Light-chain-modified rBoNT/A mutants with improved stability

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Botulinum Toxin Research
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Background

- The light chain (LC) of botulinum neurotoxins (BoNTs) is an enzyme that can be inactivated and denatured by heat and/or reactive oxygen species present in the cell and/or present during isolation, purification, and storage.
- Stabilization of the LC of BoNT/A by reducing vulnerability towards oxygen, enhancing thermostability, and/or increasing resistance against proteolytic attack would thus be advantageous for the development of BoNT-based therapeutics.
- We tackled this task with the rational design of mutations or deletions at sites critical for the stability of the LC.

Methods

LC stabilization by increasing oxidative stability

- Since the vulnerability towards oxidation of a protein residue is directly related to solvent accessibility, LC Met and Cys residues were classified into three groups according to this parameter (**Table 1**).[1]

Table 1. Mutation sites

Group	Residue	Relative ACC (%)[1]
1: Highly exposed (relative ACC ⩾ 40%)	Met 411	72.0
	Met 253	68.8
	Met 106	41.9
2: Partially exposed/buried (40% > relative ACC > 10%)	Met 344	19.7
	Cys 134	18.1
	Met 30	16.1
3: Buried (relative ACC ⩽ 10%)	Met 313	9.4
	Cys 165	3.0

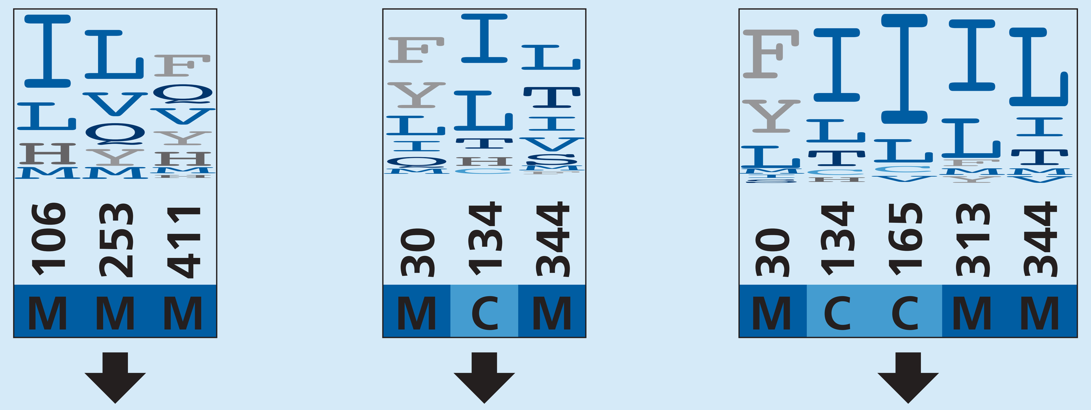
ACC, solvent accessible surface area.

- Monte Carlo searches[2] were performed to identify suitable simultaneous mutations at highly exposed LC sites (3-point mutant Oxi1) and to identify combinations of these with mutations at partially buried (6-point mutant Oxi2) and fully buried (8-point mutant Oxi3) residues (**Figure 1**).

LC stabilization through loop truncation

- Increasing the conformational stability of loops is a well-known strategy to stabilize the structure of proteins.[3] The LC is a globular protein with several loops (**Figure 2**). Loops can be stabilized by the deletion of residues with high atomic fluctuations.

Figure 1. Mutant design:[2] Monte Carlo search logo plots


106 253 411
M M M
30 134 344
M C M
30 134 165 313 344
M C C M M

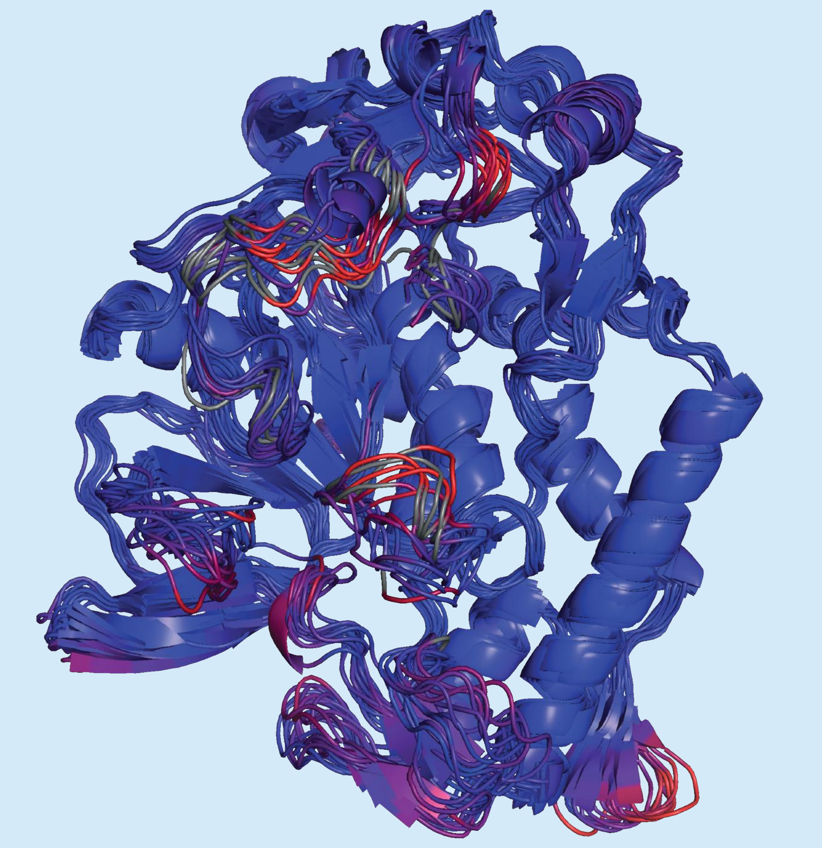
3-point mutant Oxi1: M106L, M253L, and M411Q (Group 1 mutation sites)

6-point mutant Oxi2: LC1 mutations, plus M30Y, C134I, and M344L (Groups 1 and 2 mutation sites)

8-point mutant Oxi3: LC1 mutations, plus M30F, C134I, and M344L, plus C165I and M313I (Groups 1, 2, and 3 mutation sites)

Logo plots represent the predominance and relative frequency of an amino acid residue at a given light chain (LC) position, resulting from the alignment of the most stable LC structures retrieved by the Monte Carlo search. The letters are arranged so that the most common amino acid is at the top, and the height of each letter is made proportional to its frequency.

Figure 2. Conformational stability



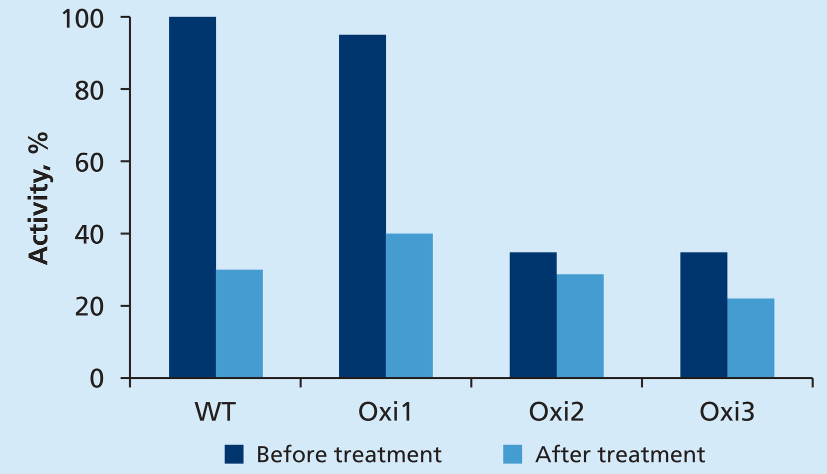
Superposition of LC molecular dynamics frames taken every 1 nanosecond (Desmond, explicit water, 14 nanoseconds, 310 K).[6] RMSD coloring: red = high, violet = medium, and blue = low. Most LC regions with high RMSD (red) are loops.
LC, light chain; RMSD, root mean square deviation.

Results

LC stabilization by increasing oxidative stability

- Oxi1 was shown to be fully active, whereas Oxi2 and Oxi3 displayed reduced proteolytic activity with respect to wild type (WT) (**Figure 3**).[4]
- Endopeptidase activity measurements after oxidative stress[5] indicated that mutants are more stable towards oxidation than WT BoNT (**Figure 3**).

Figure 3. Oxidative stability: oxidative stress test[5]

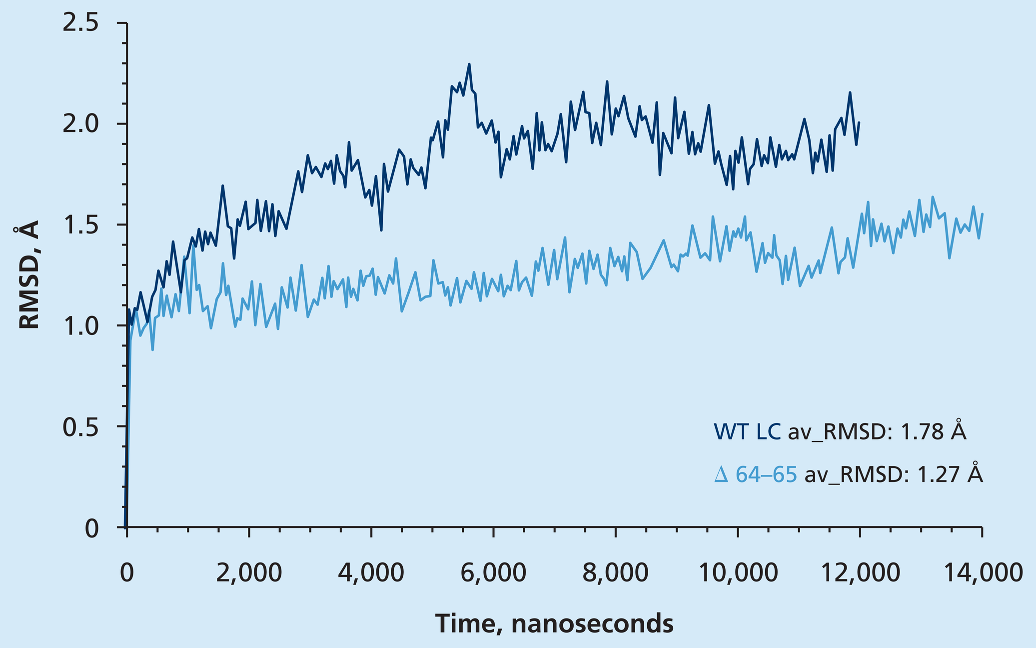

Activity, %
100
80
60
40
20
0
WT
Oxi1
Oxi2
Oxi3
Before treatment
After treatment

WT and mutants were incubated with reaction buffer containing 50 mM H_2O_2 for 16 hours.[5] After reaction quenching, the endopeptidase activity was measured.[4] Absolute activity ranking after treatment: Oxi1 > WT = Oxi2 > Oxi3. Activity ranking referred to the original activity of the respective mutant at t=0: Oxi2 (82%) > Oxi3 (63%) > Oxi1 (42%) > WT (30%). This experiment suggests that mutation of LC Cys and Met residues protects the toxin from oxidation.
LC, light chain; WT, wild type.

LC stabilization through loop truncation

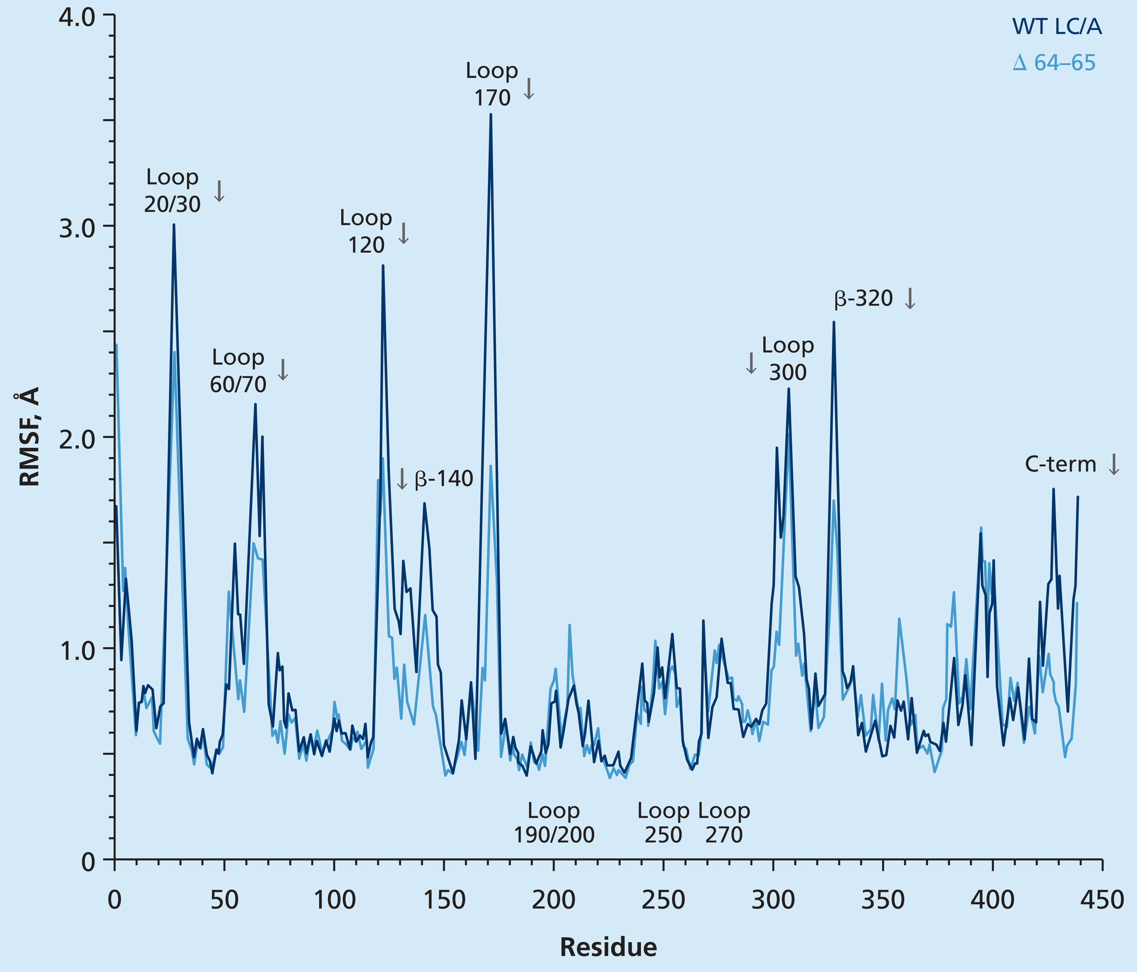
- Residues with high atomic fluctuations were identified by means of molecular dynamic simulations of the LC (**Figures 2, 4, and 5**).[6]
- Deletion of residues 64 and 65 at loop 60/70 (mutant LC Δ 64–65) led to a significant reduction of the average flexibility of the molecule (**Figure 4**).
- Interestingly, all LC flexible loops (loops with root mean square fluctuation >1 Å) were found to be conformationally more stable upon deletion of residues 64–65 (**Figure 5**).
- Deletion of highly fluctuating residues at two or more loops led to an increase of the thermal (**Figure 6**)[7] and proteolytic (**Figure 7**) stability of the LC.

Figure 4. Average global protein flexibility


RMSD, Å
2.5
2.0
1.5
1.0
0.5
0
0
2,000
4,000
6,000
8,000
10,000
12,000
14,000
Time, nanoseconds
WT LC av_RMSD: 1.78 Å
Δ 64–65 av_RMSD: 1.27 Å

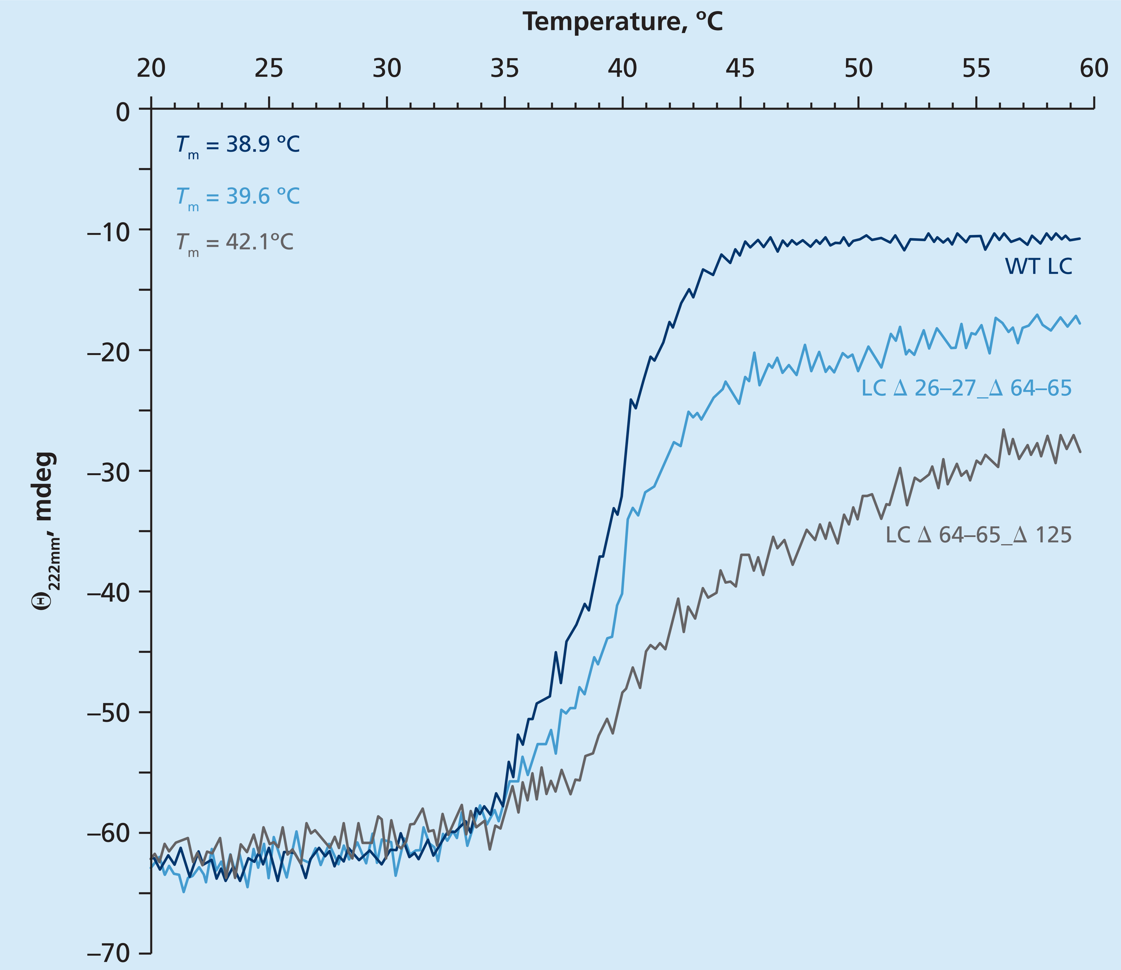
RMSD of backbone atoms as a function of the chemical time. Deletion of residues Glu64 and Ala65 at loop 60/70 led to a decrease of the molecule flexibility of ~0.6 Å, i.e. to an increase of the conformational stability of the LC.
LC, light chain; RMSD, root mean square deviation; WT, wild type.

Figure 5. Average local protein flexibility


RMSF, Å
4.0
3.0
2.0
1.0
0
WT LC/A
Δ 64–65
Loop 20/30
Loop 60/70
Loop 120
β-140
Loop 170
Loop 190/200
Loop 250
Loop 270
Loop 300
β-320
C-term
0
50
100
150
200
250
300
350
400
450
Residue

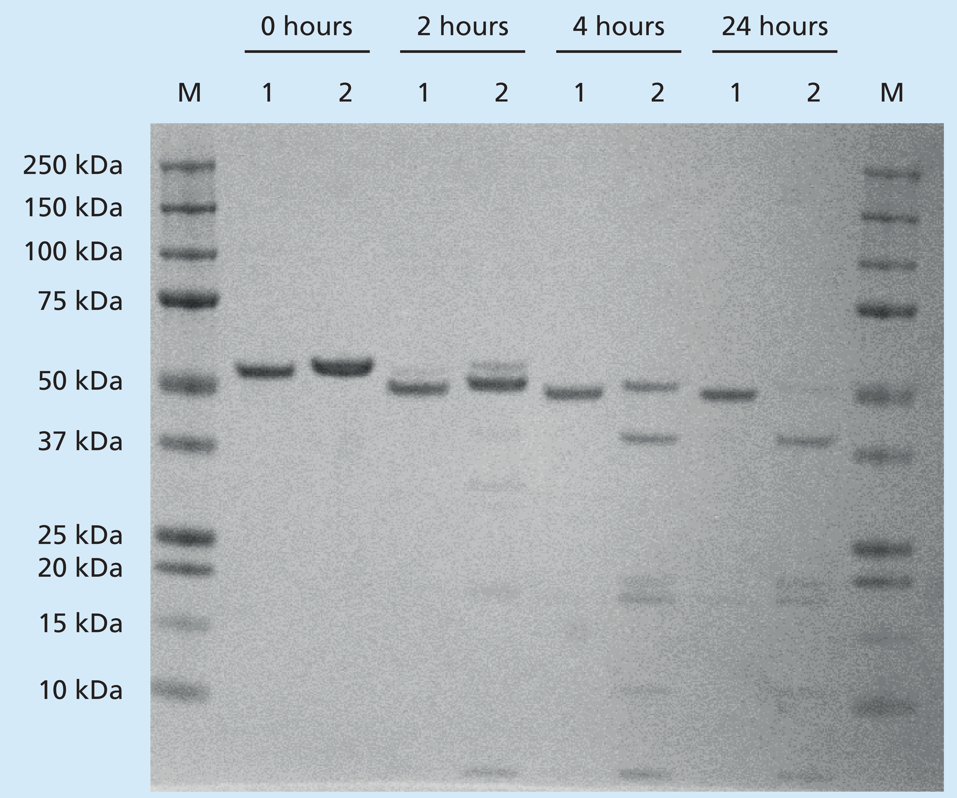
RMSF of the backbone atoms as a function of the LC residue. Deletion of residues Glu64 and Ala65 at loop 60/70 led to a rigidification of not only the modified loop but all flexible loops of the WT LC.
LC, light chain; RMSF, root mean square fluctuations; WT, wild type.

Figure 6. Thermal stability


Temperature, °C
20
25
30
35
40
45
50
55
60
0
−10
−20
−30
−40
−50
−60
−70
Θ222mm, mdeg
Tm = 38.9 °C
Tm = 39.6 °C
Tm = 42.1°C
WT LC
LC Δ 26–27_Δ 64–65
LC Δ 64–65_Δ 125

Thermal stability was assessed by thermal denaturation monitoring ellipticity (Circular Dichroism signal) at 222 nm between 20 °C and 60 °C. Ellipticity curves were analyzed assuming a two-state model (protein undergoes unfolding transition between the folded and unfolded state).[7] Loop truncation led to an increase of the T_m with respect to WT LC.
LC, light chain; T_m, midpoint of thermal transition; WT, wild type.

Figure 7. Proteolytic stability


0 hours
2 hours
4 hours
24 hours
M 1 2 1 2 1 2 1 2 M
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
10 kDa

Proteolytic stability of the LC mutant Δ26–27_Δ64–65_Δ125 (1) with respect to WT LC (2) was assessed *in vitro* by incubation with trypsin. The reaction was monitored by SDS-PAGE. WT LC was fully digested after 24 hours. No degradation was observed for the LC mutant. This experiment suggests that the stabilization of the LC conformation can enhance resistance against proteolytic degradation.
LC, light chain; M, molecular weight marker; WT, wild type.

Conclusions

- We showed that the LC of BoNT/A can be stabilized by mutating oxidation-prone Met and Cys residues, or by rigidification of flexible loops.
- A BoNT/A holotoxin with a suitably modified LC should provide a therapeutic with a higher stability and with eventually improved pharmacologic properties.

References

1. Vriend G. J Mol Graph 1990; 8: 52–56.
2. Schrödinger's biologics modeling platform BioLuminate (versions 1.6–1.8). BioLuminate, Schrödinger, LLC, New York, NY, United States, 2014–2015.
3. Russell RJ et al. Biochemistry 1997; 36: 9983–9994.
4. Jones RG et al. J Immunol Methods 2008; 329: 92–101
5. Opperman DJ, Reetz MT. Chembiochem 2010; 11: 2589–2596.
6. Bowers KG et al. Proceedings of the ACM/IEEE Conference on Supercomputing 2005; Tampa, Florida, United States, November 11–17 2006.
7. Greenfield NJ. Nat Protoc 2006; 1: 2527–2535.

Acknowledgments

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