Novel Method for Kinetic Analysis Applied to Transport by the Uniporter, OCT2

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General instructions for the MATLAB scripts, tcMMfitter and tcMMsolver (these scripts were prepared and run in MATLAB R2018b). These scripts are available on GitHub (https://github.com/secomb).

tcMMfitter.m

This script (which requires the MatLab Optimization Toolbox) calculates the J_{max} and K_t of substrate influx into a cell layer, based on the time courses of net substrate accumulation from several bulk solution concentrations of substrate. The calculations include the effect of both an unstirred water layer (line 35, which is generally fixed at 1cm; see text) above the cell layer, and the influence of mediated efflux of substrate previously accumulated by the transporter. The kinetic parameters returned by the script can reflect the presence of different concentration of a (competitive) inhibitor in the extracellular and/or intracellular solution, as well as the presence (at time zero) of substrate within the cell.

The user provides initial estimates of values for Lcell, Jl1, Jm1, and Kt1 (lines 19-22):

```
18      % initial estimates of parameters to be fitted
19 -    Lcell = 12e-4; %0.01; % Thickness (height) of cell layer in cm
20 -    Jl1 = 3.4e-7; % linear (first order) uptake rate in cm/s
21 -    Jm1 = 5e-4; % 0.001; % substrate max uptake rate in nmol/cm2/s
22 -    Kt1 = 5; % 1; % substrate uptake Michaelis constant (uM = nmol/cm3)
23
24 -    params0 = [Lcell, Jl1, Jm1, Kt1];
25      % typical values of parameters - needed for setting optimization steps
26      % use multiple of initial estimates for simplicity
27      % using a multiple gives more reliable numerical derivatives
28 -    refparams = 30.*[Lcell, Jl1, Jm1, Kt1];
29      % upper and lower bounds on parameters
30 -    ub = [1,1,1,2000];
31 -    lb = [0,0,0,0];
```

Figure 1.

The calculated values of these parameters are returned at completion of the script.

Lcell (line 19) represents the height of the cell (typically a confluent monolayer in the well of a 96 well plate). It is used in calculation of the "concentration" of accumulated substrate/inhibitor. As the "activity" of substrate/inhibitor at equilibrium (steady state) can be predicted from the energetics of the transport mechanism (for uniporters, this reflects the influence of membrane potential and substrate valence), Lcell becomes a variable used to allow accumulated substrate concentration to reflect maximum allowable activity. Consequently, to the extent that accumulated substrate is bound or sequestered within the cell, the calculated value of Lcell can exceed the true height of the cell (for CHO cells, ~3 µm) and,

so, is referred to in the text as the "Cell Partition Factor" (CFP). Steady state accumulation of MPP+ suggests that approximately 75% is effectively bound/sequestered (therefore, not chemically active), resulting in a calculated Lcell (i.e., CFP) of 12 µm (12e-4 cm), which we typically use as the initial estimate of Lcell.

Jl1 (line 20) is a first order constant that reflects the nonsaturable component of substrate accumulation (incl. diffusion, non-specific binding and incomplete rinsing of substrate prior to analysis of uptake). For polar substrates (e.g., MPP and metformin), diffusion is minimal, but the rapid rinsing procedure we use to minimize loss of accumulated substrate from the cells can leave a residual amount of extracellular (labeled) substrate that is evident as a first order, nonsaturable component of total substrate accumulation in our samples. For both MPP and metformin, the linear element is typically on the order of 3.4e-7 cm sec^{-1}, which is the usual value used for the initial estimate.

Jm1 and Kt1 are the J_{max} and K_t values for substrate influx, respectively. Here, these are set to 0.5 pmol cm^{-2} sec^{-1}, or 30 pmol cm^{-2} min^{-1}, and 5 µM), which approximates anticipated values for many comparatively high affinity substrates for OCT2 (e.g., MPP).

The script assumes upper and lower boundaries on these reference parameters (lines 30-31).

The script also assumes a set of fixed parameters. These are largely self-explanatory (Figure 2; see text).

```
33      %fixed parameters
34 -    L = 1; % Total depth of fluid in cm
35 -    LUS = 0.1; % Unstirred layer thickness in cm
36 -    c01 = 0; % initial substrate concentration in solution (uM = nmol/cm3) (temp. value)
37 -    c02 = 0; % initial inhibitor concentration in solution (uM = nmol/cm3)
38 -    c03 = 0; % initial substrate concentration in cells (uM = nmol/cm3)
39 -    c04 = 0; % initial inhibitor concentration in cells (uM = nmol/cm3)
40 -    c0 = [c01 c02 c03 c04];
41
42 -    DUS1 = 6e-6; % substrate diffusivity in cm^2/s
43 -    DUS2 = 6e-6; % inhibitor diffusivity in cm^2/s
44 -    DS1 = 0.1; % artificial high solute diffusivity in stirred layer, cm^2/s
45 -    DS2 = 0.1; % artificial high solute diffusivity in stirred layer, cm^2/s
46 -    DS3 = 1.; % artificial high intracellular solute diffusivity, cm^2/s
47 -    DS4 = 1.; % artificial high intracellular solute diffusivity, cm^2/s
48 -    DUS = [DUS1 DUS2 DS3 DS4];  %no difference in intracellular domain
49 -    DS = [DS1 DS2 DS3 DS4];
```

Figure 2.

Additional values that must be inserted into the script include:

```
53 -    Jm2 = 0; % inhibitor max uptake rate in nmol/cm2/s
54 -    Kt2 = 10; % inhibitor uptake Michaelis constant (uM = nmol/cm3)
55 -    Jm3 = Jm1; %  0.0012; % substrate max efflux rate in nmol/cm2/s
56 -    Kt3 = 5*Kt1; %10; % substrate efflux Michaelis constant (uM = nmol/cm3)
57 -    Jm4 = 0.000; % inhibitor max efflux rate in nmol/cm2/s
58 -    Kt4 = 250; % inhibitor efflux Michaelis constant (uM = nmol/cm3)
```

Figure 3.

Jm2 and Kt2 are the J_{max} and K_t values for inhibitor influx, if the presence of an inhibitor is desired in the simulation (Jm2 is typically set to zero to represent the absence of inhibitor).

Jm3 and Kt3 are the J_{max} and K_t values for substrate efflux. Although the absolute values of these parameters is typically not known, the contribution of mediated efflux can be simulated using kinetic and thermodynamic constraints that exist for uniporters (see text). Here, Jm3 is set equal to Jm1 and Kt3 is set to 5x the value of Kt1 (reflecting the influence of a -40 mV membrane potential on the equilibrium accumulation of a positively charged (monovalent, cationic) substrate.

Jm4 and Kt4 are the J_{max} and K_t values for inhibitor efflux, if the presence of an inhibitor is desired in the simulation. As with substrate efflux, these values are typically not known with any precision, but the same kinetic and thermodynamic constraints applied to substrate efflux apply to inhibitors (if transport is limited to uniporters). (Jm4 is typically set to zero to represent the absence of inhibitor).

Data Input

When run, tcMMfitter loads data from a file named "Spreadsheet1.xlsx." The file can have multiple sheets, but data will be collected from first sheet (a sample is available at https://github.com/secomb). Data in that sheet has the following layout:

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1		Time Course Kinetics												
2		Simulation												
3														
4		[Substrate]												
5		Time (sec)	0.3		1		3		10		30		100	
6														
7		0.18	0.00503		0.01483		0.03345		0.05963		0.07673		0.08527	
8		0.36	0.00998		0.02947		0.06661		0.11891		0.1531		0.17016	
9		0.9	0.02459		0.07276		0.16499		0.29544		0.38076		0.42327	
10		1.8	0.04831		0.14325		0.326		0.58574		0.75575		0.84031	
11		3.6	0.09382		0.27911		0.63837		1.15273		1.49001		1.6572	
12		5.4	0.13721		0.40912		0.93944		1.70314		2.20443		2.45263	
13		7.2	0.17875		0.53403		1.23024		2.23807		2.90048		3.22803	
14		9	0.2186		0.65427		1.51162		2.75854		3.58022		3.98482	
15		10.8	0.25694		0.77018		1.78429		3.26547		4.24327		4.72438	
16		12.6	0.29389		0.88206		2.04893		3.76035		4.89179		5.44748	
17		14.4	0.32947		0.99017		2.3056		4.24304		5.52543		6.15556	
18		16.2	0.36381		1.0949		2.55502		4.7142		6.14612		6.84927	
19		18	0.39696		1.19618		2.79731		5.17485		6.75362		7.52943	
20		21.6	0.45993		1.38932		3.26235		6.06524		7.93322		8.85232	
21		25.2	0.51893		1.57076		3.70302		6.91815		9.06856		10.1292	
22		28.8	0.57417		1.74156		4.12149		7.73664		10.1647		11.3642	
23		32.4	0.62609		1.90254		4.51964		8.52351		11.22416		12.55983	
24		36	0.67483		2.05454		4.89914		9.28186		12.25129		13.72086	
25		54	0.87923		2.7021		6.55948		12.70808		16.96947		19.09019	
26		72	1.03349		3.20401		7.90631		15.65321		21.1367		23.89246	
27		90	1.1523		3.5998		9.02229		18.23244		24.89245		28.26349	
28		108	1.24488		3.91754		9.96185		20.5275		28.32743		32.29908	
29		126	1.31812		4.17581		10.76162		22.59334		31.50274		36.06642	
30		144	1.37687		4.3875		11.4468		24.46907		34.4588		39.61112	
31		162	1.42444		4.56222		12.03907		26.18221		37.22698		42.96419	
32		180	1.4633		4.70782		12.55437		27.75478		39.8347		46.15023	
33														
34														

Additional replicates can be added:

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1		Time Course Kinetics														
2		Simulation														
3																
4		[Substrate]														
5		Time (sec)	0.3	0.3	0.3	0.3	0.3		1	1	1	1	1		3	
6																
7		14.4	0.334224	0.342376	0.320454	0.368996	0.250194		1.038014	0.917266	1.163721	0.857372	0.905361		2.318167	2.578
8		28.8	0.477447	0.576264	0.563171	0.493435	0.533348		1.593335	1.555155	1.800702	1.830875	1.911868		4.502109	5.135
9		36	0.487593	0.632443	0.554141	0.839934	0.883073		2.173111	2.378772	1.987535	1.829613	1.880624		4.745216	5.012
10		54	0.803692	1.063041	0.756385	0.827567	0.922409		3.058096	2.952818	2.857764	2.884813	2.643795		5.433717	5.469
11		72	1.0156	1.396929	0.993029	1.041748	1.166088		2.85831	3.391231	2.924282	3.259458	3.476027		8.596867	9.97
12		90	0.87062	1.180173	1.223868	1.076301	1.299297		3.331608	3.948621	2.874389	3.645725	3.339592		8.807558	8.337
13		108	1.309413	1.415064	1.505579	1.560876	1.309319		4.073118	4.348758	4.146611	4.331457	3.453306		9.709692	8.893
14		126	1.383558	1.16092	1.182236	1.280428	1.107197		4.976815	3.981992	2.980407	4.448763	4.009696		10.11742	10.61
15		144	1.323575	1.256261	1.42001	1.955786	1.407557		3.622922	4.41348	5.256837	4.583525	4.486079		10.3511	11.00
16		162	1.479712	1.137591	1.191374	1.54469	1.367345		5.502765	4.350355	5.207046	5.236427	4.448027		13.0338	14.48
17		180	1.613292	1.596199	1.770001	1.599558	1.315907		5.207468	4.845065	3.503718	4.684366	4.43562		7.909621	16.72
18																
19																
20																
21																
22																

When the script is run, final estimated parameters (paramsest) are returned in the order: Lcell, Jl1, Jm1, and Kt1.

```
rss =
    1.4614e+02

Local minimum possible.

lsqnonlin stopped because the size of the current step is less than
the default value of the step size tolerance.

<stopping criteria details>

paramsest =
    1.3748e-03    3.7500e-07    4.3284e-04    4.9528e+00
funcalls =
    108
```

Figure 4.

Where funcalls refers to the final number of iterations and rss is the final root sum of squares. In addition, at completion the script provides Figure 1 (an example is shown here as Fig. 5) that shows the experimental time courses (mean value at each time point) and calculated lines that describe the predicted time courses for each concentration expected from the calculated values for Lcell, Jl1, Jm1, and Kt1 (and the fixed values in the script).

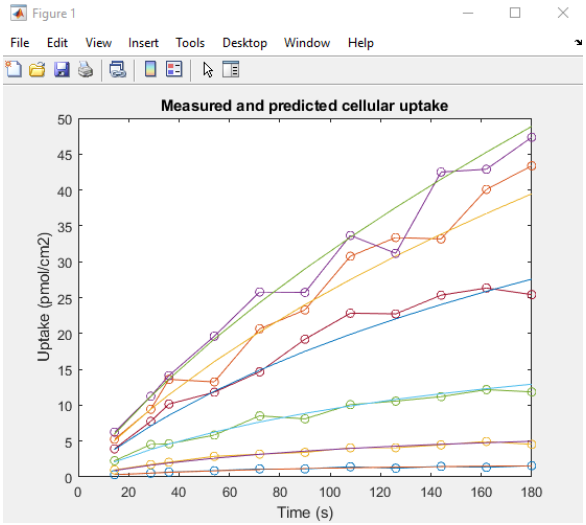


Figure 5.

tcMMsolver.m

This script simulates net substrate/inhibitor accumulation over time for a range of substrate concentrations (with or without inhibitor). There are two running options (Fig. 6), varyinhib = 0 and varyinhib = 1.

```
39 -    varyinhib = 0;
40      % varyinhib = 0 runs a range of substrate concentrations,
41      % with a fixed inhibitor concentration
42      % varyinhib = 1 runs a fixed substrate concentration,
43      % with a range of inhibitor concentrations
44      % conc_fac = [1];
45 -    conc_fac = [0.001 0.003 0.01 0.03 0.1 0.3 1 3 10 30 100 300];
46      % Runs are done for each of the above factors multiplying the substrate
47      % or inhibitor initial concentration (c01 or c02)
48      % *** Do not set conc_fac to zero ***
49 -    nconcs = length(conc_fac); % Length of conc_fac defines number of runs
```

Figure 6

When varyinhib is set to 0 (the case shown above), the script runs a series of 12 concentrations based on the input value, c01 (line 59), which is entered in µM and then, in sequence, is multiplied by each of the listed values for conc_fac (line 45). In the example, c01 was 1 µM, so the calculated concentration range ran from 1 nM to 300 µM. [note: when varyinhib is set to zero, line 45 needs to be active, and line 44 rendered inactive by a leading '%' symbol (the script reads such lines as 'comments')]. For an initial c01 value of 1 µM (and an arbitrary set of input values for the additional required parameters, described below), a figure displaying calculated rate vs. log of substrate concentration is returned as 'Figure 1' (see Fig. 7). In this figure, rate of 'substrate' uptake is shown in blue. The orange line shows the calculated rate of inhibitor uptake, had an inhibitor concentration >0 (line 60), along with suitable inhibitor kinetic parameters (lines 74 and 75) been included. In the example shown, the fixed inhibitor concentration (c02) was set to 0.

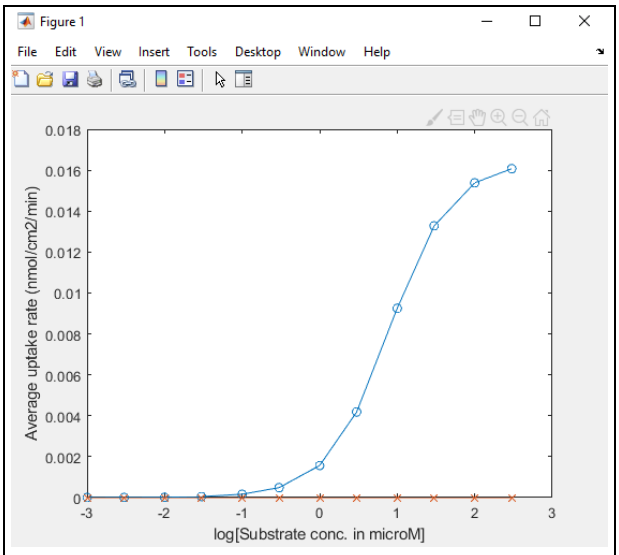


Figure 7

When varyinhib is set to 1 (and line 44 is activated by eliminating the %, and line 45 is rendered inactive), the script returns temporal profiles associated with a single concentration of substrate (line 59; see Fig. 8). [note: the length of the time course, Tmax, is entered in line 81] The script returns Figure 3 (an example is included here as Fig. 9) shows the calculated time course of net substrate uptake into the cell, reflecting the combined influence of the kinetics of influx (Jm1 and Kt1, lines 72 and 73), as well as the influence of an unstirred water later (LUS, line 58), the effective depth of the cell layer (Lcell, line 52; this is treated as the Cell Partition Factor, or CPF – see text), a first-order (nonsaturable) component of substrate accumulation (Jl1, line 71), and the kinetics of carrier mediated substrate efflux (Jm3 and Kt3, lines 76 and 77). [note: the influence of a competitive

```
50      %%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
51 -    L = 1.0; % Total depth of fluid over cell layer in cm
52 -    Lcell = 0.0012; % Thickness (height) of cell layer in cm
53 -    Cellfac = L/Lcell; % Factor to scale for difference in extracellular
54      % and intracellular compartment sizes
55 -    L00 = 0.0;  % Thickness of initial solute-free/depleted NMZ (near membrane zone)
56 -    ramp = 0; % ramp = 0 for zero conc., ramp = 1 for ramped conc. in  NMZ
57      % ramp = 2, 3 etc. for smooth (power-law) initial profile in NMZ
58 -    LUS = 0.150; % Unstirred layer thickness in cm
59 -    c01 = 1.0; % initial substrate concentration in solution (uM = nmol/cm3)
60 -    c02 = 0; % initial inhibitor concentration in solution (uM = nmol/cm3)
61 -    c03 = 0; % initial substrate concentration in cells (uM = nmol/cm3)
62 -    c04 = 0; % initial inhibitor concentration in cells (uM = nmol/cm3)
```

Figure 8.

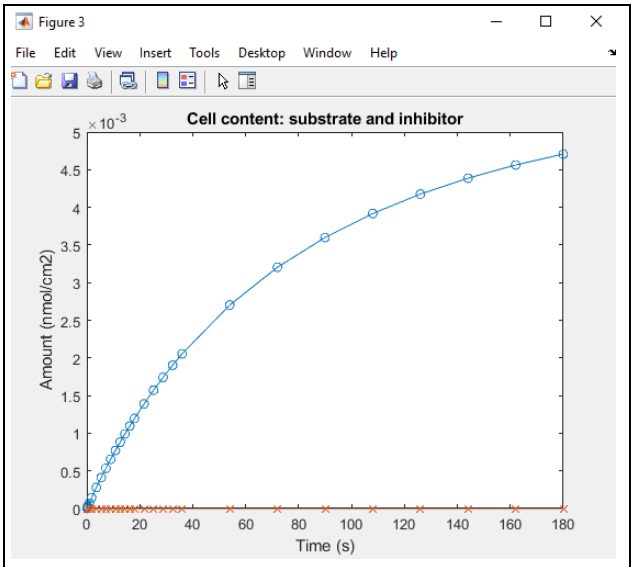
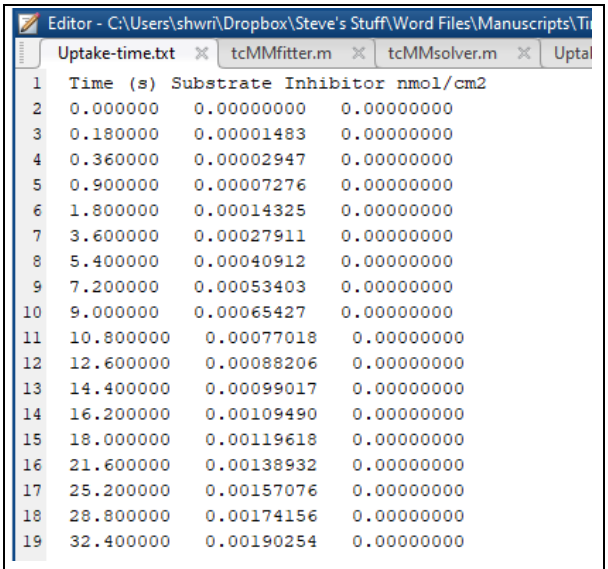


Figure 9.



	Time (s)	Substrate	Inhibitor	nmol/cm2
2	0.000000	0.00000000	0.00000000	
3	0.180000	0.00001483	0.00000000	
4	0.360000	0.00002947	0.00000000	
5	0.900000	0.00007276	0.00000000	
6	1.800000	0.00014325	0.00000000	
7	3.600000	0.00027911	0.00000000	
8	5.400000	0.00040912	0.00000000	
9	7.200000	0.00053403	0.00000000	
10	9.000000	0.00065427	0.00000000	
11	10.800000	0.00077018	0.00000000	
12	12.600000	0.00088206	0.00000000	
13	14.400000	0.00099017	0.00000000	
14	16.200000	0.00109490	0.00000000	
15	18.000000	0.00119618	0.00000000	
16	21.600000	0.00138932	0.00000000	
17	25.200000	0.00157076	0.00000000	
18	28.800000	0.00174156	0.00000000	
19	32.400000	0.00190254	0.00000000	

Figure 10.

inhibitor can be simulated by including values for the kinetics of its influx, lines 74,75, and efflux, lines 78,79].

Data for the simulated time course is in the file, Uptake-time.txt (an example is shown in Fig. 10).

Also returned by the script is Figure 2 (an example is shown in Fig. 11), which shows the temporal profile of substrate concentration at the membrane (blue circles), compared to that in the reservoir (which represents the bulk medium; orange circles), the depth of which is entered in line 51).

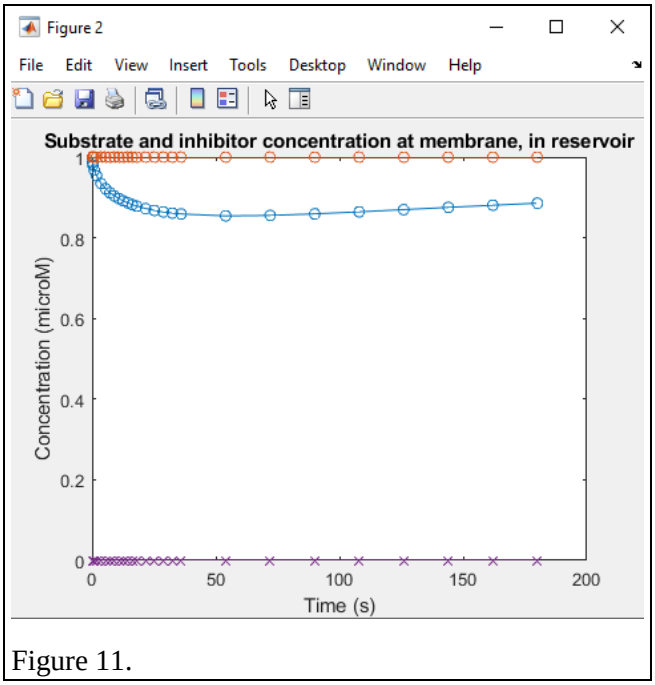


Figure 11.