided.
Null hypothesis: mock less than BHB.
Density (percentage of starting value) by treatment."),
        label="tab:ttpsv")
```

	time	p_value
1	3	0.03
2	6	0.02
3	20	0.01

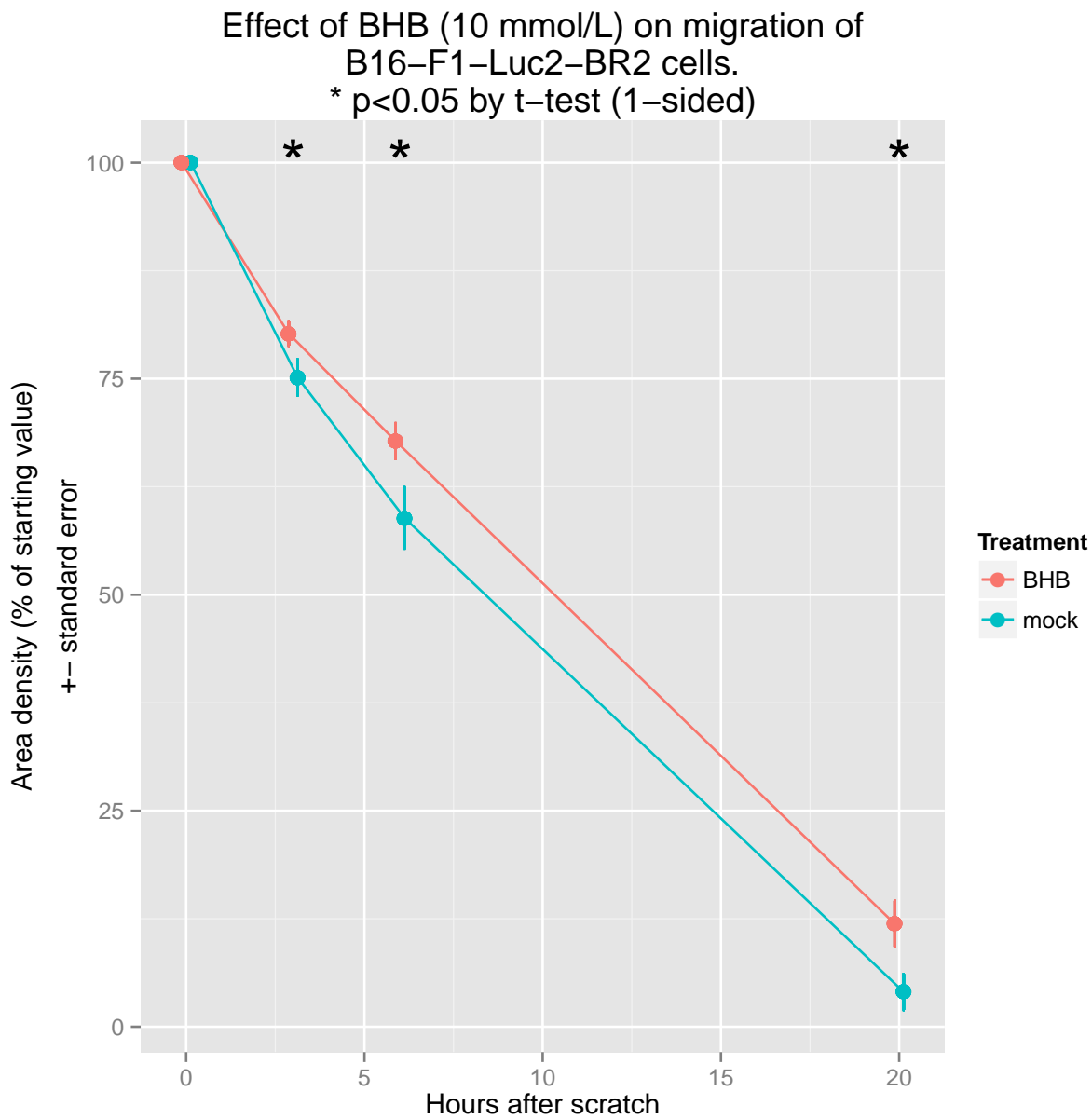
Table 4: Welch Two Sample t-test. 1-sided. Null hypothesis: mock less than BHB. Density (percentage of starting value) by treatment.

5.8 Plot of percentage of starting value

Again we plot mean and standard error, this time in terms of percentage of the starting value.

```
pd1 <- position_dodge(0.5)
ggplot(d1, aes(x=h, y=psvM,
               color=factor(tx, levels=c("b", "m"),
                               labels=c("BHB", "mock")))) +
  geom_line(position=pd1) +
  geom_errorbar(
    aes(ymin=psvM - psvSE,
        ymax=psvM + psvSE), width=0.1, position=pd1) +
  geom_point(position=pd1, size=3) +
  annotate("text", x=c(3, 6, 20), y=100, label="*", size=10) +
  ggtitle("Effect of BHB (10 mmol/L) on migration of
B16-F1-Luc2-BR2 cells.
* p<0.05 by t-test (1-sided)") +
  theme(plot.title = element_text(size=15)) +
  xlab("Hours after scratch") +
  ylab(expression(atop("Area density (% of starting value)", "+- standard error"))) +
  labs(color="Treatment")

## ymax not defined: adjusting position using y instead
## ymax not defined: adjusting position using y instead
```



5.9 Linear model

As the decrease in density appears to be linear over time and the standard errors are similar at each time point, a simple linear model appears a good approximation:

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2, \quad i = 1, 2, \dots, n \quad (4)$$

The estimated coefficients of such a model and their associated Wald statistics are shown in table 5.

We proceed to an analysis of variance, shown in table 6. While time is the more important factor, treatment is also clearly highly significant.

```
xtable(summary(l1 <- d1[, lm(d ~ h + tx)]),
  caption="Linear model. Area density depends on time and treatment.",
  label="tab:lm")
```


	Estimate	Std. Error	t value	Pr(> t)
(Intercept)	8712371.7229	245322.7426	35.51	0.0000
h	-376728.9982	19002.0241	-19.83	0.0000
txm	-1029349.9861	291259.4339	-3.53	0.0006

Table 5: Linear model. Area density depends on time and treatment.

```
xtable(anova(l1),
  caption="Analysis of variance table. Response = area density.",
  label="tab:anova",
  display=c(rep("g", 6)))
```

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
h	1	1.2e+15	1.2e+15	3.9e+02	4.423e-42
tx	1	3.7e+13	3.7e+13	12	0.0005584
Residuals	1e+02	4.1e+14	3e+12		

Table 6: Analysis of variance table. Response = area density.

6 Conclusions

In the presence of BHB, there is impaired migration of these cells.

This is apparent from a t-test at 20 hours ($p = 0.01$).

This is emphasised by comparing linear models with and without treatment ($p = 0.0006$).

Appendix

A Images

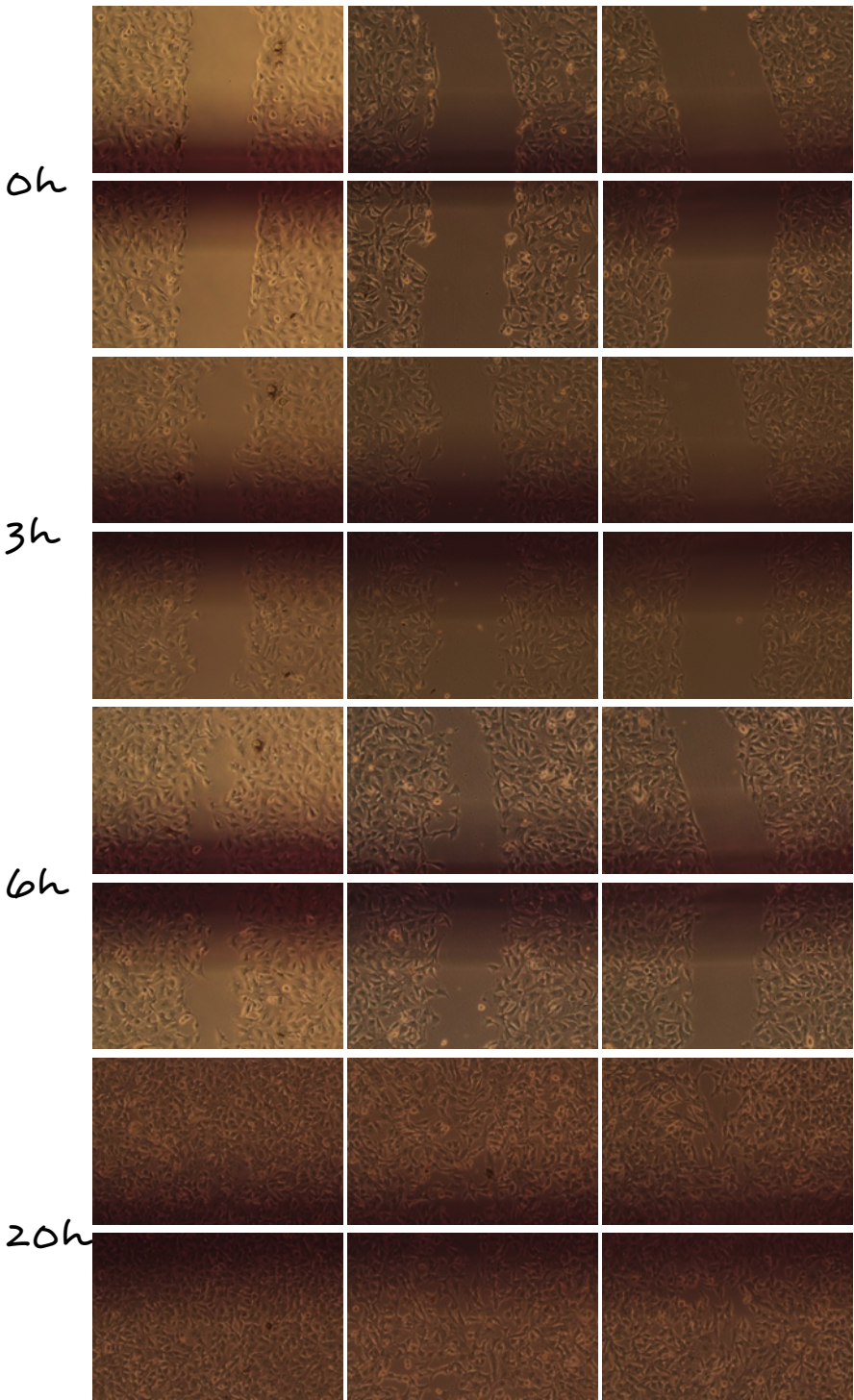


Figure 1: Mock - well 1

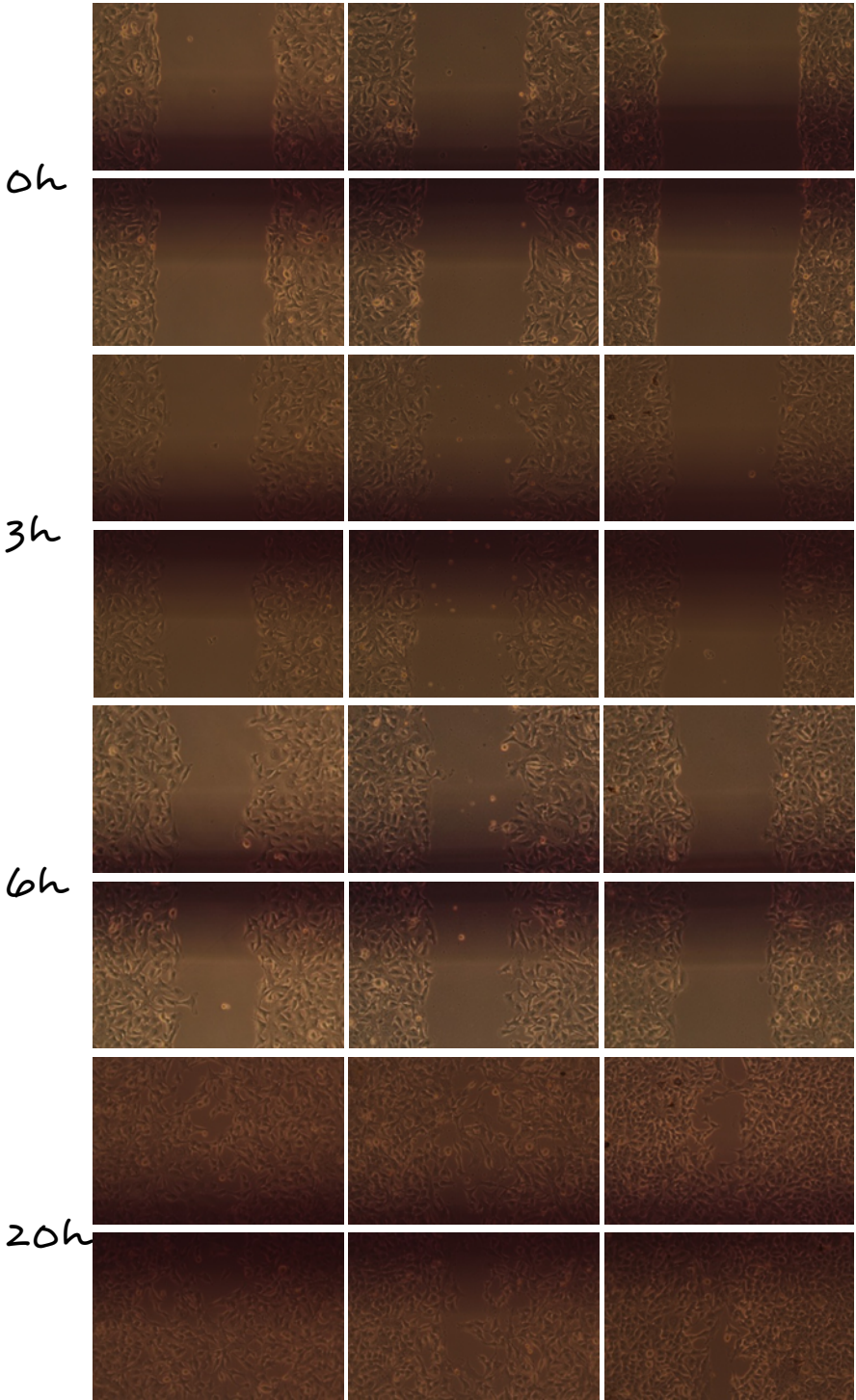


Figure 2: Mock - well 2

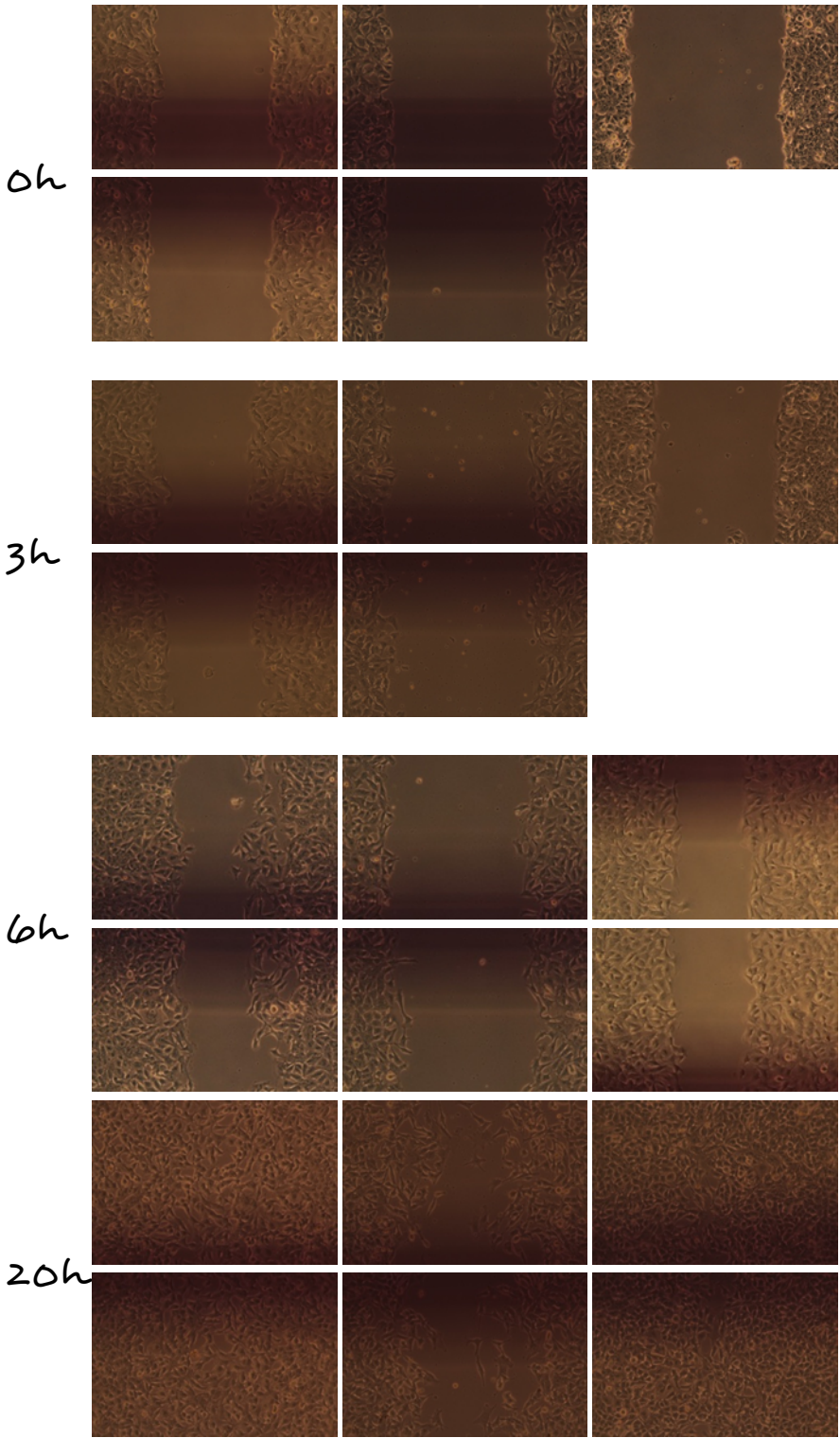


Figure 3: Mock - well 1

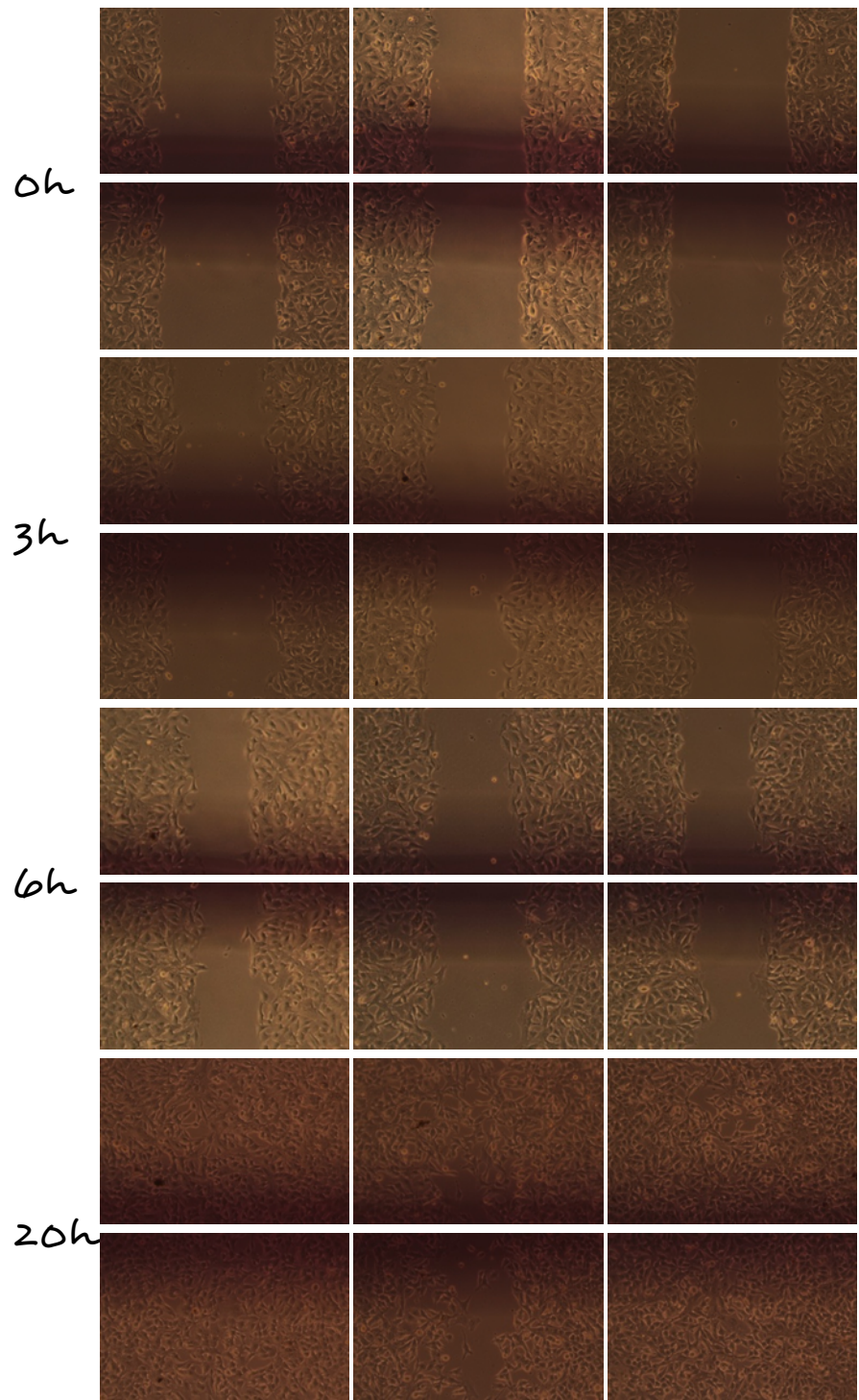


Figure 4: BHB - well 1

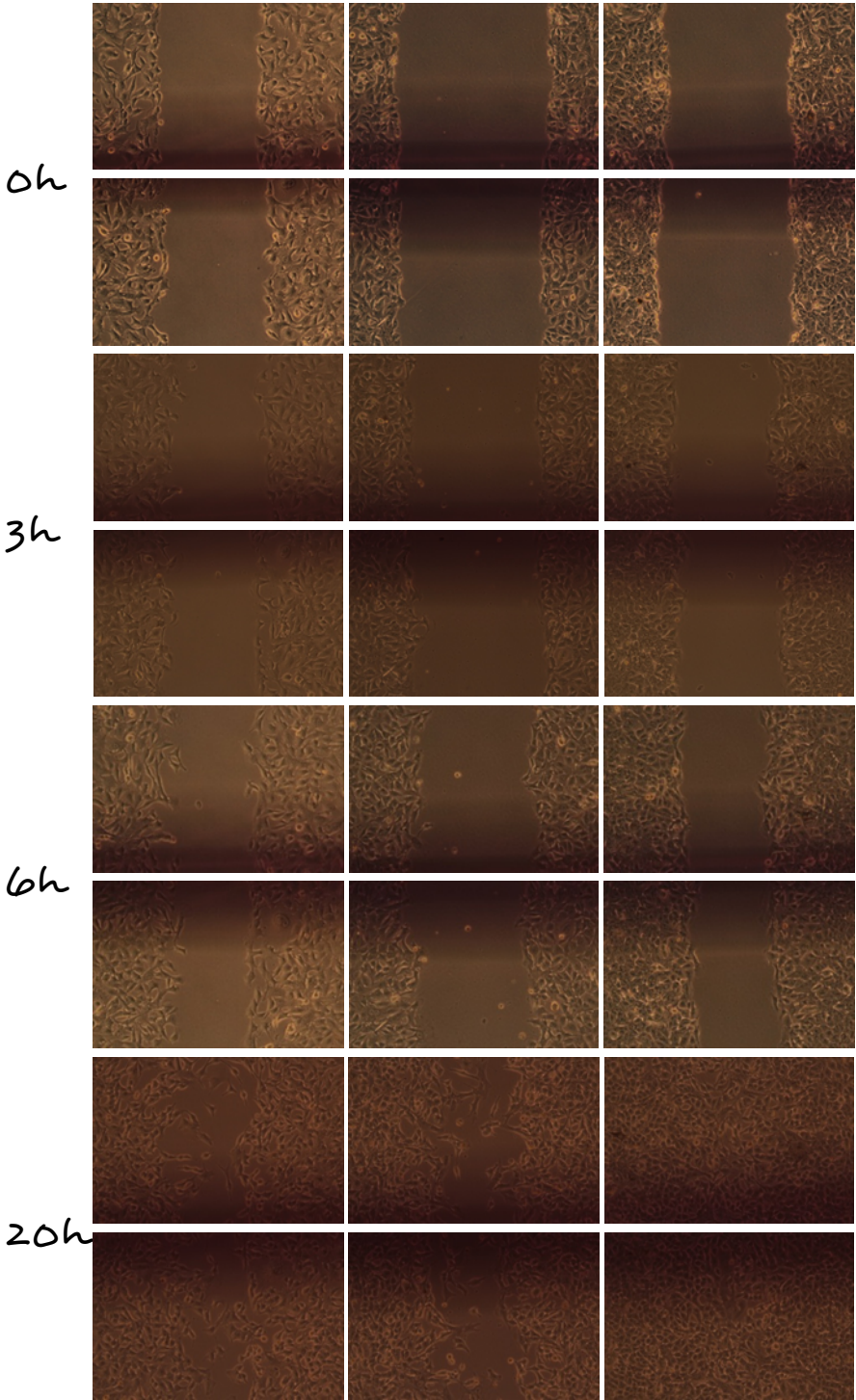


Figure 5: BHB - well 2

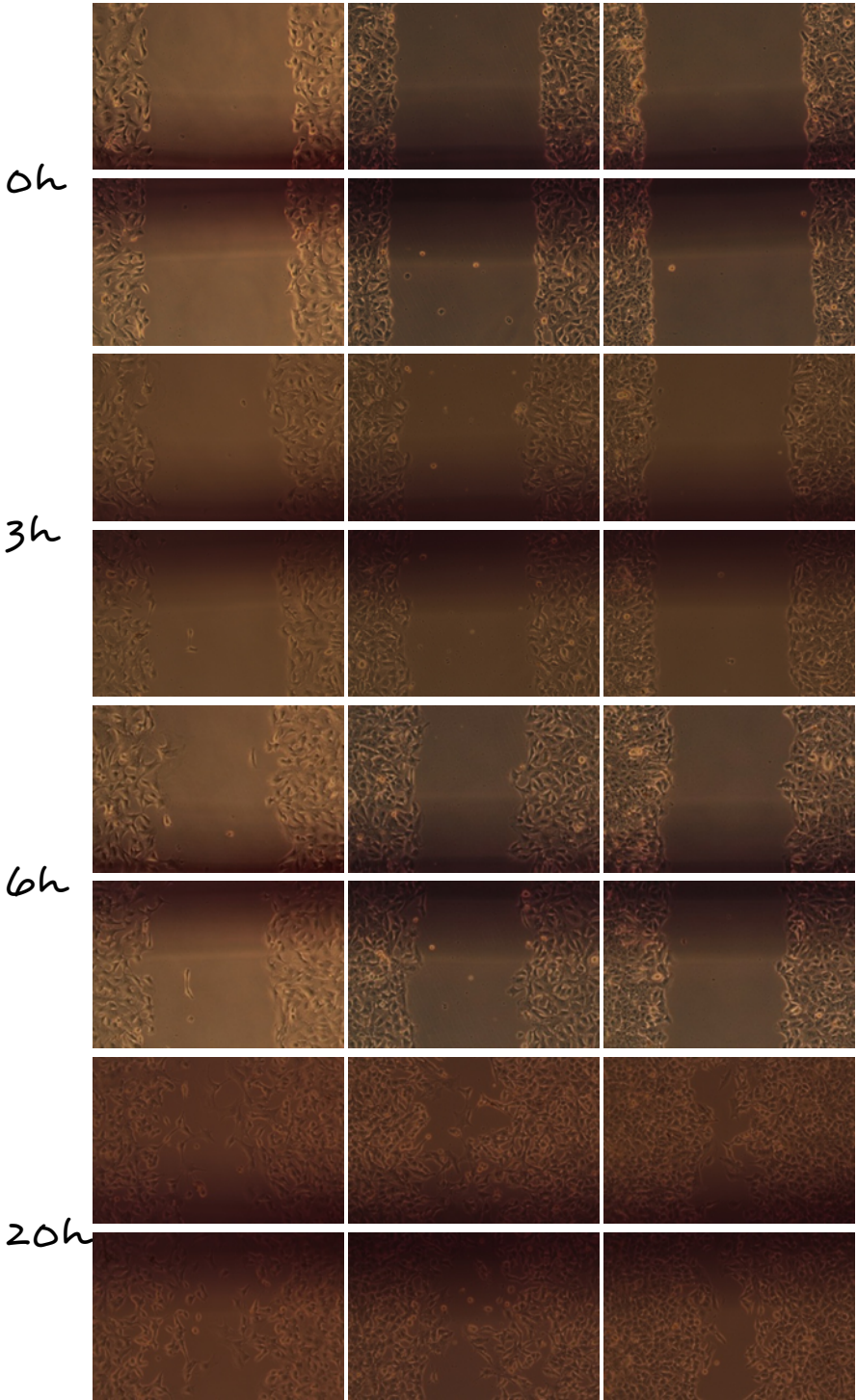


Figure 6: BHB - well 3

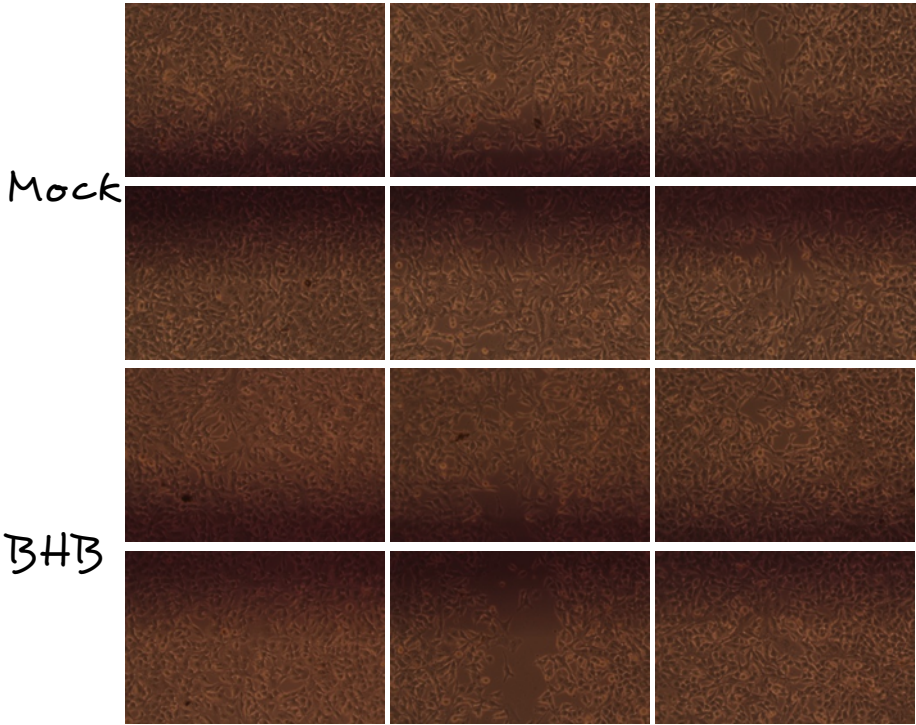


Figure 7: Images at 20h

B Key

```
## read data in from an external .csv file k1 <-
## read.csv('key1.csv', stringsAsFactors=FALSE) k1[is.na(k1)]
## <- 'NA' below is output from dput(k1)
k1 <- structure(list(Column.name = c("tx", "", "w", "pTL", "",
  "lS", "", "", "h", "d"), Long.name = c("treatment", "", "well",
  "position to line", "", "location of scratch", "", "", "hours after start of ex-
periment",
  "density"), Values = c("m", "b", "", "a", "b", "l", "c",
  "r", "", "NA"), Values...explanation = c("mock (no treatment/ control)",
  "BHB 10 mmol/L", "1 to 3", "above", "below", "left", "center",
  "right", "", "not available")), .Names = c("name", "long_name",
  "Values", "Values...explanation"), row.names = c(NA, -10L),
class = "data.frame")
```

C Data

```
## read data in from an external .csv file d1 <-
## read.csv('d1.csv') below is output from dput(d1)
d1 <- structure(list(tx = structure(c(2L, 2L, 2L, 2L, 2L, 2L,
  2L, 2L, 2L, 2L, 2L, 2L, 1L, 1L, 1L,
  1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L,
  2L, 2L, 2L, 2L, 2L, 2L, 2L, 2L, 2L, 2L, 2L, 2L, 2L, 2L,
  2L, 2L, 2L, 1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L,
  1L, 1L, 1L, 1L, 1L, 1L, 2L, 2L, 2L, 2L, 2L, 2L, 2L, 2L,
  2L, 2L, 2L, 2L, 2L, 2L, 2L, 2L, 1L, 1L, 1L, 1L, 1L, 1L,
  1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L, 2L, 2L, 2L,
  2L, 2L, 2L, 2L, 2L, 2L, 2L, 2L, 2L, 2L, 2L, 2L, 2L, 2L,
  1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L, 1L,
  1L, 1L, 1L), .Label = c("b", "m"), class = "factor"), w = c(1L,
  1L, 1L, 1L, 1L, 1L, 2L, 2L, 2L, 2L, 2L, 2L, 3L, 3L, 3L, 3L,
  3L, 3L, 1L, 1L, 1L, 1L, 1L, 1L, 2L, 2L, 2L, 2L, 2L, 2L, 3L,
  3L, 3L, 3L, 3L, 3L, 1L, 1L, 1L, 1L, 1L, 1L, 2L, 2L, 2L, 2L,
  2L, 2L, 3L, 3L, 3L, 3L, 3L, 3L, 3L, 1L, 1L, 1L, 1L, 1L, 1L, 2L,
```



```

20L, 20L, 20L, 20L, 20L, 20L, 20L, 20L, 20L, 20L,
20L, 20L, 20L, 20L, 20L, 20L, 20L, 20L, 20L, 20L,
20L, 20L, 20L, 20L, 20L, 20L), d = c(4654583L, 4975494L,
5623493L, 5896831L, 7087319L, 7129416L, 8203592L, 8033043L,
7761189L, 7730470L, 9920399L, 9827826L, 8355243L, 8767531L,
11723752L, 11763422L, 10996453L, NA, 6613000L, 6617906L,
8362343L, 8340142L, 8208323L, 8150313L, 7145124L, 7381792L,
10719451L, 10278916L, 8775864L, 9431899L, 10241518L,
10538629L, 10454921L, 10398363L, 11515045L, 11480473L,
2503584L, 3031875L, 4101049L, 4611598L, 5054676L, 5217503L,
6333081L, 6318841L, 6379680L, 6653387L, 7287418L, 7342764L,
6055806L, 6224729L, 10279446L, 10263094L, 8410908L, NA,
4998927L, 4992045L, 6512314L, 7449820L, 5927061L, 5621165L,
6337978L, 6042076L, 9005799L, 8873342L, 6635782L, 6861604L,
8326295L, 9198402L, 8310737L, 8568745L, 9615269L, 9348981L,
973066L, 1957407L, 3018767L, 3826106L, 4095748L, 4362068L,
4968700L, 5241024L, 5505523L, 5467842L, 6119653L, 6032279L,
4664637L, 4710845L, 9846330L, 9185420L, 4506993L, NA,
3904176L, 3906179L, 5393996L, 6430096L, 4989009L, 4161891L,
5817872L, 4858694L, 7892191L, 7644489L, 5526064L, 5132353L,
7824846L, 8270159L, 6890105L, 7497808L, 8380387L, 8081758L,
0L, 0L, 0L, 0L, 0L, 0L, 0L, 0L, 0L, 0L, 0L, 653391L, 1097059L,
0L, 0L, 2370259L, 3595470L, 0L, NA, 0L, 0L, 0L, 2448328L,
0L, 0L, 2059799L, 833207L, 2273643L, 2477303L, 0L, 0L,
2469428L, 1853711L, 663784L, 2351778L, 1408810L, 1915999L)),
.Names = c("tx", "w", "pTL", "ls", "h", "d"), class = "data.frame",
row.names = c(NA, -144L))
suppressPackageStartupMessages(library("data.table"))
d1 <- data.table(d1)

```

References

- [1] Abdelwahab, Mohammed G., et al. The ketogenic diet is an effective adjuvant to radiation therapy for the treatment of malignant glioma. *PloS one* 7.5 (2012): e36197.